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India & COVID-19

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Indian J Med Res 151, February & March 2020, pp 111-262

Special Issue - India & COVID-19

Guest Editors: Drs Rajesh Bhatia & Priya Abraham

Contents *Pages*

Editroial

Time to revisit national response to pandemics

Rajesh Bhatia & Priya Abraham ... 111

DOI: 10.4103/ijmr.IJMR_846_20

Viewpoint

Tracking the impact of interventions against COVID-19 in absence of extensive testing *Active surveillance for SARI is urgently needed*

Ganesan Karthikeyan ... 114

DOI: 10.4103/ijmr.IJMR_864_20

Perspectives

The research community must meet the coronavirus disease 2019 challenge

Poonam Khetrapal Singh ... 116

DOI: 10.4103/ijmr.IJMR_832_20

Public engagement is key for containing COVID-19 pandemic

Rajesh Bhatia ... 118

DOI: 10.4103/ijmr.IJMR_780_20

COVID-19: Impact on health of people & wealth of nations

Prakash N. Tandon ... 121

DOI: 10.4103/ijmr.IJMR_664_20

Ethics preparedness for infectious disease outbreaks research in India: A case for novel coronavirus disease 2019

Roli Mathur ... 124

DOI: 10.4103/ijmr.IJMR_463_20

Need for integrated surveillance at human-animal interface for rapid detection & response to emerging coronavirus infections using One Health approach

Rajesh Bhatia ... 132

DOI: 10.4103/ijmr.IJMR_623_20

Contents

Pages

COVID-19 in India: Moving from containment to mitigation

George M. Varghese & Rebecca John ... 136

DOI: 10.4103/ijmr.IJMR_860_20

Responding to COVID-19 pandemic: Why a strong health system is required

Tanzin Dikid, Sushma Chaudhary, Kapil Goel, Preeti Padda, Rajesh Sahu, Tripurari Kumar, S.K. Jain, S.K. Singh & Jai Prakash Narain ... 140

DOI: 10.4103/ijmr.IJMR_761_20

Review Articles

The 2019 novel coronavirus disease (COVID-19) pandemic: A review of the current evidence

Pranab Chatterjee, Nazia Nagi, Anup Agarwal, Bhabatosh Das, Sayantan Banerjee, Swarup Sarkar, Nivedita Gupta, Raman R. Gangakhedkar ... 147

DOI: 10.4103/ijmr.IJMR_519_20

Perspectives for repurposing drugs for the coronavirus disease 2019

Sarah S. Cherian, Megha Agrawal, Atanu Basu, Priya Abraham, Raman R. Gangakhedkar & Balram Bhargava ... 160

DOI: 10.4103/ijmr.IJMR_585_20

Biorisk assessment for infrastructure & biosafety requirements for the laboratories providing coronavirus SARS-CoV-2/(COVID-19) diagnosis

Devendra T. Mourya, Gajanan Sapkal, Pragya D. Yadav, Sujeet Kumar M. Belani, Anita Shete & Nivedita Gupta ... 172

DOI: 10.4103/ijmr.IJMR_763_20

Policy Guidelines

Guidance for building a dedicated health facility to contain the spread of the 2019 novel coronavirus outbreak

Anup Agarwal, Nazia Nagi, Pranab Chatterjee, Swarup Sarkar, Devendra Mourya, Rima Rakeshkumar Sahay & Rajesh Bhatia ... 177

DOI: 10.4103/ijmr.IJMR_518_20

Protocol

Lopinavir/ritonavir combination therapy amongst symptomatic coronavirus disease 2019 patients in India: Protocol for restricted public health emergency use

Tarun Bhatnagar, Manoj V. Murhekar, Manish Soneja, Nivedita Gupta, Sidhartha Giri, Naveet Wig & Raman Gangakhedkar ... 184

DOI: 10.4103/ijmr.IJMR_502_20

Original Articles

Prudent public health intervention strategies to control the coronavirus disease 2019 transmission in India: A mathematical model-based approach

Sandip Mandal, Tarun Bhatnagar, Nimalan Arinaminpathy, Anup Agarwal, Amartya Chowdhury, Manoj Murhekar, Raman R. Gangakhedkar & Swarup Sarkar ... 190

DOI: 10.4103/ijmr.IJMR_504_20

Contents

Full-genome sequences of the first two SARS-CoV-2 viruses from India

Pragya D. Yadav, Varsha A. Potdar, Manohar Lal Choudhary, Dimpal A. Nyayanit, Megha Agrawal, Santosh M. Jadhav, Triparna D. Majumdar, Anita Shete-Aich, Atanu Basu, Priya Abraham & Sarah S. Cherian ... 200

DOI: 10.4103/ijmr.IJMR_663_20

Strategic planning to augment the testing capacity for COVID-19 in India

Nivedita Gupta, Tarun Bhatnagar, Kiran Rade, Manoj Murhekar, Raman R. Gangakhedkar, Anu Nagar & ICMR COVID Team ... 210

DOI: 10.4103/ijmr.IJMR_1166_20

Laboratory preparedness for SARS-CoV-2 testing in India: Harnessing a network of Virus Research & Diagnostic Laboratories

Nivedita Gupta, Varsha Potdar, Ira Praharaj, Sidhartha Giri, Gajanan Sapkal, Pragya Yadav, Manohar Lal Choudhary, Lalit Dar, A.P. Sugunan, Harmanmeet Kaur, Ashok Munivenkatappa, Jayanthi Shastri, Krishnasamy Kaveri, Shanta Dutta, Bharti Malhotra, Amita Jain, Kammilli Nagamani, G.B. Shantala, Sharmila Raut, M.M. Vegad, Ajanta Sharma, Aashish Choudhary, Megha Brijwal, Anukumar Balakrishnan, Jayaswamy Manjunatha, Manish Pathak, Sivasubramanian Srinivasan, Hasina Banu, Himanshu Sharma, Parul Jain, Pakalpati Sunita, R. Ambica, Babita Fageria, Disha Patel, Gitika Rajbongshi, Neetu Vijay, Jitendra Narayan, Neeraj Aggarwal, Anu Nagar, Raman R. Gangakhedkar & Priya Abraham ... 216

DOI: 10.4103/ijmr.IJMR_594_20

Detection of coronaviruses in *Pteropus & Rousettus* species of bats from different States of India

Pragya D. Yadav, Anita Shete-Aich, Dimpal A. Nyayanit, Prachi Pardeshi, Triparna Majumdar, R. Balasubramanian, Padinjaremattathil Thankappan Ullas, Sreelekshmy Mohandas, Hitesh Dighe, Pradeep Sawant, Savita Patil, Dilip Patil, M.D. Gokhale, Basavaraj Mathapati, A.B. Sudeep, Sreekant Baradkar, Abhimanyu Kumar, Rutuja Kharde, Malvika Salve, Yash Joshi, Nivedita Gupta & Devendra T. Mourya ... 226

DOI: 10.4103/ijmr.IJMR_795_20

Severe acute respiratory illness surveillance for coronavirus disease 2019, India, 2020

Nivedita Gupta, Ira Praharaj, Tarun Bhatnagar, Jeromie Wesley Vivian Thangaraj, Sidhartha Giri, Himanshu Chauhan, Sanket Kulkarni, Manoj Murhekar, Sujeet Singh, Raman R. Gangakhedkar, Balram Bhargava & ICMR COVID Team ... 236

DOI: 10.4103/ijmr.IJMR_1035_20

Correspondences

Transmission electron microscopy imaging of SARS-CoV-2

Sharda Prasad, Varsha Potdar, Sarah Cherian, Priya Abraham, Atanu Basu & ICMR-NIV NIC Team ... 241

DOI: 10.4103/ijmr.IJMR_577_20

Contents

Pages

First isolation of SARS-CoV-2 from clinical samples in India

Prasad Sarkale, Savita Patil, Pragya D. Yadav, Dimpal A. Nyayanit, Gajanan Sapkal, Shrikant Baradkar, Rajen Lakra, Anita Shete-Aich, Sharda Prasad, Atanu Basu, Lalit Dar, Veena Vipat, Sidhartha Giri, Varsha Potdar, Manohar Lal Choudhary, Ira Praharaj, Amita Jain, Bharati Malhotra, Pranita Gawande, Kaumudi Kalele, Nivedita Gupta, Sarah S. Cherian & Priya Abraham ... 244

DOI: 10.4103/ijmr.IJMR_1029_20

Development of *in vitro* transcribed RNA as positive control for laboratory diagnosis of SARS-CoV-2 in India

Manohar Lal Choudhary, Veena Vipat, Sheetal Jadhav, Atanu Basu, Sarah Cherian, Priya Abraham & Varsha A. Potdar ... 251

DOI: 10.4103/ijmr.IJMR_671_20

Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy, Iran & China, & Italian tourists in India

Varsha Potdar, Sarah S. Cherian, Gururaj Rao Deshpande, Padinjaremattathil Thankappan Ullas, Pragya D. Yadav, Manohar Lal Choudhary, Rohan Gughe, Veena Vipat, Sheetal Jadhav, Savita Patil, Dimpal Nyayanit, Triparna Majumdar, Atul Walimbe, Shivshankar Gaikwad, Hitesh Dighe, Anita Shete-Aich, Sreelekshmy Mohandas, Deepika Chowdhury, Gajanan Sapkal, Atanu Basu, Nivedita Gupta, Raman R. Gangakhedkar, Sidhartha Giri, Lalit Dar, Amita Jain, Bharati Malhotra, Priya Abraham & NIC Team ... 255

DOI: 10.4103/ijmr.IJMR_1058_20

Indian J Med Res 151, February & March 2020, pp 111-113
DOI: 10.4103/ijmr.IJMR_846_20

Quick Response Code:

Editorial

Time to revisit national response to pandemics

The coronavirus disease 2019 (COVID-19) pandemic is not the first, and certainly not the last to savagely hit the world. Previous pandemics have also attracted global attraction[1]. There is invariably a nationwide response at the time of crisis, but the efforts wane off soon after the pandemic. Political commitments shift towards other emerging issues of national and global importance. Restoration of economic activities assumes priority. Lessons learnt from the pandemics are quickly forgotten. Funds earmarked to strengthen pandemic preparedness plans are reduced or diverted. Health system remains weak, at times getting weaker because of the impact of the pandemic and continues to be inadequately equipped to combat next such event.

History is replete with such events[2]. The last two decades of this millennium have already made us confront severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), avian flu, HINI flu pandemic and SARS-CoV-2 - all of which saw these causative viruses spread quickly and causing widespread havoc. Global response to the COVID-19 pandemic has exposed inherent weaknesses in our preparedness and response[3]. The lessons learnt and costly mistakes must make us wiser enough not to repeat these and take pre-emptive steps.

The next pandemic is imminent. An estimated 1.7 million viral species are circulating among wildlife and 50 per cent of these have the potential to cause human infections[4]. In the absence of serious efforts to preserve the environment and wildlife ecology, it is impossible to prevent the emergence of these novel pathogens from animals, a few of which will have the capacity to jump species and become swiftly transmissible among humans. This can ignite epidemics with potential to explode into pandemics. Maintaining a state of perpetual pandemic preparedness is not an option, rather a necessity for the global community to mitigate mortality and morbidity. This will prevent economies sliding back by decades and loss of lives and will minimize the ensuing disruptive social chaos.

Public health has to be pushed higher on a national development agenda. It is well established that investments in health sectors provide substantially better returns, all of which may not be calculable in economic returns. The National Health Policy of India (2017) articulates increasing investment in health to 2.5 per cent of the national gross development product (GDP) by 2025 from a meagre 1.15 per cent in 2017[5]. Even if complied with the National Health Policy, India's allocations shall be far below that of a large number of developing and developed countries[6]. COVID-19 has irrefutably demonstrated the need for greater increase in this share of GDP in health. It also beckons for this much earlier than 2025. The allocated funds should establish comprehensive services in a planned way.

Health services are usually considered synonymous with preventive, curative, protective, rehabilitative and restorative services. Without undermining the importance of these components of health systems, pandemics have frequently and forcefully reminded policymakers to allocate more funds for public health, especially in establishing and maintaining adequate capacity for early anticipation, detection, confirmation and mounting effective interventions for any outbreak using a One Health approach[7].

The National Pandemic Preparedness Plans (NPPP) were developed by all countries between 2005 and 2010 in anticipation of influenza pandemic[8]. These are great resources which can be modified in the context of experiences and gaps identified in the COVID-19 pandemic. Accordingly, anticipated technological advances can be implemented in the immediate future[8]. The NPPP should not be driven by the health sector.

It has to be a "national" and "whole-of-society" plan in the true sense with active role and participation of all sectors and communities. A national high-powered and multisectoral decision makers' platform should be created to oversee the state of preparedness, provide policy directives and infuse adequate resources into its operations. As with wars, simulation exercises for different scenarios should be regularly undertaken to validate operations, monitor readiness and carry out continuous improvements in access, coordination, quality and safety of interventions under the NPPP.

In a country like India where health is a State subject, the NPPP needs to be replicated as State Pandemic Preparedness Plan (SPPP) as well. Alignment, coordination and collaboration between NPPP and SPPPs should be ensured for efficiency, cost-effectiveness and seamless operations including capacity building in accordance with the International Health Regulation (IHR) (2005)[9]. The IHR (2005) is a legally binding international instrument that calls upon countries to report unusual events, promote the development of core capacities of health systems and work together to obviate the impact of public health emergencies of international concern[10]. The core capacities are directed to prevent, report, detect and respond effectively to epidemics and similar unusual biological, chemical and radiological events.

The IHR (2005) also enunciates best and evidence-based practices and specific measures at ports, airports and ground crossings to limit the spread of health risks to neighbouring countries, and to prevent unwarranted travel and trade restrictions so that traffic and trade disruption is kept to a minimum, thus protecting national and global economy.

The private sector is already a dominant player in the curative services. As much as 70 per cent of Indian curative healthcare services are provided through private sectors. Engagement of private sectors through public-private partnership (PPP), as has been in practice in some areas (tuberculosis control, national urban health mission, *etc.*)[11], should be encouraged and facilitated. This will strengthen public health in India through access to the vast, modern and easily accessible resources in private health sector. Guidelines and modalities for PPP in health have been developed. Their implementation for responding to epidemics can be institutionalized. The National Health Policy articulates the need for PPP[5]. Greater access of communities to private sectors provides opportunities for interaction, which can be utilized for educating public and facilitation of risk communication.

Community is the key to control pandemic. Containment of a swiftly transmissible virus demands absolute cooperation and engagement of the community. The NPPP and the SPPPs must emphasize on the development and implementation of a comprehensive risk communication strategy that addresses context-specific needs of India with diversity in sociocultural norms, educational status, languages, faiths and beliefs as well as demography. These strategies should also systematically address the multisectoral, multidimensional risks and impacts of pandemics and devise communication tools to promote prevention and response to pandemics. Trust and transparency must be fundamental to obtain absolute public engagement and to bust the myths and misinformation, ensuring that stigma and discrimination are strongly rejected[12].

COVID-19 and several earlier events have shown the misused power and impact of social media in fanning misinformation and rumours. Notwithstanding this hazard, communication strategy must utilize and direct social media to obtain community engagement and reduce fear and panic[13,14]. The reach of social media is already phenomenal[15] and is bound to increase manifold in the near future with advancement in affordable technologies.

Rich technical expertise available in a large number of academic and research institutions and the pharmaceutical industry in India has to be harnessed for the promotion of R&D in the production of local technology-driven solutions including point-of-care diagnostics, drugs and vaccines as well as innovative use of information and communication technologies for data collection and analyses and offering telemedicine. Enhancing the capacity of the national regulatory authority in promoting indigenous production of equipment, reagents and other materials and instituting fast-track approval processes for indigenous and imported material and equipment are needed for responding to the epidemics.

It is often said that global public health is driven by events in India. It is because of India's demographic and technological strength. International development partners and a large number of reputed national public health institutes are willing to work together to augment India's inherent capacity. It is time to consider

harnessing their technical expertise in establishing a near-perfect system to combat any pandemic in the future.

Conclusion

The COVID-19 pandemic has created opportunities to build an improved response mechanism for future pandemics. Concerted, well-funded, comprehensive, planned and all-encompassing activities should facilitate building sustained institutional capacity to provide swift and effective nation-wide response to disease outbreaks. This could be done through access to appropriate technologies and improved logistics for efficient supply chains. These will also promote developing multisectoral stakeholder consortia at national and State levels to coordinate actions and launch comprehensive whole-of-the-society response to emerging infections. Overall and long-term target should be to encourage and ensure convergence of all stakeholders for human health, animal health and environment to collaborate in implementing the One Health approach and protecting human life, reduce misery and avoid damage to the national economy.

These are doable actions. The national will and determination are key to mitigate the serious impact of pandemics such as COVID-19 in India.

***Conflicts of Interest*:** None.

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Viewpoint

Tracking the impact of interventions against COVID-19 in absence of extensive testing

Active surveillance for SARI is urgently needed

Several analyses using mathematical models have made predictions for the number of cases and fatalities due to COVID-19 in India[1]. Though the recommended public health interventions centred on social distancing based on these predictions, are sensible, the assumptions underlying these may not be accurate. The standard epidemiologic modelling methods [such as the susceptible-exposed-infectious-recovered (SEIR) model] assume transmission at the community level. However, the number of cases reported till recently in India was identified through screening just two populations: travellers from countries where community transmission was already ongoing, and the contacts of COVID-19-positive individuals. In such a situation, the magnitude and the time to community seeding depends on numerous, unknown variables such as the infectivity of asymptomatic individuals with infection, the efficacy of contact tracing and the time to quarantine of successfully traced individuals[2]. One estimate accounting for some of these variables suggested a time to epidemic initiation of about 45 days[3].

Not accounting for this delay in the initiation of community transmission in SEIR models may induce a sense of complacency, and result in premature withdrawal of public health interventions. The standard approach to infectious disease modelling is to calibrate the rate of community seeding to reproduce the death rates due to the disease[4]. However, for such models to be reliable, active surveillance for severe acute respiratory illness (SARI) should be operational, with all patients undergoing testing for COVID-19, with prompt notification of deaths. The sentinel surveillance system for SARI in place at the moment[5], will need to be transformed into an active surveillance system for the duration of the outbreak.

While efforts are on to scale up testing for COVID-19, given the resource constraints, it is unreasonable to expect population-level testing rates such as those seen in countries like South Korea. The most practical approach is to test symptomatic patients presenting to hospitals, and aggressive testing to identify and contain local chains of transmission. However, these data will not be representative of infection in the population as a whole, and cannot inform disease modelling. Random sampling in the community to determine the prevalence of infection over multiple points in time may perhaps be the ideal way to track the course of the epidemic. However, given the large population of India and its heterogeneity, obtaining representative prevalence data may be impractical, and will be very resource and time intensive. Given these logistic challenges, reliable data on deaths due to COVID-19 over time, can be used to obtain useful insights into the trajectory of the epidemic, and the effect of public health interventions.

Time to number of deaths as a measure of the effect of public health interventions: The pattern of cumulative deaths early in the epidemic in different countries provides insights into the success of any suppression or containment measures adopted. The Figure shows the time taken in days, for the number of deaths to increase to 10, from the day the first death was recorded in five indicative countries, contrasted with the time to occurrence of at least 100 deaths. Though the outbreak of infection was rapid in South Korea (time taken to the first 10 deaths was five days), the institution of successful measures to suppress the epidemic resulted in a marked prolongation of the time to the occurrence of at least 100 deaths (24 days)[6]. This is in contrast to the countries where the epidemic remains uncontrolled (time from 10 to

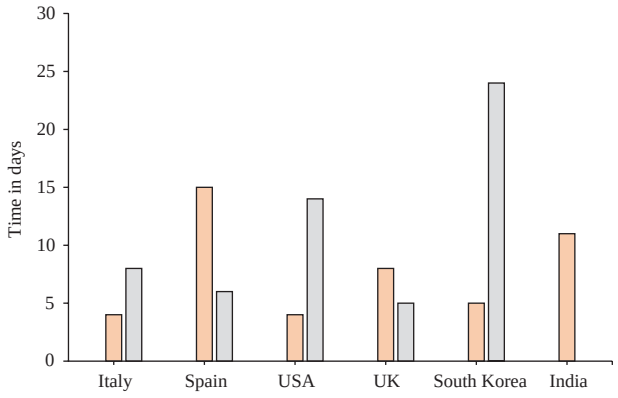

Figure. Time to 100 deaths due to COVID-19. The orange bars indicate the time from the first recorded death to 10 deaths. The grey bars show the time taken for the cumulative number of deaths to at least 100 in each of these countries. *Source*: Ref. 6.

100 deaths, 5-14 days). The number of deaths in India in the weeks following the initiation of the lockdown will provide an indication of the success of the measures adopted. The data on deaths may be expected to lag behind the infection by about three weeks.

In conclusion, the existing systems for the surveillance and testing of SARI in the country should be intensified, with provisions for the prompt notification of deaths due to COVID-19. In the absence of reliable information on the incidence and prevalence of infection in the population, the cumulative death rate will provide the only credible indicator of the effect of public health interventions on the trajectory of the epidemic.

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Perspective

The research community must meet the coronavirus disease 2019 challenge

The coronavirus disease 2019 (COVID-19) pandemic continues to unfold. The situation varies greatly from country to country. In many countries, the number of cases is still less than ten. Some countries have declining epidemics, with no reported case in weeks. Of all the cases reported globally, a substantial majority are from a handful of countries. The outbreak in Europe is accelerating. Countries that have taken aggressive measures to contain the virus, such as China, Singapore and the Republic of Korea, have had success. Evidence shows that COVID-19 can be contained.

As of March 31, 2020, the South-East Asian Region has confirmed 4,215 cases and 166 deaths from 10 countries[1]. The countries affected are Thailand, Indonesia, India, Sri Lanka, Bangladesh, Maldives, Myanmar, Nepal, Bhutan and Timor-Leste. We expect the number of cases and deaths to rise in the coming weeks. All countries must be ready to aggressively contain the virus and must be prepared for all scenarios, including community transmission.

Our knowledge of this virus is growing. The World Health Organization (WHO)-China Mission made several key findings. The vast majority of cases in China arose from close contacts of symptomatic cases. Between one and five per cent of close contacts developed COVID-19. Transmission in most settings was driven by family clusters. There were no examples of children transmitting to adults. Approximately 80 per cent of cases are mild or moderate at diagnosis, 15 per cent are severe and five per cent are critical. Children tend to have milder disease than adults. Virus shedding is highest early in the course of the disease detected as early as 24-48 h prior to the disease onset. Virus shedding usually continues for 7-12 days in mild or moderate cases, and for over two weeks in severe cases[2].

All countries must take a systematic and rigorous approach to containment. The WHO has consolidated its guidance for countries into four scenarios: those with no cases; those with sporadic cases; those with clusters and those with community transmission. For all countries, the aim is the same: to stop transmission, prevent spread and save lives[3]. For the first three scenarios, health authorities must focus on finding, testing, treating and isolating individual cases and following their contacts. In areas with community spread, testing every suspected case and tracing their contacts becomes more challenging. Action must be taken to prevent transmission at the community level to reduce the epidemic to manageable clusters. It is possible that countries will experience one or more of the four scenarios at the subnational level, requiring them to tailor their approach.

Research and innovation must continue to inform the outbreak response. On March 7, 2020, the WHO published its Global Research Roadmap to help coordinate global research to overcome key challenges. The most pressing challenge is that there are currently no proven therapeutics, vaccines or rapid point-of-care diagnostic tests for COVID-19[4]. The global imperative for the research community is to maintain a high-level discussion platform that enables consensus on strategic directions, nurtures scientific collaborations and supports optimal and rapid research to address crucial gaps, without duplication of efforts.

The Roadmap identifies the following nine core research priorities: (*i*) virus: natural history, transmission and diagnostics; (*ii*) animal and environmental research on the virus origin, and management measures at the human-animal interface; (*iii*) epidemiological studies; (*iv*) clinical characterization and management; (*v*) infection prevention and control, including healthcare workers' protection; (*vi*) candidate therapeutics R&D; (*vii*) candidate vaccines R&D; (*viii*) ethical considerations for research; and (*ix*) integrating social sciences in the

outbreak response. In each area, the WHO has identified key knowledge gaps, priorities and milestones. Researchers must focus their efforts and identify where they will have the greatest impact.

A coordinated and multidisciplinary approach is needed. The Global Research Roadmap is a critical tool, but only if transparency and collaboration are maintained. All researches must be carried out in the spirit of collaboration, solidarity and equitable access to all innovations. Research must also be applied in a context-specific way. Protocols, interventions, assessments and the translation of results must be adjusted to local needs and realities. The WHO will continue to provide Member States in the South-East Asian Region the best advice based on the best science, accounting for local contingencies.

The Indian research community has much to offer the outbreak response in India, the Region and the world. India's research community has informed evidence-based interventions against a range of infectious diseases, from TB, HIV and malaria to polio and Nipah virus. The Indian Council of Medical Research has already initiated a clinical study to ascertain the impact of repurposing lopinavir/ritonavir, which was successfully used against severe acute respiratory syndrome and Middle East respiratory syndrome coronavirus[5]. In addition to clinical treatments, the Indian research community has great potential to enhance knowledge on optimal infection prevention and control strategies, particularly in community settings. India's diverse skill set and research capability must be fully leveraged to generate information and knowledge that will contribute evidence to rapidly respond to this outbreak.

Our challenge is immense. COVID-19 is a serious threat to the health and well-being of all people in the South-East Asian Region, and across the world. The WHO will continue to support countries as they respond to every case, cluster and evidence of community transmission. The research community must continue to step up and inform the outbreak response. We must learn as much as possible as quickly as possible. Speed, focus and rigor: together we must prevail.

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Perspective

Public engagement is key for containing COVID-19 pandemic

COVID-19 pandemic has caused unprecedented human health and economic consequences. Almost all countries have been affected[1]. The spread of this novel virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues relentlessly. COVID-19 pandemic, and previous pandemics during this millennium, have demonstrated that the current state of global preparedness is inadequate for an effective response and to prevent local outbreaks from becoming international health emergencies. This is in spite of the substantial efforts and investment in enhancing a few of the core capacities as enunciated in the International Health Regulations of 2005 (IHR-2005)[2]. Among IHR (2005) core capacities, the technology-driven interventions have been preferred over strengthening of core capacity on risk communication of engaging communities and obtaining its willing and continued support in combating unusual events namely epidemics and pandemics.

COVID-19 pandemic is driving home the irrefutable evidence that mitigation of the pandemic warrants immediate application of evidence-based non-pharmaceutical interventions (NPIs) through an empowered and educated community. This is especially relevant in the context of the ongoing pandemic because of the rapid transmission of SARS-CoV-2 and non-availability of the specific antiviral medicine and vaccines[3]. The pharmaceutical interventions are not likely to become accessible to developing countries during the next few months.

Public health history is replete with examples of successful containment of pandemics using social distancing. This was the mainstay of containment policy in the pandemics of influenza in the previous millennium[4,5]. In the current millennium, as the threat of a pandemic with avian flu due to Influenza H5N1 virus emerged and subsequent Influenza H1N1 (2009) pandemic hit the world, major recommendations made by the WHO[6] were public education, social distancing, home quarantine and travel restrictions. The successful outcome of these measures is well documented[7].

NPIs comprising repeated hand hygiene, respiratory etiquettes and social distancing have been advocated to result in the interruption of transmission of SARS-CoV-2. Hand hygiene and respiratory etiquettes are individual-oriented actions. Social distancing - the key to contain pandemic by interrupting the transmission of virus - has several dimensions. These include avoiding contact with patients of COVID-19, refraining from non-essential use of public transport, working from home and avoiding large and small gatherings namely dining out, socializing and visiting other places where infections can spread easily. It is well established that if NPIs are promptly and effectively implemented during pandemics, disease transmission can be reduced[4,5].

This approach is being advocated with greater intensity in COVID-19 pandemic. It calls for sharing of factual information that can be understood and trusted by the communities in bringing about a change in their behaviour to implement efficiently desired public health actions[8]. A systematic and locally-relevant approach through comprehensive risk communication strategy (CRCS)[9] is essential. While developing CRCS for communities, especially in countries such as India with diversity in culture, social norms, educational status, language, faiths and beliefs as well as demography, it is essential to identify and appropriately address the common practical concerns of the communities in responding to a pandemic. The WHO guidelines on ethical considerations in developing a public health response to influenza pandemic can be adopted to develop comprehensive risk communication strategies in the local context and with relevant cultural values[10].

The CRCS should systematically address the multisectoral, multidimensional risks and impacts

of pandemics, and devise communication tools to promote prevention and response to pandemic. There are multiple drivers that collectively tend to increase risk vulnerability and reduce societal resilience, and all these need to be factored into CRCS[11]. Trust and transparency are fundamental to obtain absolute public engagement. There is no 'one-size-fits-all' approach. Communication strategies and actions must address the plethora of factors that influence the communication of actionable messages for the public. At the same time, these messages must bust the myths and misinformation ensuring that stigma and discrimination are strongly rejected. These are more harmful than the SARS-CoV-2 itself for any global efforts to control the pandemic.

Mental health is another major issue that is becoming critical in managing COVID-19 pandemic. Extended lockdowns have become a global norm. Panic and fear are destroying the mental peace of public with a potential to explode into irrational behaviour and social chaos, thus superseding evidence and jeopardizing the pandemic control efforts. Psychologically, when the living environment changes, people naturally feel unsafe, scared and anxious. Efforts must be made to address these and reassure public through a systematic approach led by social and mental health experts[12,13].

To maintain mental stability, public may be encouraged to augment their social communications with family and friends using modern and affordable telecommunication tools and web-based applications. With over 400 million users, India is the WhatsApp's biggest market globally. The app is deeply penetrated in the country, is free and is used by majority of Indians. Used rationally and by avoiding misinformation and circulation of fake news, this can be an excellent tool for social communication in addition to social distancing and accessing reliable information[14].

The importance of social distancing as a tool to limit disease transmission is well recognized, but there are several difficulties associated with this measure[15]. There are challenges in ensuring social distancing especially in densely populated urban slums and refugee habitations in developing countries where people are forced to occupy and live together in small and poorly ventilated homes. Health authorities face challenges in providing separate space for isolation or quarantine of contacts or suspected cases. The lack of freedom of movement facilitates transmission of virus. Clearly augmented surveillance by health system and prompt voluntary reporting by the educated communities are critical.

The COVID-19 pandemic has hit the world severely and unexpectedly. Time for effective response is at premium. Pandemic affects all. It is the responsibility of every citizen to join hands to mitigate its impact by using evidence-based NPIs and following guidance provided by the national authorities through various 24×7 mechanisms.

The WHO Director-General has recently said[16], "This is a time for facts, not fear. This is the time for science, not rumors. This is the time for solidarity, not stigma. This outbreak is a test of solidarity - political, financial and scientific. We need to come together to fight a common enemy that does not respect borders".

We must adhere to this philosophy to defeat the COVID-19 pandemic.

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Perspective

COVID-19: Impact on health of people & wealth of nations

The prophetic warning by the Nobel Laureate Joshua Lederberg[1] that "the microbe that felled one child in a distant continent can reach yours today and seed a global pandemic tomorrow" has once again proved its relevance with the emergence of coronavirus disease 2019 (COVID-19) as the latest pandemic that is affecting human health and economy across the world. COVID-19 pandemic erupted in the Wuhan City of People's Republic of China in December 2019. The PR China, under its obligations for International Health Regulations (2005), reported to the World Health Organization (WHO) that between December 31, 2019 and January 3, 2020, 44 cases with pneumonia of unknown aetiology had taken place[2]. Soon, the disease spread rapidly within and outside the Hubei Province and also engulfed a large number of countries, with Thailand, Japan and the Republic of Korea as the initially affected countries[2]. The Chinese authorities identified the virus to be a new coronavirus which was subsequently named as severe acute respiratory syndrome (SARS)-CoV-2 by the International Committee on Taxonomy of Viruses[3]. The WHO also named the disease due to this virus as COVID-19[4].

As of February 29, 2020, a total of 85,403 confirmed cases and 2,838 deaths had taken place with no respite in geographical spread, mortality, morbidity and economic loss due to the virus[5]. The data currently available indicate mild symptoms in almost 80 per cent of the infected individuals but higher vulnerability of the elderly, especially those with underlying medical condition. The case fatality ratio is less than that seen in two recent epidemics due to SARS-CoV-1 and Middle East respiratory syndrome (MERS)-CoV, but greater transmissibility and rapidity of the spread are the observed characteristics of this virus[6]. Various predictions have been made for the spread of COVID-19 including by a leading Harvard epidemiologist Marc Lipsitch who warns that the coronavirus will infect up to 70 per cent of humanity within a year[7].

History of pandemics and emergence of new pathogens

Pandemics of various infectious diseases with millions dying have been recorded in the history for the past several centuries. The most well known in the history have been pandemic due to plague[8] in Asia and several pandemics of influenza that killed millions of people[9]. The pandemics continued in the current millennium too, and COVID-19 is the latest and certainly not the last pandemic. One of the reasons for the occurrence and delayed response to pandemics is the lacklustre approach to building capacity to respond to infectious diseases. With the availability of antibiotics, even the Surgeon General of the United States of America, William Stewart, said in 1967, "The time has come to close the book on infectious diseases"[10]. But it was not to be. The past three decades have seen emergence of almost 40 new pathogens, most of which are viruses including HIV, hepatitis C virus and coronaviruses that have caused pandemics, novel-influenza viruses, *etc*[11]. Many non-technical but popular publications have also highlighted the persistence and revival of infectious diseases[12].

Pandemics and human development

It has been generally believed that poverty and underdevelopment predispose to infectious diseases. Although true to some extent, the occurrence of the ongoing COVID-19 in developed countries also highlights the fact that developed countries and rich populations are not immune to the outbreaks of infectious diseases. Sufficient evidence in support of this contention through the use of human-made weapons of mass destruction and nature's agents of mass destruction has been provided to support the aforesaid assumption[13].

There exists an inextricable relationship between human development and infectious diseases. The

United Nations Sustainable Development Goals also recognize this in its Goal No. 3[14]. This fact has been highlighted for the last several decades in popular literature[15]. There is another side of development. Ecological changes brought about by the development activities include new technology, construction of new irrigation channels, dams, deforestations, migration of people, high density of populations, emergence of urban ghettoes, globalization of food and increasing international travel. All these facilitate rapid spread of infection across the countries[16]. Some of these factors have been responsible for the rapid spread of COVID-19 across international geographical borders.

Global warming, or the climate change, is another factor that may have acted as a predisposing factor for the emergence and spread of several epidemic-prone diseases[17].

Impact of pandemics on global economy

Pandemics adversely impact the economy of all affected countries. Poor get hit the most. This has been documented earlier[18], and even the United Nations has indicated that the pandemics threaten national security[19]. A comprehensive study extending over a period between 1950 and 1991, involving 20 countries including developed, developing and underdeveloped countries, revealed that the increasing prevalence of infectious diseases will not only increase human mortality and morbidity, but also result in gradual erosion of State capacity and increase in poverty[20]. This pathogen-induced economic decline was found to have a negative effect of such measures of state capacity as fiscal resource, resilience, reach and responsiveness, autonomy and legitimacy. There has been evidence to support the claims that infectious diseases constitute a verifiable threat to national security and State power. Infectious diseases' prevalence was found to have a negative association with the ability of the state to maintain the armed forces with adverse effect on State security[21].

Many industrial units in PR China, Republic of Korea and other countries with large number of cases of COVID-19 had to be closed down within a month of onset of the outbreak. PR China having interrupted the supply chain to other countries has adversely affected its industrial production, thus undermining trade and tourism. The world tourism body has estimated the cost to world tourism to be around US$ 22 billion[22]. Economists warn of a reduced global economic growth since 2009. Concerns about the pandemic have already ruined global stock exchange markets. Both World Trade Organization (WTO) and Organization for Economic Cooperation and Development (OECD) have indicated COVID-19 pandemic as the biggest threat to global economy since the financial crisis of 2008-2009.

Conclusions

Microorganisms antedated human beings. They will continue to cause pandemics because of their ingenuity and basic survival instinct[23]. It is obvious following the spread of COVID-19 that notwithstanding the phenomenal advances in epidemiology, disease biology, molecular biology, genomics and proteomics, humanity is still unable to predict and prevent the unsuspected onset of epidemics and pandemics of infectious diseases. It is also obvious that besides their disastrous effect on human morbidity and mortality, there are equally distressing socio-economic consequences for the affected countries and the whole world. It is essential to strengthen biomedical research, improve healthcare delivery system, establish a permanent 'watch-dog' body and create an improved communication and coordination mechanism for the diverse agencies responsible for mitigating the broader adverse consequences of pandemics. This will require not only national efforts but a coordinated global response through international agencies and development partners.

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Post-script: Within a week of submitting this manuscript for publication the situation both in respect to health and economy, has rapidly deteriorated globally. Thus, as per latest information [WHO coronavirus disease 2019 (COVID-19) Situation Report - 59] the infection has spread to 147 countries, involving over 0.2 million individuals and resulting in over 8,000 deaths. Its adverse effect on economy has disturbed the political leaders of the most advanced countries, USA, UK, Germany, Japan. The President of USA indicated the need for one trillion dollars to meet the expected ravages of the pandemic. Similar thoughts have been expressed by several other nations.

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Perspective

Ethics preparedness for infectious disease outbreaks research in India: A case for novel coronavirus disease 2019

In the past decade, India has witnessed several outbreaks/epidemics (such as H1N1, H5N1, avian influenza, Ebola, SARS, Zika and Nipah) which were successfully tackled with appropriate research[1]. India with its 1.3 billion population residing in densely populated areas in resource-constrained environments is at constant risk for any emerging outbreaks that can cause rapid spread of infection. There have been considerable investments in preparedness for rapid responses and to create a conducive environment to undertake therapeutic or disease prevention research. Research is needed when the outbreak is ongoing or after it has subsided.

Ethics preparedness refers to the capability of the public health system, to protect and have the ability to quickly respond to by having in place an ethical framework that would build trust and guide measures to recover from health emergencies. This can make science more holistic, sensitive and people centric focussing on local, social and cultural values helping better protection of our population. The outbreaks could be regular and known for which the research preparation is high or these could be first time or novel for which there is no specific research preparation and requires quick adaptation. Over the last several years, the Indian Council of Medical Research (ICMR) has worked with the Department of Health Research (DHR), Ministry of Health and Family Welfare (MoHFW), Government of India, in enhancing the national capacity for early diagnosis of infections that have an epidemic potential by setting up a network of well-equipped Virus Research and Diagnostic Laboratories for managing epidemics that can lead to public health crisis[2]. The network is led by the ICMR-National Institute of Virology, Pune, which is well equipped with capability to handle highly contagious pathogens having a Biosafety Level 4 (BSL4) laboratory to set the stage for prompt action[3]. It has also been identified among the 15 laboratories across the world for diagnosis of novel coronavirus infection recently labelled coronavirus disease 2019 (COVID-19) by the WHO Director-General at the media briefing on February 11, 2020[4]. In case of COVID-19, the research areas could be varied ranging from study of disease transmission routes, incubation period, secondary attack, sequelae, susceptibility and isolation containment, *etc*., or research could be on setting up of diagnostics which may include tools for quick screening or molecular tools for confirmatory diagnosis. Research can also be on treatment or therapeutics because there is no known treatment and ways for protection or it could also be on vaccine candidate molecules. Important research needs in epidemic/outbreaks are given in Table I.

Existing governance structure that helps in ethical preparedness

Provisions that guide the implementation, measures to control, surveillance and ethical conduct of biomedical and health research or of clinical trials for approval of therapeutics in the case of epidemics/outbreaks are as listed below.

Indian Council of Medical Research's (ICMR) national ethical guidelines

The latest version of the National Ethical Guidelines for Biomedical and Health Research Involving Human Participants released in October 2017 has, for the first time, included a separate section on research during Humanitarian Emergencies and Disaster conditions and suggested ethical safeguards for any research during an outbreak[5]. This section has identified the need for pre-emptive preparation and suggested a framework for prior planning for dealing with emergency and outbreaks. It discusses how the Ethics Committee (EC) can undertake an

Table I. Types of research requiring ethics preparedness during outbreak investigation

Type of research	Examples
Epidemiological	To explain - where, when, how and who; disease transmission, susceptibility, *etc*.; causes, outcome, case control, cohort, clinical trials, operational, implementation research
Vector/agent characterization	Biology, structure, behaviour, resistance, *etc*.
Diagnostics	Methods, scaling up, validations
Therapeutics	New drugs, indications, dosage, duration, adverse events, efficacy, alternative systems
Prevention	Secondary attack, containment, vaccine development
Storage of biological samples and data	Secondary use of samples and data, privacy and confidentiality, sharing of information/ sequences/samples/isolates
Collaboration and partnerships	Regional, national or international, public and private
Monitoring	Research evaluation, surveillance, oversight

expedited review or hold unscheduled meetings in a given time frame or to connect with relevant experts through available channels, such as video or teleconference whenever physical presence may be difficult for ethical review in a time-bound manner. The guidelines provide measures for protection of the affected individuals or populations from harm and ensuring their safety, stigmatization and ostracization, while maintaining their dignity and protect their privacy or any unauthorized use of identifying information, despite the fact that such diseases are to be notified to public health authorities. The need to draft formats and research protocols in advance, including informed consent forms or plans to seek waiver of informed consent, can help speed up the responses when the need arises. A Monitored Emergency Use of Unregistered and Investigational interventions is also suggested and may be implemented with due precautions. Need for thorough scientific review, followed by an ethics review, local oversight, good manufacturing practice compliance/rescue medicines and supportive treatment accessible are some of the other safeguards suggested.

Clinical trial regulations - New drugs and clinical trial rules (NDCT)

The New Drugs and Clinical Trial Rules (NDCT), under the Drugs and Cosmetics Act, 1940, notified in March 2019, have included supportive provisions to enable fast-track approval processes for the use of unapproved Drugs in Public Health Emergencies[6,7]. Permission can be sought from the Drugs Controller General of India (DCGI) for restricted use of combination drugs which are expected to be potentially useful against the infection. Therefore, on the regulatory front, there have been major strides and a responsive enabling environment has been created for online fast-track clinical trial registration and approvals, registration of ECs on SUGAM Portal and monitoring and accreditation of ECs through National Accreditation Board for Hospitals and Healthcare Providers (NABH)[6,8]. The norms for clinical trials are updated and the NDCT Rules regulate all new drugs, investigational new drugs for human use, clinical trials, bioequivalence studies, bioavailability studies and ECs.

Clinical Trials Registry of India (CTRI)

It is a public database to improve accountability and transparency and ensure that every clinical trial can be prospectively registered. Since 15th June 2009, DCGI has made it mandatory to register all clinical trials on Clinical Trials Registry of India (CTRI) Platform run by the National Institute of Medical Statistics (NIMS) under ICMR[9]. The database collects information about the study sites, investigators, sponsors, interventions and patient groups, and registration is mandatory before any participant is enrolled. The registration also requires uploading a copy of the EC approval letter and approval from the Central Drugs Standard Control Organization (CDSCO) for clinical trials. The details are available on the website for public and these registered trials can be searched both on the CTRI and the World Health organization's (WHO's) International Clinical Trials Registry Platform (ICTRP) search portal, as well as from the CTRI[10].

Department of Health Research - Naitik portal

Chapter IV of the NDCT[7] has made it mandatory for every researcher and EC to follow the ICMR National Ethical Guidelines for research and requires registration of all ECs engaged in review of any biomedical and health research with the DHR on its portal[11]. This registration is in addition to the requirement for the EC to register with CDSCO for clinical trials[6]. Therefore, since September 2019, an Office for EC Registration has been created at the DHR, which is now registering ECs and will be coordinating and monitoring the activities of the ECs registered with it. This fulfils the existing gap related to the regulation of biomedical and health research apart from clinical trials and has helped create a framework for accountability of every EC in the country.

Integrated disease surveillance programme

This programme was started in 2004 by the MoHFW, Government of India, to strengthen the disease surveillance systems for epidemics/outbreak detection and response. It maintains a rapid response team across several locations of the country and aims to strengthen laboratory systems for early detection and diagnosis of epidemics/outbreaks. It uses a network of IT-enabled systems for quick collection, analysis, reporting on samples and dissemination of results. Policy guidance is, thus, provided to respective local, regional or national health departments[12].

Five specific areas have been identified to focus upon to improve ethics preparedness in dealing with outbreaks such as COVID-19 and urges all relevant stakeholders to recognize the same and implement various steps to guide better public health outcomes. Table II discusses the areas for ethics preparedness in case of outbreaks[5].

Building public trust: Protecting societal values, engaging with community and improving communication

Any large public health programme must be based on ethical principles of transparency, accountability and information exchange with the involved population or communities. Along with building appropriate infrastructure for scientific research, training of investigators and field workers, it is also important to simultaneously inform the public about outbreaks and to educate them about their responsibilities to curtail the spread of infection. There is also a need to dispel and clarify about the fake information floating on social and print media related to the use of unproven claims. The importance of community engagement can never be undermined; however, in an outbreak situation, it is very challenging to practically plan it in view of limited time and resources. Simple key public health messages and responsible use of social media platforms can greatly help. Recently, the MoHFW has prepared some frequently asked questions (FAQs) and fact sheets and ICMR has been preparing press statements and posting updates on COVID-19 on its website on a daily basis. The WHO has also developed a lot of educational messages and warned the public about an ongoing infodemic, discussing ways of

Table II. Key areas of ethical preparations for research in case of outbreak/epidemics

Area	Objective	Stakeholders
Building trust and communication	To create better understanding and trust between patients/representatives and healthcare professionals through better communication	Patients, public, researchers, medical professionals, ECs, Regulators, government agencies, sponsors, media, civil society, community representatives
Protection and engagement	To have better transparency, accountability and to plan activities while being sensitive to the needs of the patients/representatives	Patients, families, community, doctors, nurses, field workers
Collaboration and partnership	To plan collaboration across boundaries with public or private entities for more meaningful and timely outcomes	Scientists, medical professionals, State agencies, national agencies, governments, international agencies, pharmaceutical companies
Quality ethics review	To ensure quality ethics review by trained ethics committees in a timely and efficient manner and to guide and monitor the conduct of research	ECs, institutions, accreditors, regulatory bodies
Governance structure	To have a framework for governance of healthcare as well as research for meaningful outcomes	Scientific advisory committees, monitors, DSMB, ministry, departments

EC, ethics committee; DSMB, Data and safety monitoring board
Source: Ref. 5

combating rumours and false information on the internet[13]. Efforts must be in place to gather public support and trust and remove unnecessary anxiety, panic or scare. Media has an important responsibility to report facts in a timely manner and not create false alarms. The ICMR has partnered with the Global Health Strategies to receive guidance on communication and networking activities[14]. Nodal communication officers have been appointed in each of the institutes who coordinate with the Communication Unit at ICMR Headquarters office and public relations officers for media-related activities. A social media policy has been released and a few workshops have been held to train scientists on effective communication skills[15]. These small initiatives can go a long way in improving communication capabilities and build strong public support. Communication, engagement and consultations with various members of civil society, non-government organization (NGOs), patient and community representatives can greatly improve the understanding of the societal ethical values in planning and make interventions more sensitive to the needs of our population.

Capacity building and protection of patients, research participants, families and health workforce

Constant efforts are required at State, regional or national level to build capacity in diagnostics, infection control, outbreak management and adaptation to the international standards and ensure appropriate training of the workforce. Researchers must make sure that there is a fair selection of patients as research participants, and steps have been taken for risk minimization and equitable distribution of risks and benefits, and approvals from ECs[5]. It may be important to note that some vulnerable persons may become particularly vulnerable due to the disease condition and may need more protection. If a clinical trial is planned, the trial site capacity and country scientific capacity development is essential and local researchers should be involved in the conduct of a trial, especially at planning, review and writing stages. The health professionals and healthcare workers should look at individual needs and make efforts to not only prevent the spread and reduce the severity but also be sensitive to the impact on family or the community. Due care should be provided to treat, give supportive care, and provide comfort in isolation, or quarantine, as well. Informed consent process should be simple and in the language understood by the participant. Depending on circumstances, use of oral methods for consenting or waiver of consent can be planned with due approval of the EC in line with the sociocultural milieu. Often, research or therapeutic interventions may be using unproven or experimental therapies and there would be unclear benefits or unknown harms, which require due monitoring and follow up on a long-term basis. The researchers must be aware of plans to safely store the collected samples or information, for the specified duration, knowing who can have access, or with whom this may be shared, and for what purpose. This can help avoid any further trauma, stigmatization, discrimination or even ostracization. Any unauthorized disclosure of personal information collected during an outbreak (name, address, diagnosis, family history, caste, community, *etc*.) can expose individuals to additional risk. Use and sharing of non-aggregated surveillance data for research purposes should have the approval of well-trained ECs. Researchers as well as ECs should be alert to the issues of conflicts of interest which could be commercial (monetary gains and patents) or academic (awards, promotion and publications) and manage them. Safety of all healthcare workers should also be top priority in ethical preparedness and they should have access to good-quality basic safety and protection gear, from possible infection in the course of their interactions with infected patients/participants. It is also important to ensure the physical, social and psychosocial support to health workers who take great risk to their own health.

For ethical conduct of public health interventions or research and protection of patients/participants/families and communities ongoing efforts are required to build capacity of the health force and safe work environment for doctors, nurses, field workers or other members of the team.

Collaborations and partnerships - Including sharing samples, biobanking

Collaborations at the level of State, region, national or international level are important to ensure prompt and appropriate responses in outbreak/epidemic situations. Collective knowledge and experiences can guide the situation better and accelerate the scientific efforts to control the spread of infection or develop new therapeutics. Collaboration can be between public agencies or with private partners and in case of COVID-19, following the genome sequence released by China, global efforts were initiated to target diagnostics therapeutics

control and prevention. The WHO plays an important role in coordinating efforts to control the outbreak at the global level and brings the scientific community for various countries together for undertaking critical public health research in therapeutics, diagnostics as well as innovations. Collaborations may also be needed between international partners to closely monitor the outbreak and understand the pathogen better. Preparedness is needed to have policies in place in regard to sharing the data, samples, results of studies when protecting intellectual property and ownership, as well as storage of sample for future use. At present, there is a requirement to obtain a clearance from the Health Ministry's Screening Committee (HMSC) for any research involving international collaboration and the committee looks at the sensitivity of the research and need for collaboration[16]. For research on outbreaks/ epidemics, there should be appropriate fast-track mechanisms in place to facilitate research in international collaboration. Extensive collaborations at local, regional, national or international level involving public agencies or private entities are needed to multiply efforts to control the spread of infection and develop therapeutics to preserve lives. Ethical Considerations form an important part of the collaborative policies to allow sharing of samples/ data and recognize intellectual contributions.

Robust ethical review

All biomedical and health research must undergo ECs review before its conduct in accordance with the National Ethical Guidelines and considering the local ethical values for due protection of research participants. To review clinical trial protocol, ECs are requested to register with CDSCO, whereas others must register with DHR and can review biomedical and health research. There are many ECs that have received accreditation from NABH or even have international recognition from Strategic Initiative for Developing Capacity in Ethical Review[17]. Depending on the region of outbreak, the needful ECs in the region can be activated. The ICMR in the last few years has conducted extensive training and dissemination programmes across the country and more than 7000 persons have received an update[18]. The copy of national guidelines has been widely shared across medical colleges and research institutions to empower the decisions and functioning of the ECs. ICMR also released a brief handbook summarizing the key take-home messages from the main national guidelines[19]. The ICMR Bioethics Unit at the ICMR-National Centre for Disease Informatics and Research, Bengaluru, has also developed a web portal with all important information and resources[20]. A good EC would review all documentation looking at both scientific and ethical aspects of the research as it would concern the human participants. Besides reviewing the protocol and informed consent form, it would look at other elements as well, such as management of adverse events, provisions made for isolation, quarantine, travel restrictions, monitoring research teams for any signs and symptoms of infection, availability of appropriate training to work in such challenging situations, *etc*. It would guide payment of compensation in case of injury, look at ways for protection of confidentiality, and prevention from any stigmatization, rapid post-trial access, local roll out and surveillance, benefit sharing with the community, fair and transparent partnerships, international collaboration and publication of results in a timely manner. Ethical values must be upheld during public health emergencies[21].

Ethical review by well-trained, efficient and accredited ECs in a timely manner can not only protect the safety and well-being of patients/research participants as well as health workers from any undue harm but also improve science and quality of research outcomes. Table III summarizes the ethical issues for consideration[5].

Appropriate governance and monitoring structure

Dealing with outbreaks and emergencies efficiently and promptly would require a very robust governance mechanism where the roles and responsibilities of each stakeholder are clearly laid out. Research must be responsive to affected communities and the officials must ensure appropriate, expedient and flexible mechanisms[22]. The framework would also facilitate close oversight, appropriate monitoring to entail activities such as adequacy of informed consent, adherence to approved protocols, collection of adverse events, ensuring the integrity of the collected research data, appropriateness of participant selection. A clear organizational chart defining responsibilities and flow of information with all stakeholders would help clarify right at the beginning, on who conducts, or who reviews the protocol scientifically and who monitors the study, or appoints Data and Safety Monitoring Board to function independently. An enabling network which can share, communicate, support, issue advisories, handle media concerns, reduce panic in the public and be effective

Table III. Ethical issues for consideration during outbreaks/epidemics

Topic	Details
Social value	Public health relevance, importance of using unapproved interventions to save lives, prevent spread of infection in care of outbreaks or epidemics
Scientific design and conduct	Use of unapproved interventions after thorough scientific consultation for trial (placebo/ intervention). Clear inclusion exclusion criteria
Benefit risk assessment	Benefit and risks assessed at both individual and population level. Supportive care, adequate monitoring/follow up, management of adverse events, *etc*.
Selection of study population/ recruitment of participants	Fair selection of participants following defined methodology removing the possibility of any bias or discrimination of particular group or individual
Protection of privacy and confidentiality	Identifying information of confirmed cases to be reported to public health authorities but protected from unauthorized access to media or others
Community consideration	Efforts be made to engage with communities to communicate, discuss sensitivities involved. Release timely, accurate updates to remove panic
Qualification and site facilities	Site well equipped to deal with outbreaks/appropriate facility for isolation and quarantine. Investigators and health workers trained/qualified to understand ways of infection control
Informed consent process	Prior approvals with dummy protocol/informed consent forms. EC may be requested to allow oral consent/waiver of consent depending on situation, efforts be made to simplify informed consent in local language
Collaboration	Urgent need to join hands to make collective efforts for infection control. Adequate safeguards needed and sharing data, sample storing for future use, biobanking, *etc*., and fast-track systems to facilitate collaboration
Governance mechanism	Well informed, responsive governance mechanism involving all stakeholders who can work towards a common goal of outbreak control and guide research

Source: Ref. 5

in controlling the crisis in a time-bound manner can be of great value in an outbreak situation. It will cover provisions for data collection and analysis while ensuring that the conflicts of interests are managed. Since it is not possible to predict the region for outbreak or its extent in advance to support prior planning, holistic, well informed, and fast-track approaches at the central level are critical. Dealing with an outbreak may require an inter-ministerial master plan involving not only MoHFW but also other Ministries, Departments and Agencies. In ICMR, the Division of Epidemiology and Communicable Diseases has developed action plan in consultation with expert groups. The responsibilities of multiple stakeholders including those of sponsors, industry, NGOs, government and others must be clearly defined[23]. A governance framework for rapid response with collaborative planning and coordination can help define responsibilities of each stakeholder, encourage judicious use of resources, better planning and coordination to control the infection[24].

An ethically conscious, well informed and updated governance framework which identifies the relevant stakeholders, defines their roles and responsibilities, lays down an implementation plan and a monitoring strategy, can safeguard the ethical values of the society, promote good science and deliver better outcomes.

Conclusions

Ethics preparedness is an important component of plan for dealing with public health emergencies or outbreaks because it helps ensure best standards and quality of deliverables without any compromise on human safety and the ethical values. Availability of trained health workers with strong psychosocial support, research-enabled environment, good ethics review, prior community needs assessment and possible adoption to the present requirements are measures that can help develop interventions that are robust and at par with the international standards. The preparedness also helps the government initiate immediate response and fulfil the expectations from the public and build their confidence and trust. Along with well-equipped laboratories to tackle highly contagious organisms, appropriate updates on training of health personnel are important. Prior plans for data sharing, storage of samples and biobanking can be developed and the network of laboratories and the governance structure can enable them to initiate timely

action and improve quality of outcomes. Fair and transparent accountable collaborations are important. The strong political support along with preparedness activities can lead to the successful conduct of public health interventions that may be required and pave the way for future trials in the country. Outbreaks stimulate all partners and agencies to collaborate their efforts to establish best socially acceptable responses that ensure ethical safeguard for the population. Together countries can achieve a lot in tackling the outbreaks which need prompt public health action to prevent their spread and save human lives. Ethics preparedness can guide various stakeholders involved in the public health emergency for fair decision-making based on moral reasoning in the best interest of the population and thereby help make outcomes more ethical and acceptable to the public.

***Conflicts of Interest*:** None.

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Quick Response Code:

Perspective

Need for integrated surveillance at human-animal interface for rapid detection & response to emerging coronavirus infections using One Health approach

The World Health Organization (WHO) on January 30, 2020 declared the coronavirus disease (COVID-19) event as the Public Health Emergency of International Concern. The event that commenced in Wuhan, Peoples' Republic of China, in December 2019, continues to spread relentlessly. Till February 28, 2020, the WHO has reported 85,403 confirmed cases of COVID-19. Of these cases, spread over 53 countries, 2,924 have died[1]. The causative agent of COVID-19 has been designated by the International Committee on Taxonomy of Viruses[2] as severe acute respiratory syndrome- coronavirus-2 (SARS-CoV-2) because of genetic similarities of this virus with the corona virus (SARS-CoV-1) that caused SARS.

The United Nations had earlier linked national security with pandemics[3]. COVID-19 has also assumed immense global implications for human health, economy and development. The spread of the virus seems to be unstoppable. Swift international travel seeds the virus in hitherto virgin areas. Explosive human-to-human transmission becomes a possibility because of non-immune status of almost the entire population. The inherent characteristic of the coronavirus further fuels rapid transmission. Several other factors such as overcrowding, lack of awareness on proper use of non-pharmaceutical measures, weak health system and inadequate resources for isolation of patients and contacts and infection prevention and control practices in health facilities further facilitate the spread of the virus.

Recent pandemics

The past four decades have seen emergence and spread of several new viral diseases. These have transformed the microbial landscape of global public health. A large number of human infectious diseases arise from animals; 60 per cent of these are transmitted from animals, and 75 per cent of emerging infectious diseases originate from animals[4]. Many of the viruses originated from animals have caused pandemics associated with substantial mortality, misery, social chaos and colossal economic losses. Non-availability of specific antivirals or vaccines during these crisis periods made it extremely difficult to provide pharmaceutical interventions to combat these emerging viruses.

During current millennium itself, apart from Influenza H1N1 pandemic of 2009 due to Influenza H1N1 pdm 2009 virus, avian flu (Influenza H5N1), SARS (SARS-Cov-1), Middle East respiratory syndrome (MERS)-CoV and COVID-19 (SARS-CoV-2) have severely hit the world[5]. Nipah virus outbreak in Kerala, India, is yet another example of a zoonotic infection causing social upheaval requiring emergency public health actions[6]. One of the major reasons for these epidemics to spread rapidly is the delay in early detection of appearance of viruses because of weak surveillance at human-animal interface.

Three major events during the current millennium (SARS, MERS and COVID-19) have been due to coronaviruses. There are many more corona and other viruses lurking among wild animals. Most of these have the potential to jump the species and cause novel infection in human beings, which may explode into uncontrollable pandemic. It has been estimated that "there are over 1.6 million unknown viral species in mammalian and avian populations, of which approximately 700,000 have the potential to infect and cause disease in humans"[7]. Compared to just over 260 viruses that are currently known to cause diseases in humans, the unknown viruses represent 99.9 per cent of potential zoonoses[8]. We need to be better prepared to detect these viruses through an efficient surveillance and characterize such significant viral threats available for spillover from animal reservoirs.

Strong surveillance of these viruses for early detection is critical to contain these viruses during initial phase of emergence of virus only.

Need for a sensitive surveillance system at human-animal interface

The recent events have reinforced the need for a global sensitive surveillance system that can detect these viruses during early phase of outbreak and facilitate mounting of appropriate non-pharmaceutical interventions to prevent their spread and amplification. Since these viruses have originated from human-animal interface, a system that integrates surveillance by human health and animal health sectors needs to be evolved in true spirit of One Health for early detection and efficient response to spillover of such viruses. The WHO in cooperation with international animal health agencies (Food and Agriculture Organization of the United Nations and OIE-World Organisation for Animal Health) has been encouraging "collaboration, networking and technical consultation for the purpose of jointly analyzing epidemiological, virological and human-animal interfaces and promptly sharing and distributing public health information"[9].

The Global Virome Project (GVP), an innovative 10 years partnership, is striving to detect the majority of the unknown viral threats. GVP discoveries can catalyze activities that facilitate proactive preparations for them. It may be the beginning of the end of the pandemic era[7].

Surveillance is a core capacity agreed to under legally binding International Health Regulation (IHR) (2005)

In 2005, the International Health Regulations (IHR 2005) were adopted as WHA Resolution 58.3[10]. The scope and purpose of IHR (2005) has been to prevent, protect against, control and provide a public health response to the international spread of diseases in ways that are commensurate with and restricted to public health risks. Since its entry into force in 2007, signatory States have been working, individually and collectively, to meet their core capacity requirements under the new framework. Surveillance is one of the important core capacities within the framework of IHR (2005)[10].

The WHO and the international organizations in charge of animal health are working together to strengthen the contribution of the veterinary sector in the implementation of the IHR (2005) and surveillance of zoonotic infections[10]. Tools have been developed through joint efforts and assessments in countries have been undertaken. The results of these assessments have unequivocally demonstrated the need for a greater interface between human and animal health sectors to benefit global health security[11].

International Health Regulation (2005) and One Health approach

In consonance with IHR (2005), One Health approach that is a validated, integrated and holistic concept is being advocated by the WHO, the Food and Agriculture Organization of the United Nations (FAO)[12] and the World Organisation for Animal Health (OIE)[13] for combating health threats to humans and animals through human-animal-plant-environment interface. A tripartite agreement[14] between these three organizations has been in vogue since 2010 to apply One Health approach. This needs to be percolated down to the field level where surveillance at human-animal interface should take place.

One Health concept warrants multi-sectoral, multi-disciplinary, multi-institutional and multi-specialty coordination, in all aspects of response to outbreaks. Joint surveillance by the human health and animal health can detect emergence of new viruses from animals at initial phase thus helping in early containment[15].

There have been several barriers to successful implementation of One Health approach including fragmented and disconnected governance of health, animal health and environment, lack of clarity about the definition, concept and scope of One Health approach, under-recognition of its economic benefits, absence of an agreement between professionals on way forward and inadequate training activities. At the same time, successful outcomes have been observed in implementation of One Health in developing countries, namely, Rwanda and Zambia[16,17].

It is imperative that all those working in the fields of human, animal and ecological health with focus on surveillance must agree on operational aspects which are coordinated through a governance mechanism run by senior policy makers. Interdisciplinary training on surveillance may encourage cross-disciplinary collaboration[18].

Countries may consider adopting the framework for effective implementation of One Health that

incorporates political commitment, policy formulation, sustainable financing, programme development, knowledge sharing, institutional collaboration, capacity enhancement, research to generate evidence, engagement of civil society and active participation of the communities[19,20]. A beginning can be made with integrated surveillance.

Animal and public health authorities should collaborate to develop protocols for surveillance, and capacity building for responding to zoonotic infections. In addition, appropriate research needs to be undertaken and results of national and international research be integrated into surveillance and response protocols[21], so that evidence-based surveillance and response be undertaken. Data and science should be the cornerstones of planning, implementation and monitoring epidemiology of pandemic-prone diseases.

Conclusions and way forward

In the early phase of future emergence (early warning) of coronaviruses from animals, veterinarians and stakeholders play an important role in early detection at the human-animal interface. Principles of one health must be applied in these settings. Although One Health is a simple and powerful concept, it has an extremely complex implementation process which has to overcome well-established silo approaches in all countries. It is imperative to bring about a change in the narrative in national response to zoonoses, especially integrated surveillance. The success of One Health implementation shall depend on the extent of attainment of institutional collaboration, joint planning and coordinated comprehensive surveillance for the early detection and prevention of zoonoses, especially coronaviruses to mitigate any future outbreaks due to these viruses.

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Perspective

COVID-19 in India: Moving from containment to mitigation

In late December 2019, a cluster of cases with pneumonia of unidentified aetiology was reported in Wuhan city, Hubei province of People's Republic of China. This was soon identified to be caused by a novel strain of coronavirus which was named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the disease caused by it as the coronavirus disease (COVID-19)[1]. Since then, an epidemic of acute respiratory tract infection has been set in swing with the rapid transmission of infection primarily through droplets, respiratory secretions and direct contact[2]. By the end of February 2020, the infection had spread over 54 countries, infected more than 85,403 individuals across the world and resulted in an approximate 2,924 deaths[3]. The exponential rise in the number of cases being witnessed daily has compelled the World Health Organization (WHO) to title this outbreak a pandemic[4].

In India, the first COVID-19 case was reported in Trissur, Kerala, on January 30, 2020[5]. Current evidence suggests that the incubation period may last for 1-14 days, with a mean duration of 5-7 days[2]. The peak viraemia occurs at the end of the incubation period and before the onset of symptoms, suggesting that transmission begins 1-2 days before the onset of symptoms[6]. The rapid spread of infection is augmented by the potential for transmission by asymptomatic or minimally symptomatic patients[7]. Until now, our national strategy in tackling the COVID-19 has been predominantly one of containment, an approach typically utilized when a pathogen has slow transmission capacity or is brought in from external sources. This allows for the implementation of measures to limit its spread such as quarantine of individuals coming from a high transmission area, isolation of infected individuals, contact tracing as well as reducing the movement of people in areas that have a high case load. The containment strategies adopted by Kerala have helped in slowing the spread of infection into the community by the end of March[8]. However, once the infection starts to spread in the community with evidence of sustained local transmission, it becomes impossible to isolate all the infected individuals. In such situations, mitigation measures are needed with the aim to slow down the spread of infection. These procedures include closure of schools and a ban on public mass gatherings[9]. With the escalating number of COVID-19 cases being detected in our country over the last few weeks, it is time for India to shift its efforts to slow the spread of the SARS-CoV-2 virus from containment to community mitigation.

The community mitigation strategies are called 'flattening the curve' in epidemiological terms. The curve refers to the projected number of people who will contract COVID-19 over a given time frame. The shape of the curve varies according to the rapidity with which the infection spreads in the community. Infection curves with a steep rise also have a steep fall. This results in an overloading of the local healthcare systems beyond their capacity, leading to higher case fatality rates (Figure). The highest priority at this stage is to keep the mortality as low as possible. If individuals and communities take appropriate steps to slow the spread of the virus, the cases would be stretched out across a longer period of time, thereby flattening the curve and avoiding overburden of the existing healthcare systems. It also buys time to potentially develop newer drugs and vaccines targeted at the virus.

As the disease spreads in the community beyond the primary contacts, strategies to prevent further spread within communities is the need of the hour. We discuss below the possible interventions to help attain this.

Social distancing

Timely implementation of aggressive strategies that create social distance and reduce close contact

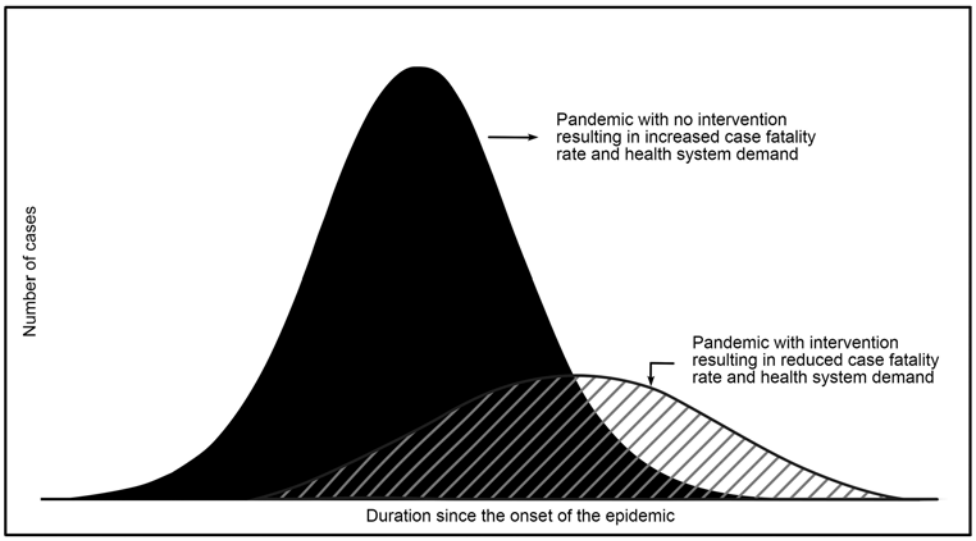

Figure. Flattening the epidemic curve.

of people has proven effective in delaying the rates of transmission and reducing severe illness and death in times of pandemic. The influenza outbreak of 1918 has proved that non-pharmaceutical measures such as social distancing are as important as drugs and vaccines in controlling a pandemic[10]. The lockdown in Wuhan to contain the COVID-19 outbreak in China showed a positive impact with significantly decreased growth rates and increased doubling time of cases[11]. Social distancing measures include isolation of infected people, quarantine of their contacts, options for people to work from home, the closure of schools and the cancellation of large gatherings. Such measures allow our healthcare system to handle the additional burden in a phased manner. The WHO recommends a minimum distance of at least 1 m (3 feet) to be maintained between individuals to prevent the spread of the infection through respiratory droplets[12].

Personal protection measures

Individual protection measures, an integral part of infection control, reflect a level of personal commitment and action above and beyond governmental policies towards containment and mitigation of the disease. Evidence from earlier outbreaks advocate that face masks and hand hygiene reduce respiratory illnesses in shared living settings and in turn allay the impact of pandemics[13]. Frequent hand washing with soap and water may significantly reduce the chance of acquiring and transmitting the infection. Individuals are encouraged to practice respiratory hygiene. In case a person develops respiratory symptoms, using a medical mask is recommended. However, wearing rubber gloves out in public is discouraged and does not replace the need for appropriate hand hygiene. Disinfection and cleansing of frequently touched surfaces should also be carried out daily[12,14,15].

Home isolation when sick

Patients with suspected COVID-19, following triage at the point of first healthcare contact, can be managed at home if presenting with a mild illness, and there is no concern of rapid deterioration. Patients can be managed symptomatically with oral paracetamol. Such patients need to be placed in a well-ventilated single room, with their movements limited within the house and their shared space minimized. A single caregiver should optimally be chosen from among the household members, and visitors should not be permitted until the patient has a complete recovery. Respiratory masks are to be worn by both the patient and the caregiver, and dedicated linen and eating utensils should be assigned separately for the patient. All individuals in close contact with the patient with suspected or confirmed COVID-19 should be considered for quarantine and their health should be monitored for 14 days from the last day of interaction[16].

Care of the vulnerable population

A critical facet of COVID-19 has been the disproportionately higher mortality seen among individuals more than 60 yr than the young adults or paediatric population[17]. Those above 80 yr were noted to have the highest case fatality rate at 14.8 per cent[17]. This was brought out in one of the largest data analyses conducted in China involving 72,314 patient records

which comprised 44,672 (61.8%) confirmed cases, 16,186 (22.4%) suspected cases, 10,567 (14.6%) clinically diagnosed cases and 889 asymptomatic cases (1.2%). While patients with no prior co-morbid conditions had a case fatality rate of 0.9 per cent, the case fatality rate was higher among those with cardiovascular disease (10.5%), diabetes (7.3%), chronic respiratory disease (6.3%), hypertension (6.0%) and cancer (5.6%)[17]. These findings make it imperative to protect individuals belonging to these highly susceptible groups more strongly. As children may often be asymptomatic transmitters of the disease, their interaction with elderly should be limited[18]. Help in grocery shopping and delivery of food, medicines and essential services and supplies will go a long way in minimizing unnecessary exposure of our vulnerable population. Such rationally layered social-distancing interventions may be a more acceptable long-term solution to the current pandemic[19].

Widening the testing and treatment capacity

Initially, testing facilities for COVID-19 by reverse transcription-polymerase chain reaction (RT-PCR) laid with government facilities alone, with the Indian Council of Medical Research (ICMR) recommending the testing of only those symptomatic patients with a history of international travel to affected countries or a history of close contact with a laboratory-confirmed positive case[20]. India has now scaled up the diagnostic and laboratory testing for SARS-CoV-2 virus considering the dire urgency for enhanced case detection to curb the dissemination of the disease. There is a need to secure adequate clinical infrastructure in the country with ample supply of personal protective equipment for the healthcare professionals. The ICMR-National Institute of Virology, Pune, has succeeded in retrieving the SARS-CoV-2 strain from infected patients, confirming a homology of 99.98 per cent with the strain from Wuhan[21]. This could imply that similar patterns of disease transmission can be expected if robust strategies to tackle the infection are not in place. It also gives hope to the possible development of newer diagnostics, therapeutics and vaccines to combat COVID-19. A comprehensive approach is needed to break the chains of transmission, and more aggressive testing, early diagnosis and isolation along with adequate treatment seem to be the way forward in tackling this infection in the future.

As India prepares for a worst case scenario, it is of utmost importance for all its citizens to follow strict hygiene practices to ensure self-protection and prevent the further spread of the infection within the community. The success of mitigation strategies will depend on public adherence to these. Clear, coordinated management guidelines need to be communicated consistently to healthcare professionals as well as to the public to avoid unnecessary fear and anxiety. With these measures, we can hope to tide over the pandemic as early as possible.

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Perspective

Responding to COVID-19 pandemic: Why a strong health system is required

Background

The incidence of emerging infectious diseases in humans has increased in the recent past and will continue to do so in the near future. The emergence of novel coronavirus (CoV) [severe acute respiratory syndrome-CoV-2 (SARS-CoV-2)] which causes CoV diseases or COVID-19 was first identified in Wuhan city of China in December 2019, when a cluster of unusual respiratory illnesses dominated by pneumonia was reported[1,2]. The initial cases were linked to a seafood wholesale market in Wuhan city; subsequently, many cases rapidly emerged with no linkage to the seafood market, indicating a person-to-person spread[3]. On January 30, 2020, the World Health Organization (WHO) declared the CoV epidemic or COVID-19 centred in Wuhan in Hubei province 'a public health emergency of international concern' (PHEIC)[4]. The WHO Director-General Dr Tedros Gabreseus said, "the decision to announce a PHEIC was made because of signs of human-to-human transmission outside China and WHO's concern regarding what might happen if the virus were to spread in a country with a weaker health system"[4]. On March 11, 2020, the WHO characterized COVID-19 as a pandemic[5].

Genetic analysis early in the outbreak in China revealed that the virus was similar to, but distinct from, SARS-CoV-1, however, the closest genetic similarity was found in a CoV that had been isolated from bats[6]. CoVs are a family of large, enveloped, positive-strand RNA viruses that can be divided into four genera: alpha, beta, delta and gamma, of which alpha- and beta-CoVs are known to infect humans. Four HCoVs (HCoV 229E, NL63, OC43 and HKU1) are endemic globally and account for 10-30 per cent of upper respiratory tract infections in adults[7].

In the 21st century, two highly pathogenic human CoVs - SARS-CoV-1 and Middle East respiratory syndrome (MERS)-CoV - emerged from animal reservoirs to cause outbreaks in many countries with alarming morbidity and mortality. The CoVs are ecologically diverse, with the greatest variety seen in bats, suggesting that they are the reservoirs for many of these viruses[7,8]. Peri-domestic mammals serve as intermediate hosts, facilitating recombination and mutation events, furthering the expansion of genetic diversity[7].

Evolving global situation

The COVID-19 pandemic situation is at present rapidly evolving. The situation began in Wuhan in December 2019, an alert was issued by the Wuhan Municipal Health Commission on December 31, and the same day, a notification was made to the WHO[3]. On January 7, 2020, the causative pathogen was identified as a novel CoV[9]. Realizing the threat of rapid spread, China implemented a lockdown in Wuhan city, the epi-centre, to prevent further spread, even as cases started rising and more Chinese provinces were affected[3], leading to declaration of a PHEIC on January 30, 2020[4].

Till March 21, 2020, a total of 292,142 confirmed cases of COVID-19 had been reported to the WHO from 192 countries, with 12,784 deaths[10]. China reported a total of 113,702 confirmed cases and 4,012 deaths from 27 provinces. Of these, 83 per cent were reported from Hubei province alone. In provinces other than Hubei, the cases occurred either sporadically or human-to-human transmission was observed in clusters among families. Following the lockdown, the cases and deaths in China started to decline (Figure)[3,11]. The data also suggested that 80 per cent of cases in China were of mild nature and the severity increased with age, indicating that the elderly and those with underlying chronic conditions were at a higher risk of severe disease and dying[11].

Over the recent weeks, a global spread of the infection at large, and at times a sharp increase in cases

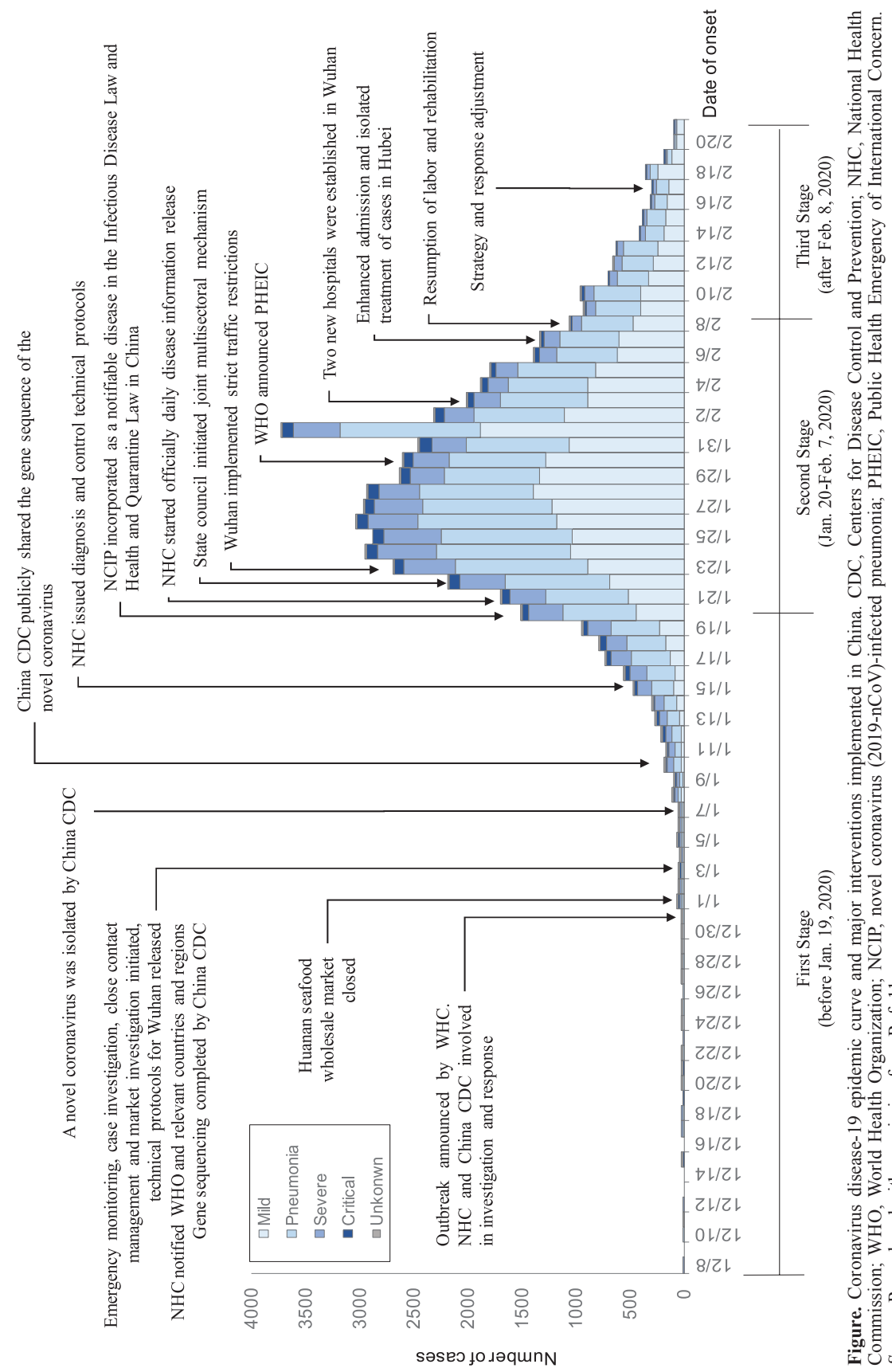

Figure. Coronavirus disease-19 epidemic curve and major interventions implemented in China. CDC, Centers for Disease Control and Prevention; NHC, National Health Commission; WHO, World Health Organization; NCIP, novel coronavirus (2019-nCoV)-infected pneumonia; PHEIC, Public Health Emergency of International Concern. *Source:* Reproduced with permission from Ref. 11.

and deaths is occurring in countries outside China, namely South Korea, Iran, Italy and Spain. These include now cases with no clear link to China. A more focal community transmission may be presently underway in these countries, as well as in cruise ships. Therefore, the global outbreak is now undergoing a remarkable and ominous shift. While cases are declining in China, the pandemic caused by the novel virus is engulfing several countries of the world, both developed as well as developing ones[12]. COVID-19 is currently confirmed in nearly 192 countries around the world. The WHO revised the risk assessment on February 28, to very high, at regional as well as global level[12].

The spread of COVID-19 to African region has been an issue of concern due to the relatively vulnerable health system[13]. Africa had remained unaffected till February 13, 2020, but now, it has 37 countries affected with more than 600 confirmed cases[13]. These recent developments have led to deep concerns that the pandemic will have grave health, social and economic consequences around the world.

India's response

In India, 283 confirmed cases and four deaths have been reported as of March 21, 2020, with many having had travelled to affected countries[10]. A few cases have occurred in a cluster, among family members. However, there are no reports of community transmission so far in the country.

While intensifying preparedness for the unprecedented threat posed by COVID-19, the Government of India (GOI) has constituted an inter-ministerial committee represented by the ministers of the Ministry of Health and Family Welfare, External Affairs, Home, Civil Aviation as well as the National Disaster Management Authority[14]. Several measures have been undertaken by the government for the early detection of cases including thermal screening at the international airports, major and minor seaports and land crossings. So far, more than half million incoming airline passengers and thousands coming through seaports have undergone the screening procedure. To engage the community, *Gram Sabhas* are organizing awareness drives regarding clinical presentation of the novel CoV diseases, preventive measures, and the need for reporting especially in the villages in border districts[15].

The Integrated Disease Surveillance Programme (IDSP) based at the National Centre for Disease Control (NCDC), Delhi, has been conducting entry screening for all passengers arriving from other countries and has augmented community surveillance for severe acute respiratory illnesses. The national guidelines on surveillance, case investigation and laboratory detection of COVID-19 cases have been developed by the NCDC and made available to all States[16].

The relevant laboratories across the country are working as a part of network of testing facilities for SARS-CoV-2 in accordance with the national testing guidelines developed by the Indian Council of Medical Research (ICMR)[17]. A 24×7 call centre has been operational at the NCDC from an early stage of COVID-19 outbreak which provides information and guidance about testing facilities and their location, sample collection facilities, as well as other aspects of the pandemic. The States/Union Territories (UTs) are on alert, and stock of personal protection equipment and face masks is progressively being augmented[18].

The general public is advised to avoid travel to affected countries and visas of foreign nationals traveling from affected countries have been cancelled. The government has organized daily briefing for media with updates on the novel CoV situation as is evolving in the country.

The planning and preparedness for the next phase of the outbreak is indeed critical, currently seen in many countries reporting focal clusters of cases in the community and rapid escalation of the situation for the worst. Therefore, preparedness and readiness to have a public health surge capacities is urgently needed in the country. These would include active surveillance and expanded testing, contact tracing, isolation and management of cases after triage. In order to strengthen the national capacity for surveillance and outbreak investigation, we need to mobilize and effectively utilize the field epidemiology workforce, which is already trained and has skills in epidemiological analysis and disease control.

The India Epidemic Intelligence Service or India EIS programme run by the NCDC, in collaboration with the US Centers for Disease Control and Prevention (CDC), has been actively supporting the COVID-19 response. Established in 2012[19], this two-year, post-graduate, applied field epidemiology training programme is modelled after CDC's EIS programme and has now expanded to two additional hubs - the WHO India Country Office and ICMR's National Institute of Epidemiology, at Chennai. At

present, 45 EIS officers (32 alumni and 13 currently in the programme) are supporting various States in this pandemic containment exercise, including Kerala, Maharashtra, Nagaland and Uttar Pradesh. The India EIS Programme has so far graduated 64 officers (across 21 States) through the end of 2019. During 2020, over 60 officers will be in training across the three hubs.

India EIS officers work in national or State government health programmes to evaluate disease surveillance systems; investigate outbreaks; respond to disasters, emergencies and mass gatherings and conduct epidemiological evaluations. The programme has great potential in strengthening epidemiological capacity in the country to respond effectively to public health emergencies such as COVID-19; strengthening core capacity in disease surveillance, early detection and rapid response and generating evidence that can be used for policymaking and implementation.

Challenges amidst uncertainties

Clearly, SARS-CoV-2 is not the first and would not be the last challenge to confront India. There is a need to report, promptly and openly, cases of any disease with the potential for international spread. In 2003, delay in reporting of early cases of SARS originating in China led to the virus spreading to 17 countries as far as North America and causing 774 deaths[20]. The outbreak was brought under control swiftly, within four months. The outbreak was, however, associated with considerable direct costs due to intensive medical care and control interventions, but the cost of social disruption and economic losses was clearly much higher than the medical cost.

To enable the world to deal with a situation such as this, the revised International Health Regulations (IHR) (2005) were adopted by the World Health Assembly in 2005, which called for developing core capacities in each member country to combat PHEIC[21]. The IHR (2005) core capacities are required to detect, assess, notify and report events and respond to public health risks and emergencies of national and international concern, as stipulated in Articles 5 and 13, and Annex 1, of the regulations[22]. The IHR (2005) requires all countries to be prepared at all times by strengthening their surveillance and response capacities to be able to deal with the new and emerging pathogens.

The COVID-19 pandemic is unfolding at a rapid pace. There are uncertainties how it will evolve in the coming days and what impact it will have. Almost on a day-to-day basis, new information is becoming available, helping us to better understand the new virus in terms of its natural history, incubation period, the period of communicability and case fatality rates. It has been clear from the beginning that the novel CoV is more infectious but less deadly compared to SARS[23]. New data are emerging which suggest that transmission can occur while the person is either at a pre-symptomatic phase or has only a mild disease[24], which has major implications for disease transmission in communities, for identifying cases and contact tracing.

Investing in preparedness and health system strengthening

To respond to the ongoing pandemic and such events in the future, identification of critical public health measures and systemic investment in those is of utmost importance and includes the following:

First, we need to focus on preventing new infections and saving lives. A strong real-time national surveillance to detect COVID cases, respond rapidly by active case finding, prompt treatment and isolation of cases and contact tracing, thereby preventing an outbreak from happening or limiting its spread to the general population by immediate containment measures. Other than the ICMR-National Institute of Virology at Pune, the NCDC at New Delhi and other laboratories, it is necessary to expand network of virus diagnostic laboratories in private sector with adequate surge capacity to respond swiftly to the current and future events. Preparedness for ensuring skilled and trained human resources at all levels of health care by building their capacity is essential especially before the community transmission of the novel coronavirus occurs. The healthcare workers, who are at a high-risk of infection, need to strictly follow infection control procedures while managing critically ill patients. The WHO has recently issued guidelines on 'Infection Prevention and Control (IPC) for Novel Coronavirus (COVID-19)'[25]. Adequate amount of essential medical supplies such as personal protection equipment (PPEs), N95 face masks, hand sanitizers, test kits for diagnosis and ventilators should be made available.

Second, we need to empower individuals and community for efficient response through information, education and communication. At personal level, one needs to take precautions to avoid getting himself/herself infected with the virus. These precautions include 'staying at home' especially when sick, and thus not infecting others; covering your mouth or nose while coughing or sneezing; avoiding touching eyes, nose or

mouth; regular and thorough hand washing with soap and water at least for 20 sec; and cleaning the frequently touched surfaces such as table, door handle *etc*., with a disinfectant, social or physical distancing at community level to protect those at high-risk of severe disease such as elderly and those with underlying chronic conditions and healthcare workers.

Finally and importantly, we need to ensure a resilient and responsive health system which can run itself in a sustainable manner. For this, adequate and sustained financing of health is required. The most important lesson we have learnt during the present pandemic as well as the past pandemics is that investing in preparedness can cost little and could save millions of lives.

The evolving situation calls for a stronger public health systems in the country. Message is loud and clear that a more resilient and responsive health system can help ensure early detection and prompt reporting of cases. A strong health system will be able to detect an unusual occurrence quickly.

In the final analysis, it is clear that investing in robust surveillance, laboratory testing, a comprehensive communication strategy in consonance with the IHR (2005), and a strong public health system could help save lives and avert grave socio-economic disruption that may likely be caused by this virus or those that may strike in the future. The threat is real and the time for action is now.

***Conflicts of Interest*:** None.

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Review Article

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The 2019 novel coronavirus disease (COVID-19) pandemic: A review of the current evidence

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A novel coronavirus (nCoV) spillover event, with its epicenter in Wuhan, People's Republic of China, has emerged as a public health emergency of international concern. This began as an outbreak in December 2019, and till February 28, 2020, there have been 83,704 confirmed cases of novel coronavirus disease 2019 (COVID-19) globally, with 2,859 deaths, resulting in an overall case fatality rate of 3.41 per cent (95% confidence interval 3.29-3.54%). By this time (February 28, 2020) 58 countries or territories and one international conveyance (Diamond Princess Cruise Ship) were affected. As a part of the global response to manage and contain the pandemic, major emphasis was placed on generating research intelligence to guide evidence-based responses to contain the virus, which was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), owing to its genetic similarities with the SARS virus. This review summarizes the emerging evidence which can help guide the public health response, particularly in India. Key areas have been identified in which research needs to be conducted to generate critical intelligence for advising prevention and control efforts. The emergence of SARS-CoV-2 has once again exposed the weaknesses of global health systems preparedness, ability to respond to an infectious threat, the rapidity of transmission of infections across international borders and the ineffectiveness of knee-jerk policy responses to emerging/re-emerging infectious disease threats. The review concludes with the key learning points from the ongoing efforts to prevent and contain COVID-19 and identifies the need to invest in health systems, community-led response mechanisms and the need for preparedness and global health security.

Key words COVID-19 - epidemic - MERS-CoV - novel coronavirus - pandemic - quarantine - severe acute respiratory syndrome coronavirus 2 - transmission

Introduction

Coronaviruses (CoVs) represent a major group of viruses mostly affecting human beings through zoonotic transmission. In the past two decades, this is the third instance of the emergence of a novel coronavirus, after severe acute respiratory syndrome (SARS) in 2003 and

Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012[1,2]. The repeated emergence and global scale of transmission, significant number of deaths, infection and mortality of care providers and healthcare workers (HCWs), and higher risk of death in vulnerable or susceptible groups, have been the major causes of concern. Integrated early warning and response systems are an effective way to raise a timely alarm about these emerging and re-emerging pathogens, but few tools are available to enable pre-emptive prediction of such diseases. The Global Virome Project has been initiated with the objective of creating a global atlas of pathogenic viruses, with the specific objective of identifying spill-over events[3,4]. The project has not been without its critics, and is not yet close to providing evidence which can be translated into preparedness action[5]. This underscores the importance of preparedness of the health system to deal with dangerous pathogens and better control of endemic infections.

The process of naming the novel coronavirus (2019-nCoV) which emerged in Wuhan, China, in December 2019, has created some controversies[6]. In this review, the WHO convention of referring to the disease condition as novel coronavirus disease (COVID-19) has been followed[7]. The virus will be referred to as SARS-related CoV-2, or SARS-CoV-2[8].

COVID-19 has been labelled as a public health emergency of international concern (PHEIC)[9], and the epidemic curves are still on the rise[10]. Here, we summarize the clinical and public health aspects of COVID-19 and SARS-CoV-2, and the lessons gleaned from the global responses so far. As more data continue to emerge, the epidemiology of the disease will come into sharper focus.

Agent: Severe acute respiratory syndrome-coronavirus 2

The SARS-CoV-2 is a beta-coronavirus belonging to the family of *Coronaviridae*. Essentially a zoonotic disease, the first human coronavirus outbreak was recorded in 1965 - HCoV-229E, followed by two outbreaks of similar capacity - SARS-CoV and MERS-CoV in 2003 and 2012, respectively[2,11-13].

Meta-genomic sequencing of RNA samples isolated from the bronchoalveolar lavage (BAL) fluid of patients suffering from severe acute respiratory illness (SARI) in the city of Wuhan identified a novel RNA virus as the causative pathogen. Till now, 11 complete genome sequences of SARS-CoV-2 isolates are available. Six of the whole genome-sequenced SARS-CoV-2[14,15] were isolated from different parts of China and five were isolated from Japan[16]. Genome sequences of different isolates are highly similar and showed more than 99 per cent sequence identity. The genome of SARS-CoV-2 harbours 10 coding sequences (CDS), which encode polyprotein, surface glycoprotein, membrane glycoprotein and nucleocapsid phosphoprotein (Fig. 1).

The orf1ab polyprotein, encoded by the genome of SARS-CoV-2 virus isolated from the Japanese patients, has 24 nucleotide deletion. Genome deletion in the Japanese SARS-CoV-2 virus was also observed at the UTR locus and extreme 3'end of the genome. Phylogenetic analysis using complete genome sequence of SARS-CoV-2 revealed that its genome sequences are very similar (~90%) to the SARS-like CoVs. Analysis of receptor binding domains suggests that SARS-CoV-2 possibly uses angiotensin-converting enzyme-2 (ACE-2) as a cell receptor to infect the host[17].

Phylogenetic analysis suggests that although bats may act as the original reservoir for SARS-CoV-2, there is a possibility of yet another unidentified intermediate host, which was likely being sold at the seafood market in Wuhan before the outbreak[18]. When the first cases emerged in December 2019, bats were likely in hibernation, and if there was an intermediate host, it might have played a role in continuing local transmission of the virus. Hence, despite there being about 89 per cent similarity with the genomic sequence

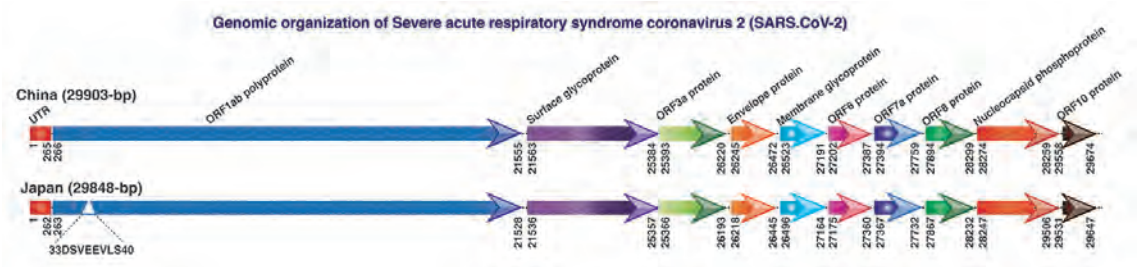

Fig. 1. Genomic organization of severe acute respiratory syndrome-coronavirus 2. ORF, open reading frame. *Source*: Refs 14, 16.

of bat-SL-CoVZC45 and bat-SL-CoVZXC21, there still remain doubts regarding its direct ancestors. It has been hypothesized that game animals, the consumption of which is culturally acceptable in China, may also represent a bridging host which instigated the spread of the virus from bats to human beings[19].

The ICMR (Indian Council of Medical Research)-National Institute of Virology (ICMR-NIV) has carried out extensive data collection from bats, which may provide critical insights for the ongoing spillover event. However, given the current state of the evidence, it remains difficult to say whether this virus will become entrenched, with endemic, seasonal or annual epidemics (like pandemic H1N1 influenza)[20,21], or it would extinguish like SARS. The knowledge base around developing robust signals which can predict or detect the emergence of viruses of this group, or their mutant forms, is still developing. The gaps in the current evidence leave us no choice but to prepare for combatting epidemic spillovers in the years ahead.

Speculations have been rife about the virus being artificially created, however, evidence accumulated by mining the genomic data of the emergent virus has failed to substantiate such claims of a human-modified origin[22,23]. The ambiguity regarding the transmission pathways and intermediate host(s) has further fueled conspiracy theories[24].

Epidemiology of COVID-19

Clusters of COVID-19, first reported from the Wuhan Metropolitan in People's Republic of China, in December 2019, have rapidly assumed a global form[25-28]. The data reported in the current review are based on the real-time updates available through the WHO Situation Reports and the Johns Hopkins University Center for Systems Science and Engineering (JHU CSSE) data visualization site[29] till February 28, 2020. All confidence intervals (CIs) reported here have been computed as the exact central CI of a proportion[30].

As of February 28, 2020, there have been 83,704 confirmed cases of COVID-19 globally, with 2,859 deaths[31]. Most cases (78,824 of 83,704; 0.9416 - 95% CI 0.94 to 0.9433) and deaths (2,790 of 2,859; 0.9758 95% CI 0.9696 to 0.9809) have been reported from mainland China. Of the 36,654 recovered cases reported, 36,268 (0.9895 95%CI 0.9884 to 0.9905) hailed from Mainland China. Outside of Mainland China, most cases were registered in South Korea (2,337 cases), on board the Diamond Princess (705) and Italy (655). The highest number of deaths outside China were reported from Iran (26 deaths), Italy (17 deaths) and South Korea (13 deaths)[31].

COVID-19 remains a highly infectious disease, with reproductive number (R_0) estimates ranging from 1.4 to 3.5. The early WHO estimate of R_0 was 1.4 to 2.5[10]. Preliminary studies, conducted at the beginning of the outbreak, reported higher estimates of R_0, in the range of 2.24-3.58[32]. Two recent estimates place it in the range of 2.0-3.1 and at 3.11 (95% CI 2.39-4.13)[33,34]. All the estimates of transmissibility indicate that self-sustaining human-to-human transmission is the only plausible explanation for the magnitude of the on-going outbreak[35]. The case fatality rate (CFR) of COVID-19 has been seen to be higher in China (2.1%) than outside (0.5%)[29]. Mortality in Wuhan was even higher at 4.9% while it was 3.1 per cent in the Hubei province. A significant proportion of deaths in China (26%) occurred in elderly people, aged over 60 yr. However, at this juncture, when the epidemic is still evolving, temptations to make policy decisions based on mortality data should be reined in[36].

On February 12, 2020, there was a spike in cases, with 14,840 cases reported overnight[29] (Fig. 2). This rapid escalation of numbers was attributed to the fact that in the WHO situation report-24[10], in addition to the laboratory-confirmed cases, clinically diagnosed cases, which accounted for 13,332 (90% of the cases reported overnight), were also added. Previously reported as probable or suspected cases, the introduction of the clinically confirmed case has been reflected in the numbers reported since February 12, 2020.

The high mortality observed in China, at the beginning of the outbreak, was only part of the whole story. The differences could be accounted by missed cases in the initial days, and the effectiveness of critical care protocols and aggressive management techniques utilized outside China[36]. In any case, as epidemiologic experience from outbreak research shows, as long as the epidemic is ongoing, CFR is likely to change, especially as case detection becomes more accurate, and less severe cases are also accounted for[37].

The mean incubation period was 5.2 days (95% CI 4.1-7.0 days) in a study covering 425 cases, and the median incubation period was 3.0 days (range 0-24 days) in another study based on 1,324 cases[25,38]. It might be possible that the single case, with an outlying incubation period of 24 days, was actually a second exposure, rather than a single

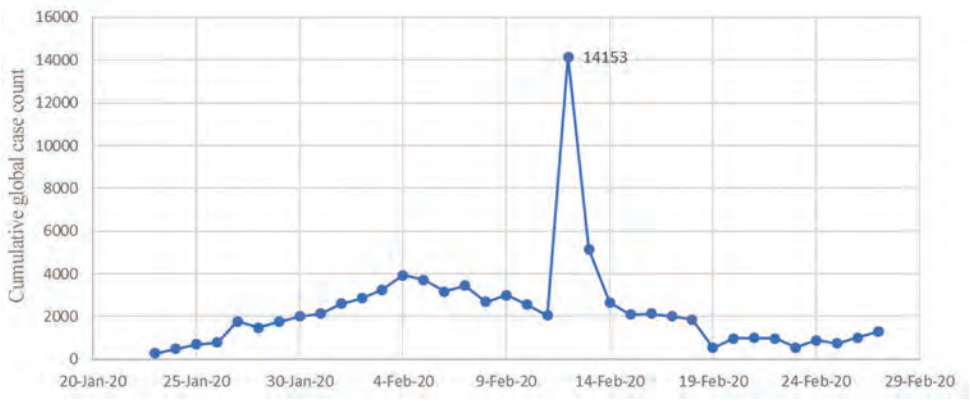

Fig. 2. Cumulative global case counts (February 29, 2020). *Source*: Ref. 29.

infection incubation period. This assertion, made at a WHO Press Conference (link of video: *https://youtu.be/a0Nu5MURFe4?t=2166*), has led WHO to reinforce the current recommendations regarding isolation and quarantine[38].

The incubation period for COVID-19 remains comparable to other recent epidemic viral diseases - SARS (2-7 days)[39] and MERS-CoV (2-14 days)[40], but it is slightly longer than swine flu (1-4 days) and seasonal influenza (1-4 days)[41]. A study looking at 88 cases of importation or travel-related spread estimated the mean incubation period to be 6.4 days (2.1-11.1 days)[42]. The estimates generally show a lot of variance based on sample size and epidemiologic profile of patients, and as more data become available, more accurate estimates are likely to emerge. As of February 28, 2020, COVID-19 has been reported from 58 countries and territories around the world, and one international conveyance, the Diamond Princess Cruise Ship[43].

The first four cases to be identified were linked to the Huanan (Southern China) Seafood Wholesale Market and were picked up by local healthcare facilities running the surveillance programme for 'pneumonia of unknown aetiology'[25,44]. The transmission chain analysis undertaken by WHO indicates that except for 16 cases for whom no clearly established epidemiological link could be identified, all other cases were associated with the ongoing transmission in China[10]. However, given the paucity of the evidence around this group of zoonotic viruses, it remains difficult to predict if the disease will eventually have a seasonal pattern. The four cases needed to set off the epidemic in China may not be representative, especially if the initial cases have been missed. The critical force of infection needed to establish a propagated epidemic is also unknown at this point, although it may be possible to model the effects based on assumptions. This also has direct bearing on the transmissibility of the disease, the specific estimate of which is yet to converge. In addition to the uncertainties around the agent and host factors associated with SARS-CoV-2, there are significant unknown factors about the environmental stability of the virus, and how effective fomites are in its transmission, especially in tropical countries. Bats have been implicated as reservoirs[45], and given their wide flight range in Asia, specific host control is difficult and unrealistic.

Case definitions

Case definitions being used currently are based on the WHO's interim guidance documents[46].

SARI - An acute respiratory infection with a history of fever or measured temperature >38°C, and cough, onset within 10 days and requiring hospitalization.

Surveillance case definitions for SARS-CoV-2 - A person with SARI with no other aetiologies with one of the following: (*i*) History of travel to Wuhan, Hubei Province, China, in the last 14 days; and (*ii*) Patient is a HCW who has been caring for patients with SARI of unknown aetiology.

Patient with acute respiratory illness and at least one of the following: (*i*) Close contact with a confirmed or probable case of SARS-CoV-2 in the 14 days before illness onset; and (*ii*) Worked or attended a health care facility in the 14 days before onset of symptoms where patients with hospital-associated SARS-CoV-2 infections were reported.

A sensitive and specific definition for community-based surveillance remains elusive. The indicators for referral and their outcome impact are yet to be ascertained systematically. Questions around

the need to quarantine children, minimum period of quarantine and its mental and socio-economic costs, relative to the current outbreak, remain poorly explored.

Clinical manifestations

The most common symptoms at illness onset are fever (99%), fatigue (70%), dry cough (60%), myalgia (44%) and dyspnoea[26,27,46]. Less common symptoms are headache, dizziness, diarrhoea, nausea and vomiting[47]. Symptoms such as pharyngeal pain, dyspnoea, dizziness, abdominal pain and anorexia are more likely to be present in patients with severe illnesses[27]. In addition, patients who are elderly, have underlying co-morbidities including hypertension, diabetes, cardiovascular disease and cerebrovascular disease are more likely to have adverse outcomes.

The most common laboratory abnormalities among patients hospitalized with COVID-19 are marked lymphopenia, prolonged prothrombin time, elevated lactate dehydrogenase and elevated D-dimer. These laboratory abnormalities are similar to the ones seen in SARS-CoV and MERS-CoV infections. Bilateral patchy shadows and ground-glass opacities are seen on chest imaging. The most common complications of COVID-19 are acute respiratory distress syndrome, arrhythmias, acute cardiac injury, shock and acute kidney injury[47-49]. The in-hospital transmission of the virus is very high with rates as high as 40 per cent. Of the hospitalized patients, the mortality rate is around 4-5 per cent[47-49]. There is adequate descriptive evidence in the published literature to develop a complete clinical picture of the disease. However, there is a need for planned constructions for providing multidisciplinary care in an integrated, single-service area. Further, designing and building these isolation wards, using humane and helpful esthetics, is also an essential step in empowering health systems to mount an adequate response to the surge in cases.

Diagnosis

Patients who satisfy clinical case definition and are epidemiologically linked to a history of travel from the city of Wuhan in the last 14 days, or have come in contact with a reverse transcription (RT)-PCR confirmed case or with a patient who is under investigation for SARS-COV-2 within the same period, are considered to be suffering from COVID-19[50]. As the asymptomatic transmission of the virus has been established[51,52], persons with epidemiological risk exposure should practice strict adherence to standard precautions and control of contact-based transmission.

Preferred clinical samples for establishing the laboratory confirmation of a suspected case include nasopharyngeal and oropharyngeal swabs collected using Dacron swabs, expectorated sputum, BAL fluid, endotracheal aspirate and tissue. The clinical sample is to be collected in a sterile container with normal saline which covers the sample; serum samples are collected in pairs in red cap vials (plain vials) with clot activators during both the acute phase and the convalescent phase of the illness[53]. For the transportation of samples to the laboratory, the swabs should be placed in a commercially available viral transport medium. The guidelines recommend triple packaging of the sample[54].

Appropriately filling the laboratory request form is vital once a clinical sample is collected from a suspected patient. Information regarding the patient's demographic details, date, time and anatomical site of the sample collection, tests required and the clinical history, symptoms, and risk factors need to be mentioned to mitigate risks of transmission if the sample turns out to be positive. The sample package must be labelled with UN3373 for Category B Biological Substances[55]. The receiving facility must be informed beforehand about the case and the transport of the sample.

The WHO recommends that the culture of the virus must be done in a BSL-3 laboratory and the RT-PCR be done in a BSL-2 laboratory[53,56]. While handling specimens of SARS-COV-2, one must ensure that neither the sample nor the HCW is contaminated to minimize any risks and to ensure accuracy of diagnosis. Isolation of SARS-COV-2 can be done in cells lines and the diagnosis has to be confirmed by RT-PCR. Charité Berlin, from Germany, was the first to develop the assay and standardize the protocol for real time RT-PCR[57]. The test detects the presence of three genes- *E, RdRp* and *N*. This is done in a step -wise process, with the three genes tested in sequence only if the one before is positive.

In laboratory-confirmed case of COVID-19, two samples collected from anatomically distinct sites or two samples collected from the same site during two different days of illness, are positive in two different assays or on repeat PCR[58]. The seroconversion of the disease is seen by detection of antibodies in convalescent phase serum, after a negative result in acute phase serum sample or a four-fold rise in antibody titres between the acute and convalescent phases. Seroconversion can be confirmed by ELISA or indirect fluorescent antibody test (IFA)[59].

Prevention of transmission

SARS-CoV-2 spreads *via* respiratory droplets and physical contact. It is essential to practice precautionary measures to prevent transmission. Standard precautions consist of hand hygiene, use of personal protective equipment (PPE) and respiratory and cough etiquettes. Hand hygiene should be done with alcohol-based hand rubs (ABHRs) containing 60-80 per cent ethanol. Hand washing following the correct steps with soap and water should suffice. Cloth towels should be avoided for drying hands and disposable tissue papers should be preferred. PPE consists of the medical masks or particulate respirators, face shields or goggles, gowns, gloves and shoe covers[60,61]. For droplet and contact-based transmission, medical masks or procedure masks with head straps should suffice. This should be worn before entering the patient area and should be taken off only after leaving the same. It is mandatory for persons in the community settings who are symptomatic, the patients who are in home care setups and suspected cases of COVID-19 with mild respiratory symptoms and healthcare workers (due to their elevated risk of exposure) need to wear medical masks at all times followed by hand hygiene and correct disposal[61]. Particulate respirators (NIOSH-certified N95, EU standard FFP2 or equivalent) should be used by HCWs involved in aerosol-generating procedures (AGPs). Face shields/goggles are to be used by all HCWs while performing AGPs. Long-sleeved, sterile, waterproof gowns, made of non-absorbable materials are to be worn. When gowns are not available, waterproof aprons should be used. Powder-free, latex gloves should be worn while handling infected patient's material. This should not be considered as a replacement of hand hygiene. Shoe covers should also be used in healthcare settings to prevent contamination of clothes. Respiratory and cough etiquettes should be adhered to: covering the nose and mouth while sneezing and coughing, using disposable tissue paper instead of cotton cloth, and if nothing else is available, using the flexed elbow, followed by appropriate hand hygiene.

Symptomatic patients in the community settings should be discouraged from congregating in public or crowded areas. Information, education and communication (IEC) messages should encourage self-deferral and self-containment for patients who are symptomatic. For home care, patients should be placed in a well-ventilated room. In healthcare settings, the patient should be placed in a negative pressure room.

Quarantine

According to WHO, "The International Health Regulations (IHR) are an international legal instrument that is binding on 194 countries across the globe, including all the Member States of WHO. Their aim is to help the international community prevent and respond to acute public health risks that have the potential to cross borders and threaten people worldwide"[62]. The IHR defines "the rights and obligations of countries to report public health events and establish a number of procedures that WHO must follow in its work to uphold global public health security"[62]. In line with the principles outlined in IHR, the Ministry of Health and Family Welfare, Government of India, has issued travel advisories from time to time, considering the surge in cases of COVID-19 in China. The travel advisory states, "Indian travellers are hereby advised to refrain from travelling to China. Existing visas (including eVisa already issued) are no longer valid for any foreign national travelling from China. People travelling to China henceforth will be quarantined on return"[63].

The medium- and long term impact of such travel bans remain to be seen, but modelling studies suggest that in the short-term, these are unlikely to have meaningful impact on global transmission of SARS-CoV-2, unless sustained 90 per cent travel restrictions are implemented in combination with more than 50 per cent reduction in local transmission[64]. Such bans may only provide a symbolic shield unless the ongoing outbreak is staunched. Ethical concerns of imposing such travel bans have also been questioned[65].

Diamond Princess, a cruise ship docked off Yokohama in Japan, was quarantined for two weeks after a tourist who disembarked at Hong Kong tested positive for SARS-CoV-2[66,67]. The cruise ship had over 3,700 passengers and crew, of whom 705 were tested positive for SARS-CoV-2, making it the second largest site of outbreak outside China at one point[68].

On January 23, 2020, the Government of the People's Republic of China imposed a lockdown on Wuhan to quarantine and prevent the spread of the disease[69]. This was a drastic public health measure[65,70]. While the benefits of such a move remain to be seen, the long-lasting negative impacts of such a measure should not be underplayed[71]. Such drastic measures can lead to social, psychological and economic stressors on the whole population, leading to long-lasting adverse health outcomes[72]. Instead of coercive top-down quarantine approaches, which are driven by the authorities,

community and civil-society led self-quarantine and self-monitoring could emerge as more sustainable and implementable strategies in a protracted pandemic like COVID-19[73].

Therapy

Like SARS, SARS-CoV-2 also uses the ACE2 receptor for entry into the cell[74]. This potentially opens up the possibility of using the same therapeutic strategies that were effective in blocking SARS. Currently, there are no definitive, proven treatments, although multiple pharmacological options are being explored. A spate of clinical trials has been initiated in the wake of the outbreak. Some of the trials which have initiated recruitment of patients are looking at the effectiveness of using washed microbiota transplantation[75], remdesivir[76,77], ritonavir-lopinavir combination[78], vitamin C infusion[79], darunavir and cobicistat[80], hydroxychloroquine for pneumonia[81], umifenovir[82] and traditional Chinese medicines[83], to name a few options. The Chinese guidelines of using α-interferon combined with the repurposed lopinavir/ritonavir combination (*Kaletra*) have also been used widely for the treatment of hospitalized patients[84]. Improvement in the first US patient of COVID-19 after treatment with remdesivir[85], and subsequent experience of clinical response in animal models has generated interest in the agent[86].

Treatment of COVID-19 is mostly supportive based on the organ systems affected. The setting of patient management, *i.e.,* intensive care unit or high dependency unit versus general wards, should be decided early on in the course of the disease, considering the high mortality rate among hospitalized patients and the facilities available for containment of infection. Published evidence from preliminary therapeutic experiences indicated that patients requiring hospitalization were managed with broad spectrum antibacterial antibiotics and glucocorticoids[26]. The treatment course may warrant management of respiratory failure with non-invasive ventilation, mechanical ventilation and extracorporeal membrane oxygenation (ECMO). Additional intensive care therapies such as vasopressors and renal replacement therapy may be required while managing SARS-COV-2 infections.

Vaccine

The WHO R&D blueprint and its Working Group conveyed an informal consultation on prioritization of vaccine candidates against SARS-CoV-2 in Geneva on January 30, 2020[87,88] and identified at least five leading candidate vaccines for SARS-CoV-2[89].

As on February 13, 2020, the WHO expert group did not release a prioritization list, nor did the US Clinical Trials registry show any registered clinical trials on vaccines against SARS-COV-2. Among the different candidates in the pipeline, nucleic acids and viral vectored vaccine are being tried. INO-4800 is one of the leading candidates developed by Inovio Pharmaceuticals and Beijing Advaccine Biotechnology based on a DNA plasmid vaccine Electroporation device. Inovio aims to begin phase I clinical trial in the US simultaneously with Beijing Advaccine in China[90]. Clover Biopharmaceuticals is developing a recombinant subunit vaccine based on the trimeric S protein (S-Trimer)[91]. All the vaccine studies are currently in the preclinical phase.

WHO at the core of global response

WHO and the Global Research Collaboration for Infectious Disease Preparedness hosted a two-day meeting at WHO Headquarters in Geneva on February 11-12, 2020, which brought together major research funders and scientists from across the world "to assess the current level of knowledge about the new COVID-19 disease, identify gaps, and work together to accelerate and fund priority research needed to help stop this outbreak and prepare for any future outbreaks"[92]. A Global Surveillance for human infection for COVID-19 has been established by WHO, and globally, 16 laboratories have been identified for confirmatory referral testing. In South-East Asia Region, two laboratories in Thailand - NIH Nonthaburi and Armed Forces Research Institute of Medical Science Bangkok, and one in India - ICMR-NIV, have been identified for referral testing[93].

The WHO has developed interim guidance documents for laboratory diagnosis[53,94], home care for patients with suspected novel CoV[95], advice on the use of masks during home care and in healthcare settings in the context of COVID-19 outbreak[61], clinical management, infection prevention and control in healthcare settings[96], risk communication and community engagement, and global surveillance for human infection with COVID-19[97]. WHO has also developed an online course to provide general introduction to emerging respiratory viruses, including COVID-19, meant for people at large as well as healthcare workers[98]. For quickly setting up emergency isolation and quarantine facilities, WHO has also prepared a disease commodity package that includes an essential list of biomedical equipment,

medicines and supplies necessary to care for patients with COVID-19[99].

The 2003 SARS outbreak was hypothesized to have originated from a mutated coronavirus from small carnivorous animals sold in a live animal market in Guangdong, China[100], the likely source(s) potentially including masked palm civets, raccoon dogs and Chinese ferret badgers[101-103]. Similarly, the 2012 MERS-CoV outbreak was found to have originated from dromedary camels[1,104]. In anticipation that COVID-19 cases might be linked to the exposure to a live wild animal market, the Chinese Government has banned wild animal business on January 21, 2020[19]. These spillover events further highlight the importance of adopting the One Health framework in approaching the pre-emption and prevention of novel and emerging dangerous pathogens[105,106].

HCWs are always exposed to an elevated risk of exposure to infectious diseases and may contribute to the morbidity and mortality, as seen in previous outbreaks of Nipah and Ebola Virus Disease[107-111]. Transmission of infection from asymptomatic patients has been a major concern as exemplified in the incident where a patient undergoing surgery infected 14 HCWs before the onset of fever[112]. An early case series identified that hospital-associated transmission infected 40 healthcare workers, and 17 hospitalized patients, who represented 29 and 12 per cent of all cases in the series, respectively[26]. In addition to the infection threat posed by SARS-CoV-2, the mental health issues of dealing with a lethal infectious disease have also been substantial, with generalized anxiety disorders, depression, poor sleep emerging as major issues[113,114].

In the aftermath of the SARS-CoV-2 outbreak, many countries, including India, initiated the travel bans and visa suspensions, with subsequent reports of incidents of stigma and discrimination against Asians, or Asian-appearing people. This phenomenon has been observed globally[115]. In response, WHO, in collaboration with the International Federation of Red Cross and Red Crescent Societies and United Nations Children's Fund, has developed a guide for preventing and addressing social stigma[116]. In addition, in the statement released by the second meeting of the IHR (2005) Emergency Committee for the Novel Coronavirus outbreak, WHO has cautioned Member States against engendering any policies which promote stigma and discrimination: "Countries are cautioned against actions that promote stigma or discrimination, in line with the principles of Article 3 of the IHR"[9].

Summary

There have been several lessons to glean from the global response to the SARS-COV-2 threat. Most responses have been reactive, with little preparedness investment in health systems and through community engagement and empowerment[117]. However, the emphasis on data sharing, the rapid development and distribution of interim guidance documents by WHO and open-access pre-print sharing of rapidly emerging evidence reflect a paradigmatic shift in providing a data-driven global-epidemic response[118]. This unprecedented effort at providing information to global practitioners has led to a more concerted response, helping to mount international, multi-country, mitigatory actions[119]. However, there have been elements of imposed travel restrictions and red-lining of affected areas, the long-term impacts of which, on sectors such as economy, agriculture and mental health remain to be seen. In this run to devise technological and medical solutions to yet another PHEIC, we have not focussed on opportunities to strengthen health systems and community resilience, through people-centric approaches.

The original source of the outbreak, the intermediate host, an effective treatment regimen, tools for early diagnosis in asymptomatic patients and tools to predict emergence of novel pathogens all remain elusive. Clinical trials have begun to identify vaccines and effective and safe treatment regimens, but efforts to identify drugs that can be repurposed and used, off-label, remain limited. Further, epidemiologic determinants and reservoirs which are likely responsible for the recent explosive case counts in Italy and Iran are yet to be identified.

The response mounted to the COVID-19 threat has largely been reactive. The lack of a reliable Early Warning, Alert and Response System, inability to mount transparent containment measures, lack of community engagement for self-deferral and isolation, and overdependence on quarantining measures have exposed the fissures in the ability of health systems across the world. It has clearly demonstrated the weak preparedness against emerging and re-emerging dangerous pathogens across the world. Despite the enforcement of the IHR (2005), strengthening international capacity to respond to PHEICs remains a hurdle. Further, initiation of militarized control efforts, discriminatory travel restrictions and poor coordination

and planning, has shown the limited ability to handle an outbreak with pandemic potential across the world.

The infectious disease threats of our times are far from over, and if these are to be contained with lower magnitudes of loss to human life and economy, we need to invest in building up people-centric health systems, which pre-empt and prevent, rather than work in reactive, feedback loops driven by the burden of human misery[120,121].

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Review Article

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Perspectives for repurposing drugs for the coronavirus disease 2019

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The newly emerged 2019 novel coronavirus (CoV), named as severe acute respiratory syndrome CoV-2 (SARS-CoV-2), like SARS-CoV (now, SARS-CoV-1) and Middle East respiratory syndrome CoV (MERS-CoV), has been associated with high infection rates with over 36,405 deaths. In the absence of approved marketed drugs against coronaviruses, the treatment and management of this novel CoV disease (COVID-19) worldwide is a challenge. Drug repurposing that has emerged as an effective drug discovery approach from earlier approved drugs could reduce the time and cost compared to *de novo* drug discovery. Direct virus-targeted antiviral agents target specific nucleic acid or proteins of the virus while host-based antivirals target either the host innate immune responses or the cellular machineries that are crucial for viral infection. Both the approaches necessarily interfere with viral pathogenesis. Here we summarize the present status of both virus-based and host-based drug repurposing perspectives for coronaviruses in general and the SARS-CoV-2 in particular.

Key words Coronavirus - COVID-19 - drugs - host-based - repurposing - severe acute respiratory syndrome coronavirus 2 - virus-based

Introduction

Coronaviruses (CoVs) belong to the family *Coronaviridae* and are enveloped, single-stranded, positive-sense RNA viruses[1]. The CoVs are seen to be distributed in mammals as well as in humans causing mild infections. However, the severe acute respiratory syndrome CoV (SARS-CoV) and the Middle East respiratory syndrome CoV (MERS-CoV) from zoonotic sources in 2002 and 2012, respectively, were responsible for high infection and mortality rates[2]. A novel CoV named as SARS-CoV-2, causative agent of the CoV disease 2019 (COVID-19), has caused 750,890 confirmed cases globally with 36,405 reported mortalities[3]. The SARS-CoV-2 belongs to the beta CoV genus which also includes the SARS-CoV-1 and the MERS-CoV. The lack of approved effective drug therapeutic protocols for CoVs would be a challenge for the treatment of the newly emerged COVID-19 infections worldwide.

Drug repurposing, which is defined as identifying alternative uses for approved or investigational drugs outside their defined indication, could be a possible

way to overcome the time limitation of research and development needed to design a therapeutic drug to combat the pathogen[4]. Apart from having a lower risk of failure, most repurposed drugs have cleared phase I trials and require lower investment, but above all, the drug repurposing strategy drastically reduces the time frame for development[5]. The drug repurposing or repositioning approach thus can facilitate prompt clinical decisions at lower costs than *de novo* drug development. Though drug repurposing is sometimes based on chance observations, target-based repurposing of drugs depends on prior understanding of the precise molecular or cellular element that is recognized by the proposed drug[6,7]. The target may or may not essentially have the same mechanism of action in both the diseased states. Antivirals that can target the viral proteins or the key events in the viral life cycle, including virus-host cell interactions, replication, assembly and egress, would belong to this class. Drug repurposing to identify candidate drug compounds centred on the target-based criteria can thus be generally distinguished into virus- and host-based therapeutics. This review outlines the present status of both virus-based and host-based drug repurposing evaluations against the CoVs. The focus would be on the Food and Drug Administration (FDA)-approved marketed drugs or those under clinical trials against the CoVs in general, and the SARS-CoV-2 in particular.

Virus-based drug repurposing for coronaviruses

Virus-based antiviral agents target specific proteins of the virus. The major open reading frame, ORF1ab, of the SARS-CoV genome encodes the large replicase polyprotein pp1ab which forms the non-structural proteins, nsp1-16, while the structural proteins include S, E, M and N[8-10]. The viral replication is facilitated by a replicase complex that involves processing of pp1ab by two cysteine proteases, namely the main protease (Mpro) or the 3C-like protease (3CLpro) and the secondary papain-like protease 2 (PL2pro)[11,12] (Figs 1 and 2). Mpro cleaves at 11 sites in the central and C-terminal regions, while PL2pro cleaves at three sites in the N-terminal regions of the polyprotein. Majority of the proteins and enzymes of CoVs vital for the replication process are potential drug targets.

Main protease (Mpro)/ 3CLpro inhibitors - Lopinavir and/or lopinavir-ritonavir, cinanserin, herbacetin, rhoifolin and pectolinarin

The Mpro is a promising viral target for the design of drugs against SARS/MERS, as the polyprotein cleavage by the Mpro facilitates the formation of the RNA-dependent RNA polymerase (RdRp) and the helicase which are the major proteins of viral replication[8,11,12,22,23]. Various classes of protease inhibitors, such as halomethylketones, phthalhydrazide ketones, α, β-epoxyketones, glutamic acid and glutamine peptides with a trifluoromethylketone group, zinc or mercury conjugates, C2-symmetric diols, peptidomimetic-α, β-unsaturated esters, aldehydes, anilides, nitriles, pyrimidinone and pyrazole analogues, benzotriazole, N-phenyl-2-acetamide and biphenyl sulphone, are reported to inhibit the SARS-CoV-1 Mpro/ 3CLpro[24,25] (Fig. 2). Of these prospective Mpro inhibitors, the common FDA-approved ones are well-known HIV-1 protease inhibitors[26]. Among these, lopinavir and/or a ritonavir-boosted form of lopinavir has been reported to have anti-CoV activity *in vitro* and also has shown improved outcomes in non-human primates infected with MERS-CoV and in non-randomized trials with SARS patients[27]. Both lopinavir and ritanovir are under phase II/III clinical trials for MERS-CoV (NCT02845843)[28]. These are also reported to have activity against HCoV-229E, HCoV-NL63 and animal CoVs[29].

Cinanserin (SQ 10,643) a serotonin antagonist, demonstrated antiviral activity against SARS-CoV-1, and the inhibition of replication was probably by blocking the activity of Mpro[14]. Flavonoids, herbacetin, rhoifolin and pectolinarin that are known to possess antioxidant effects associated with diseases such as cancer, Alzheimer's disease and atherosclerosis were also noted to efficiently inhibit SARS-CoV-1 Mpro[15].

Papain-like protease (PLpro) inhibitor - Disulfiram

Disulfiram, which is an approved drug for the treatment of alcohol dependence, demonstrated *in vitro* inhibition of the PL2pro enzyme of SARS and MERS[30]. The study also provided future directions for the development of fragment-linked inhibitors for improving its potency[31].

RNA-dependent RNA polymerase (RdRp) inhibitors - Ribavirin, immucillin-A/ galidesivir, remdesivir and acyclovir

The RdRp which is critical for CoV transcription and replication is involved in producing the genomic and subgenomic RNAs. Nucleoside analogues such as favipiravir, ribavirin, penciclovir, remdesivir and galidesivir are well-known RdRp inhibitors. A guanosine analogue, ribavirin, showed broad-

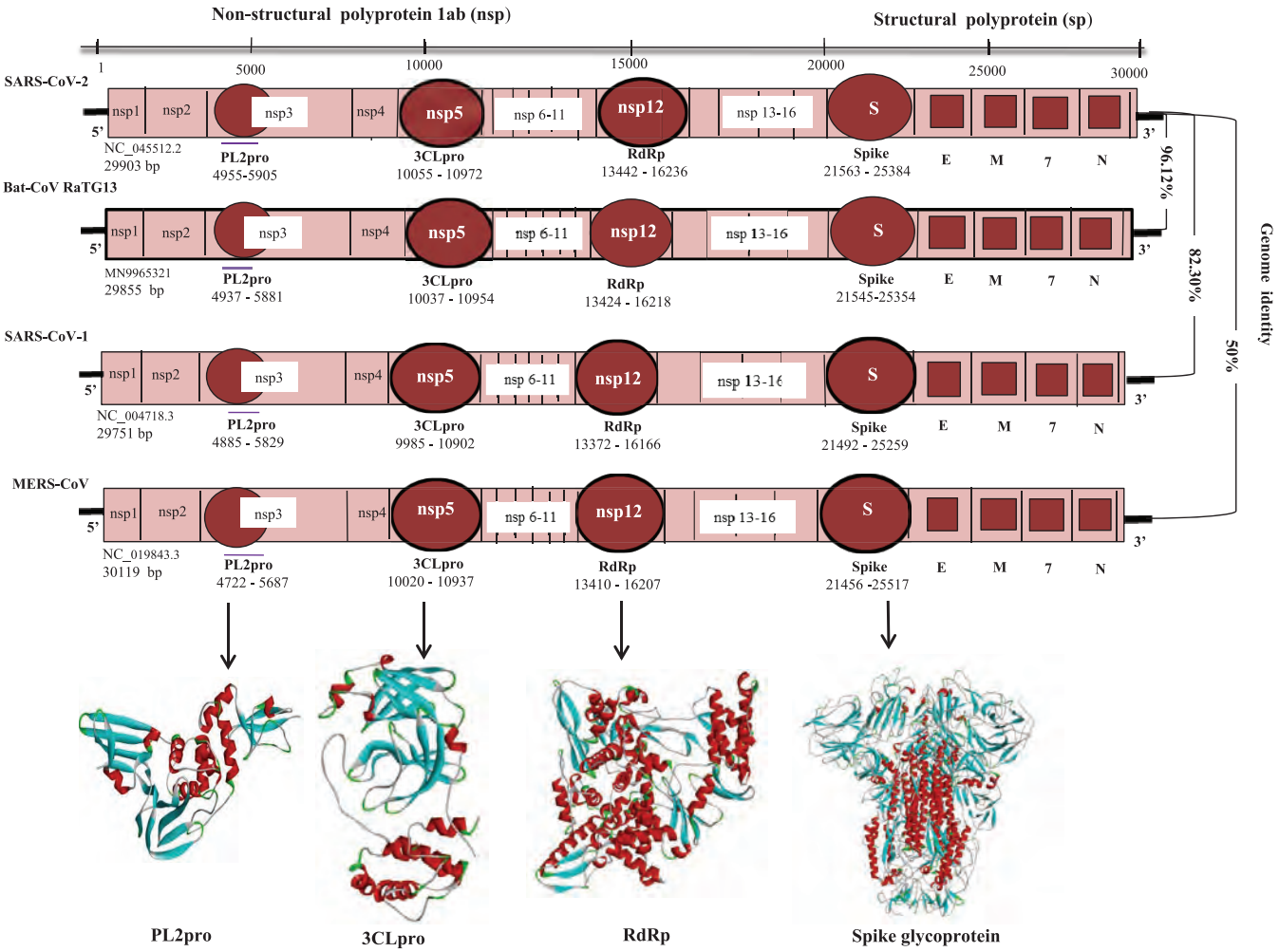

Fig. 1. Schematic representation of the genomic organization of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in comparison with bat-CoV RaTG 13, SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV). Below are the modelled three-dimensional structures of the major virus based antiviral targets [3C-like protease (3CLpro), RNA-dependent RNA polymerase (RdRp) and papain-like protease (PL2pro)] based on SARS-CoV-1 templates obtained from Protein Data Bank. Also depicted is structure of the spike glycoprotein of SARS-CoV-2 released recently (6VSB.pdb). Per cent identity between coding regions of the specific viral genomes depicted was calculated using p-distance method of MEGA software v7.0 (*https://www.megasoftware.net/*). *Source*: Refs 9, 13.

spectrum antiviral activity against several viruses including respiratory syncytial virus, hepatitis C and E viruses (HCV, HEV), chikungunya and viral haemorrhagic fevers[32,33]. Though the mechanism of action is not fully understood, it is hypothesized that the drug may be involved in the inhibition of mRNA capping or viral RNA synthesis. The *in vitro* antiviral activity of ribavirin was demonstrated against SARS-CoV-1 and MERS-CoV[34] and in rhesus monkeys infected with MERS-CoV[35]. The drug has been used in the treatment of SARS and MERS patients, though the benefits are ambiguous. Further, in severely infected CoV patients, there could be side effects associated with high doses[36].

Immucillin-A (galidesivir), an adenosine analogue, has been shown recently as a broad-spectrum RdRp inhibitor against several RNA viruses, such as paramyxoviruses, flaviviruses, togaviruses, bunyaviruses, arenaviruses, picornaviruses, filoviruses and also against SARS/MERS-CoVs[37]. Though it has been reported as a treatment option during the 2014-2016 West Africa Ebola virus epidemic, no data for animal/human were reported for CoVs until recently for the SARS-CoV-2[16].

Sheahan *et al*[38] showed that another nucleoside analogue, remdesivir (GS-5734), presently under clinical trials for the Ebola virus, demonstrated inhibition of the replication of SARS-CoV-1 and

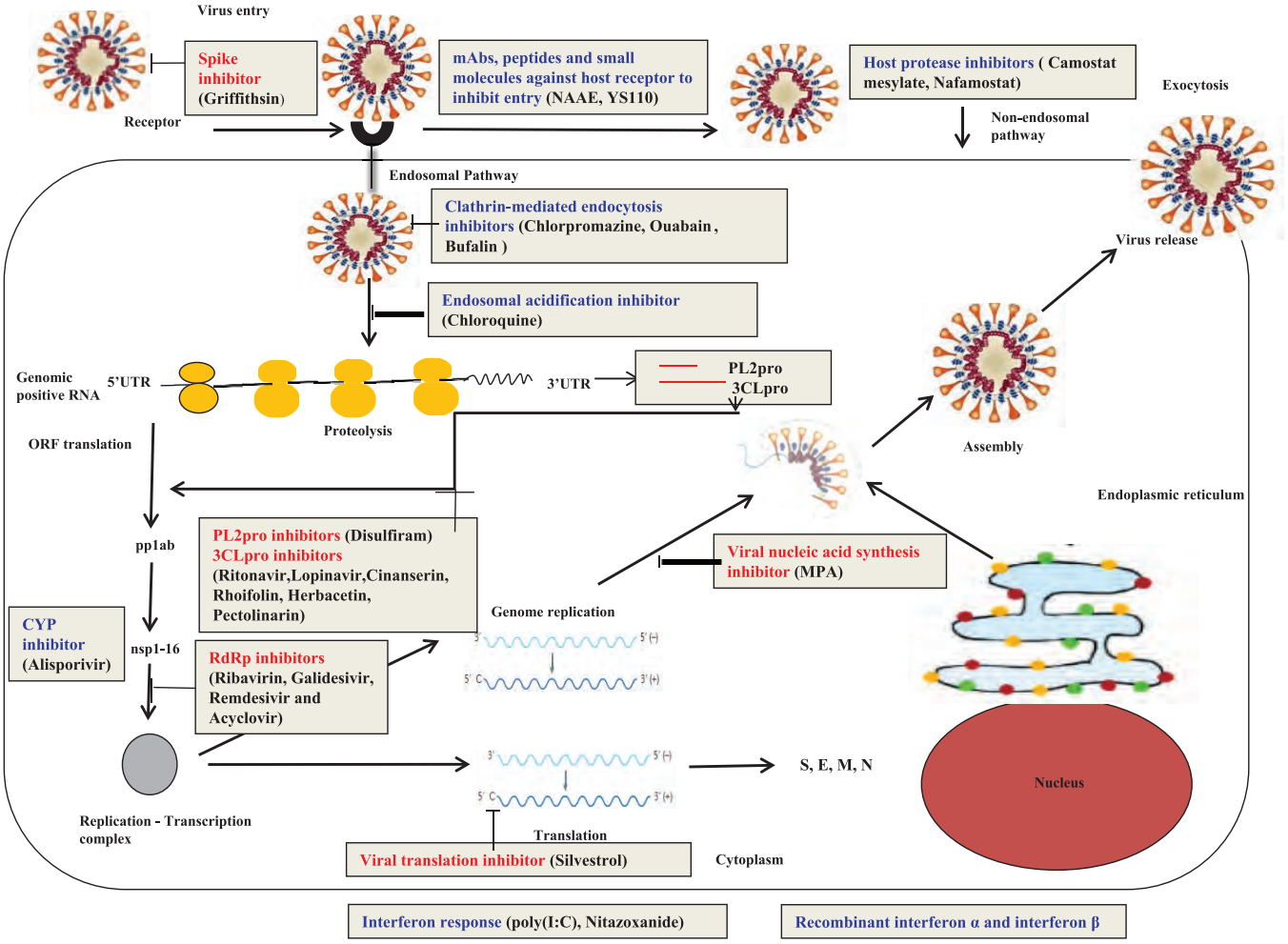

Fig. 2. Schematic representation of the coronavirus replication cycle depicting the potential therapeutics against different virus-based (red) and host-based (blue) targets for coronavirus drug repurposing. The drugs effective against the various targets are mentioned in the brackets. 3CLpro, cysteine-like protease; PL2pro, papain-like protease; nsp, non-structural protein; RdRp, RNA-dependent RNA polymerase; pp1ab, polyprotein ab; M, membrane protein; E, envelope protein; S, spike protein; N, nucleocapsid protein; UTR, untranslated region; ORF, open reading frame; MPA, mycophenolic acid; ERK-MAPK, extracellular signal-regulated kinase mitogen-activated protein kinase; poly(I:C), polyinosinic: polycytidylic acid; NAAE, N-(2-aminoethyl)-1-aziridine-ethanamine; YS110, a recombinant humanized IgG1 anti-DPP4 mAb; DPP4, dipeptidyl peptidase 4; CYP, cyclophilin. *Source*: Refs 8, 10, 14-21.

MERS-CoV in primary human airway epithelial cells. They also demonstrated broad-spectrum anti-CoV activity against bat-CoVs and human CoVs in primary human lung cells[17,38]. In another recent study, remdesivir was shown to possess better *in vitro* antiviral efficacy against MERS-CoV in comparison to lopinavir and ritanovir[17,39]. In mice, remdesivir improved pulmonary function with lower viral loads in the lungs both as a prophylactic and as a therapeutic[17,40].

Another nucleoside analogue, acyclovir that was modified by incorporating fleximers to increase its binding affinity has been reported to be effective *in vitro* against MERS-CoV and HCoV-NL63[39,41], though to the best of our knowledge, no animal or human data are available.

Inhibitors of spike glycoprotein - Griffithsin

CoVs possess a surface structural spike glycoprotein (S) which is vital for interaction with the host cell receptor and subsequent virus entry into the cell. The S protein constitutes two subunits, the S1 (receptor-binding) and the S2 (membrane fusion) domains[40]. Griffithsin, a lectin extract red algae, has been reported to bind to oligosaccharides on the surface of various viral glycoproteins, including HIV glycoprotein 120 and SARS-CoV glycoproteins[41].

Other inhibitors with unknown site of action - Resveratrol, amodiaquine, mefloquine, loperamide

Resveratrol, a natural compound from grape, which is in a clinical phase for heart and other diseases, was also reported to effectively inhibit MERS-CoV *in vitro* by downregulation of the apoptosis induced by the virus[42]. The possible site of action was suggested to be the nucleocapsid protein. Amodiaquine and mefloquine, antimalarial drugs, were also found to be effective against MERS-CoV[43]. Loperamide, an antidiarrhoeal agent that was identified by the screening of an FDA-approved compound library, showed *in vitro* antiviral activity against MERS[44].

Inhibitors of viral nucleic acids - Mycophenolic acid

Viral nucleic acids are mainly composed of nucleosides and nucleotides. The drugs that target these have mycophenolic acid (MPA) as the active compound and inhibit inosine monophosphate dehydrogenase and guanine monophosphate synthesis[45]. Broad-spectrum activity has been reported by MPA against a broad range of viruses including orthohepadnaviruses (hepatitis B), flaviviruses (HCV), arboviruses and CoVs. MPA possessed anti-MERS-CoV activity *in vitro*, though it was shown to result in a worsened outcome in the marmoset primate model[26]. Treatment of renal transplant recipients with MPA resulted in severe MERS[46]. Combination therapy with interferon beta-1b (IFN-β-1b) was, however, reported to be synergistic *in vitro*[47], implying that monotherapy with the drug might not be useful for treating CoVs.

Host-based drug repurposing for coronaviruses

Specific host factors are utilized by CoVs for entry and replication. The anti-CoV potential of monoclonal antibodies (mAbs) evoked against the receptor binding domain (RBD) of S1 subunit and fusion inhibitors which target the S2 subunit has been reported in *in vitro* and/or *in vivo* studies[48-50]. SARS-CoVs and HCoV-NL63 preferably utilize the angiotensin-converting enzyme 2 (ACE2) host receptor while dipeptidyl peptidase 4 (DPP4) is used by MERS-CoV[51,52] for entry. The further entry of CoVs into host cells includes the cell surface and/or endosomal pathways which are *via* host proteases such as transmembrane protease serine 2 (TMPRSS2) that cleave and activate viral S protein[53]. Inhibitors of these host proteases can prevent this proteolytic cleavage, partially blocking cell entry. Further, a group of drugs can target the endocytosis or cell entry[44] (Fig. 2).

The innate IFN response of the host also has therapeutic potential as it controls viral replication after infection[18,54]. Additional pathways of cell signalling have also been noted as possible therapeutic targets for CoVs[55]. These classes of inhibitors are discussed below.

Inhibitors targeting endocytosis or cell entry - Chlorpromazine, ouabain, bufalin, chloroquine

Chlorpromazine, an antipsychotic/tranquilizer drug, is also known to affect the assembly of clathrin-coated pits at the plasma membrane[44]. It showed broad-spectrum *in vitro* activity against viruses such as HCV, alphaviruses, SARS-CoV-1 and MERS-CoV. Ouabain and bufalin, examples of a class of steroids which bind sodium- or potassium-transporting ATPase subunit α1, also inhibited the endocytosis of MERS-CoV mediated by clathrin[56]. However, very high EC_{50}/C_{max} (half-maximal effective concentration value/peak serum concentration level) ratios at the typical dosages or toxicity, limit the clinical use of these endocytosis inhibitors. Acidification of the endosome can also affect endocytosis. Chloroquine, an antimalarial drug, can increase the intracellular *p*H by directing protons into the lysosomes[57]. It possesses broad-spectrum *in vitro* antiviral activities against flaviviruses, HIV, Ebola, Nipah and numerous CoVs[58]. However, it did not show activity in SARS-CoV-infected mice[59]. The anti-CoV activity of different endocytosis inhibitors thus need further *in vivo* evaluation.

Inhibitors of host receptor mediated viral entry - N-(2-aminoethyl)-1-aziridine-ethanamine (NAAE), peptides, mAb YS110

Specific peptide inhibitors and monoclonal or polyclonal antibodies can be used to target the host receptor[48]. N-(2-aminoethyl)-1-aziridine-ethanamine, a small-molecule inhibitor and synthetic ACE2-derived peptides showed inhibition of ACE2 activity and cell fusion *via* the S protein of SARS-CoV-1 *in vitro*[60,61]. However, these inhibitors have not been tested in CoV patients. Monoclonal antibodies (mAbs) such as anti-dipeptidyl peptidase 4 (DPP-4) have also been reported to block cell entry of MERS-CoV *in vitro*[62]. YS110, an anti-DPP4 recombinant humanized IgG1 mAb, used in a phase I clinical trial, was found to be well tolerated in patients with advanced malignancies[19]. However, considering that host cell receptor usage differs in different CoVs, the anti-CoV activity of these agents may be narrow-spectrum. Further, based on the vital biological functions of these receptors, the risks of immunopathology such as

blood pressure regulation, glucose metabolism *etc*., would need assessment[52].

Inhibitors of host proteases used for viral entry - Camostat mesylate, nafamostat

Camostat mesylate, a synthetic serine protease inhibitor, that is used to treat patients with chronic pancreatitis, works against the serine protease TMPRSS2[63,64]. It has shown broad-spectrum activity against enveloped RNA viruses such as CoVs and paramyxoviruses. Camostat mesylate is reported to inhibit SARS and MERS in *ex vivo* studies and improves the survival of mice infected with SARS[64,65]. Nafamostat, another serine protease inhibitor used to treat disseminated intravascular coagulation and pancreatitis, blocked MERS-CoV infection by inhibiting TMPRSS2 in human airway epithelial Calu-3 cells[65,66].

Enhancers of host innate immune response - Interferons, polyinosinic: polycytidylic acid [poly(I:C)] and nitazoxanide

Though on viral infection suppression of the IFN response is an integral part for immune evasion, several viruses and CoVs are noted to be susceptible to IFN treatment. The effectiveness of recombinant IFN-β over IFN-α has been demonstrated by *in vitro* studies against both SARS and MERS[67]. IFN-α mediated reduction of viral titres was observed in SARS-CoV-infected *in vivo* models[35,59], while IFN-β administration *via* different routes was found to be effective in MERS-CoV *in vivo* models[26]. Combinations of IFN-α/β, ribavirin and lopinavir/ritonavir-boosted lopinavir for treatment of SARS/MERS patients, demonstrated varying benefits[33,36,68].

Another type I IFN enhancer, polyinosinic: polycytidylic acid [poly(I:C)], a dsRNA synthetic analogue, demonstrated reduction in viral load in MERS-CoV-infected BALB/c mice[69]. In phase II clinical trials, poly (I:C) was shown to be beneficial for patients suffering from malignant gliomas[70].

Nitazoxanide, a synthetic derivative of nitrothiazolyl-salicylamide which is used as a treatment for parasitic infections, is an effective type I IFN inducer[71]. It has been shown to exhibit antiviral activities against several viral families and canine CoVs. Nitazoxanide was found to be safe in phase II and III clinical trials against HCV and influenza[72].

Inhibitors of signaling pathways involved in viral replication - Cyclosporine, trametinib and others

Drugs interfering with the viral replication signaling pathways are noted to have broad spectrum activity against several viruses such as HCV, HIV, vesicular stomatitis virus, human papilloma virus, vaccinia virus and CoVs[55]. Cyclosporine, a calcineurin pathway inhibitor, inhibited a broad range of CoVs *in vitro* by interacting with the nsp1 protein and modulating immune response mediated by T cells[73]. The clinical application of this drug is, however, restricted due to immune-suppressive effects and a higher EC_{50}/C_{max} ratio at standard dose levels. Other calcineurin inhibitors such as alisporivir, have demonstrated activity against HCoV-NL63[20].

The extracellular signal-regulated kinase (ERK) pathway mediates intracellular signals from membrane-associated Ras to the cytoplasmic kinase cascade Raf, Mek and Erk[74]. The kinase signaling pathway inhibitors, such as trametinib (Mek inhibitor), selumetinib (Erk inhibitor), everolimus, rapamycin, dasatinib and imatinib have also demonstrated anti-CoV effects through inhibition of early viral entry or post-entry events[75]. However, their toxicities may be a concern in severe infections.

Targeting viral translation - Silvestrol

Initiation of translation in many viruses happens through the usage of the host eukaryotic initiation factors (eIFs)[76]. The helicase eIF4A unwinds 5′-untranslated region of the mRNA, facilitating assembly of the translation pre-initiation complexes. A natural compound, silvestrol, being an inhibitor of eIF4A and reported to show anti-cancer activity[77], demonstrated inhibition of MERS-CoV and HCoV-229E translation and replication in MRC-5 lung fibroblast cells[78].

Current perspectives for COVID-2019

Comparison of the coding regions of SARS-CoV-2 showed that it possessed a similar genomic organization when compared to bat-SL-CoVZC45 and SARS-CoV-1[9] (Fig. 2). Sequence analysis further revealed good sequence identity with the bat and human CoVs in the different coding regions. Except for the spike glycoprotein of SARS-CoV-2 that differs from the other CoVs including SARS-CoV-1 spike protein[13,79], the catalytic pockets in the

major non-structural viral enzymes are conserved at both the sequence and protein structural level across CoVs. Hence, repurposing of the promising MERS and SARS inhibitors for SARS-CoV-2 is a practical strategy[16].

In vitro evaluations to test the antiviral potency of marketed drugs ribavirin, penciclovir, nitazoxanide, nafamostat, chloroquine and broad-spectrum RdRp inhibitors, remdesivir (GS-5734) and favipiravir (T-705) against SARS-CoV-2 were recently undertaken[80]. The findings have shown that remdesivir and chloroquine are more efficacious in comparison to the others. A patient from USA with COVID 2019 who was treated with remdesivir intravenously was reported to have recovered[81]. Phase III trials (NCT04252664, NCT04257656) of intravenous remdesivir are currently ongoing to assess the efficacy in patients with SARS-CoV-2. Chloroquine is under an open-label trial for SARS-CoV-2 (ChiCTR2000029609). In addition, randomized clinical trials have been initiated for SARS-CoV-2 with favipiravir (ChiCTRChiCTR2000029544, ChiCTR2000029600) and ribavirin in combination with pegylated IFN (ChiCTR2000029387).

Results following rapid sequencing of the SARS-CoV-2, combined with molecular modelling based on homologous templates[82] have identified certain compounds along with lopinavir and ritonavir that may be efficacious. Phase III clinical trials have also been initiated to test the HIV protease inhibitors including lopinavir (NCT04252274, NCT04251871, NCT04255017, ChiCTR2000029539), ritonavir (NCT04251871, NCT04255017, NCT04261270), darunavir and cobicistat (NCT04252274) in patients infected with SARS-CoV-2[21]. Another HIV protease inhibitor, ASC09F, in combination with oseltamivir is also in phase III clinical trial for SARS-CoV-2 (NCT04261270).

Arbidol (Umifenovir), a wide-spectrum antiviral drug inhibiting several flaviviruses and influenza viruses, whose mechanism of action is based on blocking crucial steps in virus- host cell interactions[83], is under phase IV clinical trial for SARS-CoV-2 (NCT04260594, NCT04254874, NCT04255017). Oseltamivir, an influenza neuraminidase inhibitor[84] is also under phase IV trial for SARS-CoV-2 (NCT04255017).

In the direction of host-based treatment strategies, randomized trials are underway for SARS-CoV-2 using recombinant IFNs (NCT04251871, ChiCTR2000029638)[19]. In another study, an artificial intelligence-based knowledge graph comprising systematically curated medical data, was searched for approved drugs against SARS-CoV-2[85]. Baricitinib, a janus kinase inhibitor, that was consequently identified, is a high-affinity AP2-associated protein kinase 1-binding drug which also interacts with a kinase regulator of endocytosis. Baricitinib has thus been suggested as a potential treatment for COVID-19 disease as it has the ability to reduce viral infection in lung cells.

Molecular docking studies undertaken

We analyzed the binding potential of HIV-1 protease inhibitors, lopinavir and ritanovir against the 3CLpro of SARS-CoV-2, using computational docking studies. This would help gain insight into the molecular mode of action of these drugs which are under clinical trials against the SARS-CoV-2 and also estimate the comparative inhibitory potency of the FDA-approved HIV protease inhibitors to the SARS-CoV-2.

The Mpro of CoVs cleaves substrates by recognizing the sequence motif (small)-X-(L/F/M)-Q↓(G/A/S)-X (X → any amino acid; ↓ cleavage site) and specifically the P1 site of the substrate requires a Gln (Q)[86,87]. The X-ray structure of SARS-CoV-1 3CLPro dimer bound with aza peptide epoxide (APE) as an inhibitor, (2A5K.pdb) was used for the modelling studies. The peptide showed major specificity to the S2 subsite and partial specificity to the S4 subsite of 3CLpro[12]. We detached the APE from the crystal structure complex and re-docked it computationally using the same protocol as for the two selected study inhibitors to obtain the docking score and it was found to be −8.27 Kcal/mol. The two inhibitors in this study had better binding potential (Fig. 3) when compared to APE. Comparison of the docked poses reveals that lopinavir occupies the S1' and S1 subsites with excellent complementarity while ritanovir occupies the S3 and S4 subsites with excellent complementarity through the benzene and 2' isopropyl thiozole groups respectively. These structural features indicate the possible mechanism by which these inhibitors can block the function of the SARS-CoV-2 3CLpro. The peptide substrate cleavage sites for SARS-CoV 3CLpro are noted to be at P1↓ P1' and P3↓P4[88,89], the occupancy at the respective active site cavities would be crucial for competitive inhibition of the polyprotein substrate.

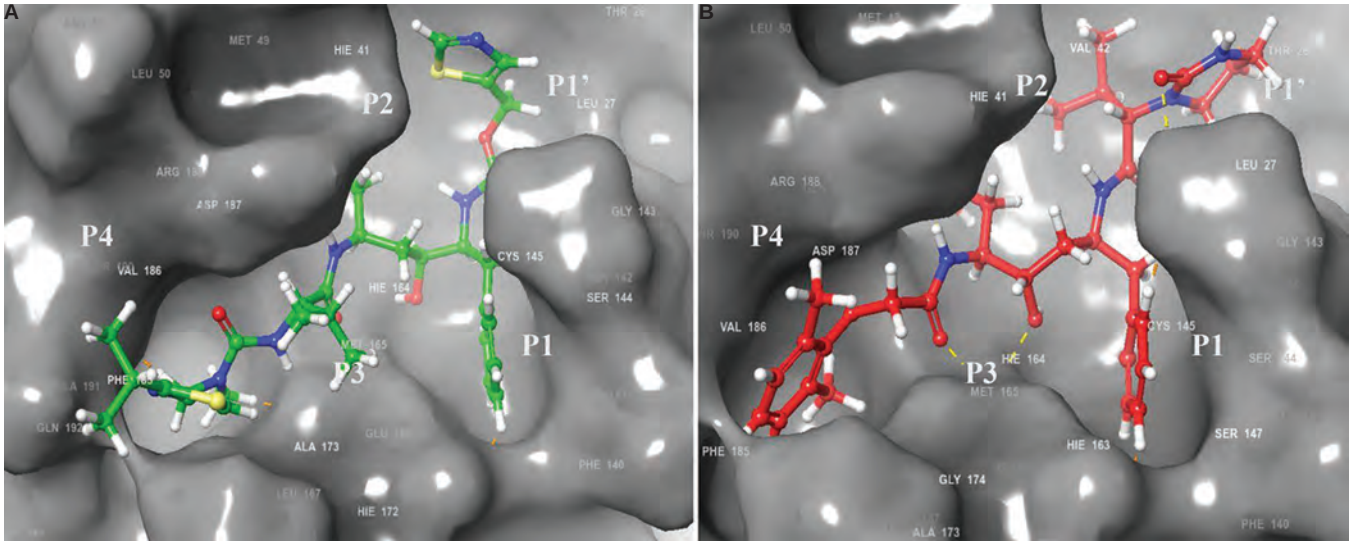

Fig. 3. Docking interaction analysis of HIV inhibitors in the substrate binding cavity of modelled severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease (3C-like protease). (**A**) ritonavir (Docking score = −11.29 kcal/mol); (**B**) lopinavir (Docking score = −9.6 kcal/mol). The P4–P1' side chains of the inhibitor are labelled. Comparison of the docked poses of the inhibitors reveals that the occupancy at the respective active site cavity subsites corresponding to P1' and P1 for lopinavir and P3 and P4 for ritanovir that are crucial for competitive inhibition of the polyprotein substrate, are good.

Based on this requirement, the findings are suggestive that ritonavir and lopinavir may have good potential for repurposing as SARS-CoV-2 protease inhibitors. Molecular dynamics simulation studies for the complexes obtained in this study would be essential to identify specific interactions between the enzyme and drug in the stable complexes and observe the hydrogen bond pattern, especially in the presence of solvent molecules. Additionally, studies need to be undertaken for the binding analyses of the other protease inhibitors, specific RdRp inhibitors and inhibitors of other enzymatic targets. The results would help gain an in-depth understanding of the relative binding affinity and design of derivatives with greater binding potential at the enzyme active site.

Conclusions

This review presented the information with respect to repurposing of FDA-approved drugs as well as those under clinical trials for SARS-CoV-1 and MERS-CoVs, wherein a lot of effort had gone in during the last decade or more. This knowledge has in fact, formed the basis for efforts towards drug repurposing for the SARS-CoV-2 as well. As highlighted in this review, phase III clinical trials of a few drugs have been initiated, though most of these are notably targeting the virus directly, essentially the RdRp or the chymotrypsin-like protease 3CLpro. The spike glycoprotein also needs be explored as a target for the SARS-CoV-2 as the S1 domain of this virus deviates from the other human CoVs. It is thus important that the spike protein should be considered as a potential SARS-CoV-2 therapeutic target. On the other hand, considering that the strategy of targeting viral proteins is vulnerable to the emergence of viral resistance, other coronavirus targets such as the papain-like protease, helicase *etc*., also need to be attempted for drug repurposing. Further, several more of the potential SARS and/or MERS host-based inhibitors should be assessed against SARS-CoV-2. The ongoing vigorous efforts would help develop broad-spectrum anti-CoV agents against SARS-CoV-2.

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Review Article

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Quick Response Code:

Biorisk assessment for infrastructure & biosafety requirements for the laboratories providing coronavirus SARS-CoV-2/(COVID-19) diagnosis

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Novel coronavirus infection [coronavirus disease 2019 (COVID-19)] has spread to more than 203 countries of various regions including Africa, America, Europe, South East Asia and Western Pacific. The WHO had declared COVID-19 as the global public health emergency and subsequently as pandemic because of its worldwide spread. It is now one of the top-priority pathogens to be dealt with, because of high transmissibility, severe illness and associated mortality, wide geographical spread, lack of control measures with knowledge gaps in veterinary and human epidemiology, immunity and pathogenesis. The quick detection of cases and isolating them has become critical to contain it. To meet the increasing demand of the diagnostic services, it is necessary to enhance and expand laboratory capabilities since existing laboratories cannot meet the emerging demand. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a BSL-2 (Biosafety Level 2) agent and needs to be handled in biosafety cabinet using standard precautions. This review highlights minimum requirements for the diagnostic laboratories opting testing of material for the diagnosis of COVID-19 and associated biorisk to the individuals and to the community.

Key words Biorisk - biosafety - diagnosis - infrastructure - laboratories - novel coronavirus

Introduction

Coronaviruses are enveloped viruses with non-segmented positive-sense RNA, widely distributed in humans and animals[1,2]. Initially, infections caused by several human coronaviruses (HCoVs) were only mild and hence were considered as neglected pathogens. After the emergence of highly pathogenic severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) (2002 and 2003) and Middle East respiratory syndrome coronavirus (MERS-CoV, 2012)[3], it has become obvious that coronaviruses can cross the species barrier and cause life-challenging infections in human, thus needing greater attention than the initial HCoVs[4].

Recently, another pathogenic HCoV was identified in Wuhan, People's Republic of China, which was initially named as 2019 novel

coronavirus (2019-nCoV)[5]. But later, it has been named as SARS-CoV-2 by the International Committee on Taxonomy of Viruses (ICTV)[6]. This virus causes coronavirus disease 2019 (COVID-19). The clinical presentation of infection ranges from asymptomatic to very severe pneumonia with acute respiratory distress syndrome, septic shock and multi-organ failure resulting in death[7]. As of March 2020, SARS-CoV-2 has spread to more than 203 countries of various regions including Africa, America, Europe, South East Asia and Western Pacific alarming public health authorities around the world[8].

Since, the first case reported on December 31, 2019, the WHO has been notified with more than 100,000 confirmed cases including 3,380 deaths globally as on March 6, 2020, of whom 90 per cent (3,045) were from China itself, while the remaining 10 per cent (335) were from other countries[9]. The SARS-CoV-2 is now one of the top-priority pathogens to be dealt with, because of high fatality rate in severe cases, spread in a wide geographical area, lack of control measures and knowledge gaps in its epidemiology, immunity and pathogenesis. Currently, there are no licensed vaccines or therapies specific to COVID-19. Hence, the WHO has initially declared COVID-19 as the global public health emergency[10] and subsequently as pandemic[11].

Because of the rapid spread of this virus, it has become necessary to enhance laboratory capabilities to provide immediate diagnostic assistance as the load of samples from suspected patients is increasing on a daily basis and existing laboratories cannot meet the demands. The objective of this review is to highlight various requirements for the diagnostic laboratories involved in the testing of SARS-CoV-2 and also describe measures to mitigate the risk factors involved in laboratories that are providing molecular diagnosis, so that more laboratories become available for providing quick diagnosis under all safety precautions.

Sample of choice

Coronaviruses are mainly responsible for respiratory tract infections resulting in symptoms such as common flu. Choice of sample for detection will be respiratory samples including clinical material from the upper and lower respiratory tracts depending on the symptoms and condition of the patient[12,13]. For SARS-CoV-2, shedding patterns are not well understood and further investigations are required to understand the timing, compartmentalization and magnitude of virus shedding. However, the virus may be detectable in other specimens including blood and urine as in cases of SARS-CoV-1 and MERS-CoV[14-16]. The mean incubation period for SARS-CoV-2 is 5.2 days; however, it may vary widely depending on severity of illness[17].

Specimen collection

Only trained staff should be allowed for appropriate specimen collection, storage, packaging and transport, ensuring that adequate standard operating procedures in consonance with the national or the WHO guidelines[18] are in use, and all specimens should be treated as potentially infectious.

Diagnosis

Suspected cases should be tested for the virus with nucleic acid amplification tests, such as real-time reverse transcription - polymerase chain reaction (RT-PCR) with confirmation by nucleic acid sequencing when needed. Viral RNA extraction should be done in a biosafety cabinet in a BSL-2 or equivalent facility, which will be used further for amplification of genes targeted including nucleocapsid (*N*), spike (*S*), envelope (*E*), and RNA-dependent RNA polymerase (*RdRp*)[18]. Serological tests are still under development, and once these become available, field surveys will aid in better understanding of the outbreak, implementation of control measures and also understanding cross-reactivity with other viruses.

Infrastructure needed

Suspected samples should be handled at initial phase in a biosafety cabinet by well-trained staff with respect to standard BSL-2 facility. National guidelines on laboratory biosafety should be followed in all circumstances[19]. At present, very limited information is available on the risk posed by COVID-19, therefore, all procedures should be undertaken based on risk assessment.

Specimen handling for molecular testing of COVID-19 would require BSL-2 or equivalent facilities. These facilities include separate hand and eye wash sinks, and these also need to have automatic door locking systems. The BSL-2 laboratories should have access to facility of decontamination, including an autoclave[19].

It is recommended that good microbiological laboratory practices and universal precautions must be followed in all laboratories where primary specimens

(such as sputum, throat swab, nasopharyngeal swab, oropharyngeal swab, and stool) that may contain SARS-CoV-2 virus, are handled. While working with suspected patient's samples, laboratory personnel should be supervised by staff competent in handling infectious agents and related standard procedures[19]. The list of basic laboratory equipment and reagents required for providing laboratory diagnosis for COVID-19 is provided in Table I.

Table I. List of laboratory equipments and reagents required for laboratory diagnosis of coronavirus disease 2019 (COVID-19) and personal protective equipment (PPE) for carrying out COVID-19 molecular test

Sr. No.	Details of PPE and equipment
1.	Disposable, back closure laboratory gowns
2.	Face mask and head cap
3.	Disposable gloves
4.	Closed-toe footwear
5.	Protective eyewear
6.	Protective laboratory coats
7.	Disposable shoe covers
8.	Centrifuge tube 15 ml sterile (250 tubes/pack)
9.	Centrifuge tube 50 ml sterile (150 tubes/pack)
10.	Microcentrifuge tube (1.5 ml)
11.	Micropipettes of variable volumes
12.	Sterilized filter tips
13.	Vortex
14.	Mini spin
15.	Small high-speed centrifuge for RNA extraction process
16.	Cold centrifuge for sample processing
17.	Plate spinner
18.	Real-time PCR machine
19.	Biosafety cabinet class 2 type II

Biosafety measures

In most cases, SARS-CoV-2 is transmitted from human to human through inhalation or deposition on mucosal surfaces of large respiratory droplets. Other routes identified are contact with contaminated fomites and inhalation of aerosol, generated during handling of large volumes, *etc*[20]. For the laboratories involved in the diagnosis of COVID-19, it is necessary that staff should be well trained in the implementation of appropriate biosafety measures. The rational, correct and consistent use of available personal protective equipment (PPE) and appropriate hand hygiene help to reduce the spread of the pathogens. Though PPE is considered as a primary prevention strategy, it should not be completely relied upon for complete prevention for virus transmission. The effectiveness of PPE depends upon proper handling of PPEs by trained staff, hand hygiene practices and human factor[21-24]. Immunization policy for influenza would also help in

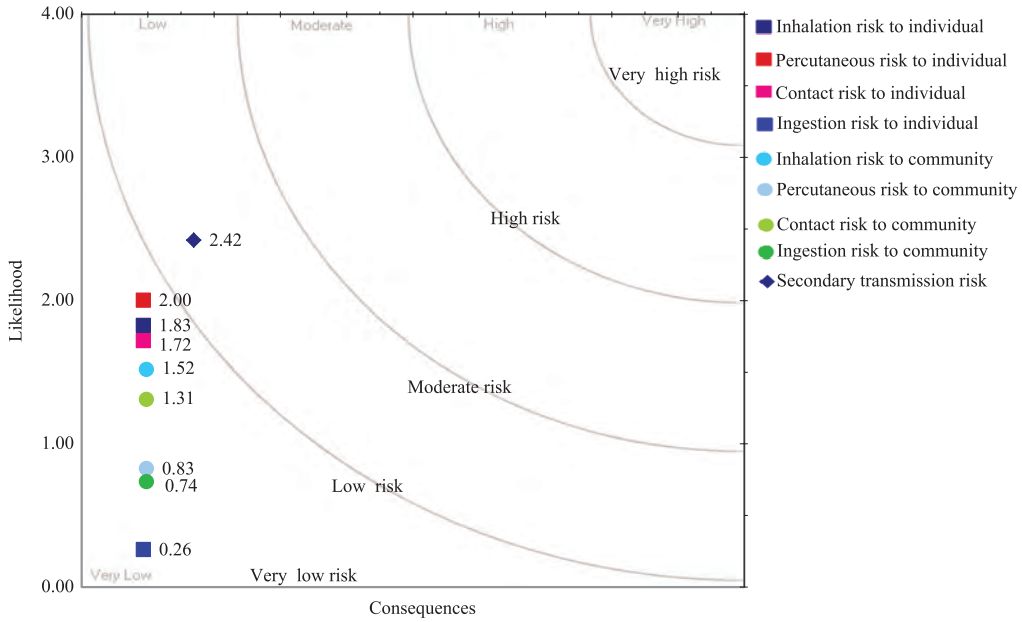

Figure. Biorisk assessment for individual laboratory personnel and community with regard to providing molecular diagnosis for coronavirus disease 2019. Likelihood of secondary transmission to human is on moderate risk, in case laboratory personnel get infected while handling infected material.

Table II. Basic biosafety requirements for the laboratories (some important features of procedures and processes to be followed during processing samples for coronavirus disease 2019 laboratory diagnosis)

Sr. No.	Requirements
1.	Personnel wear dedicated laboratory clothing (*e.g*., scrubs) which should not be worn outside the laboratory, anteroom or change room
2.	Primary containment devices should always be used in this procedure, these should be validated/certified and well-maintained and there are procedures in place for proper use
3.	Type of material to be used in this procedure for diagnostic samples should be up to 250 ml volumes. Absorbent materials should be used on the bench or BSC to contain spills and reduce splashing
4.	Proper practices for reducing/eliminating aerosols should be identified in the laboratory procedures; should be taught and verified on a regular schedule
5.	The measures should be in place to reduce infectious aerosols exiting the laboratory, all the aerosolization procedures and processes should be conducted in the biosafety cabinets and, during open bench, proper PPEs should be worn; depending on the risk assessment, respirators (*e.g*., N95, N100 and PAPR), goggles and face shield should be used
6.	Since all such procedures will be performed in biosafety cabinets, and being small volumes of samples handled, there will be very low potential and extent of a splash or spill in this procedure, however, personnel must be trained on biosafety and should have laboratory procedures in place during spill or splash
7.	Biosafety cabinets should always be used, these should be routinely validated/certified and well-maintained and there are procedures in place for proper use
8.	Contaminated waste should be safely and efficiently treated within laboratory and should be stored in the laboratory, till disposed properly
9.	No sharps should be used in these laboratory procedures
10.	All surfaces in the laboratory should be easy to clean and decontaminated. No equipment should be maintained or repaired without decontamination, and the process should be documented and validated
11.	The laboratory should have a complete and well-maintained inventory system. It should also have an active shipping and receiving programme and well-defined procedures and plans in place
12.	There should be medical surveillance programme in place
13.	Laboratory should implement standard laboratory practices for safety
14.	There should be defined procedures in place for entry into the laboratory
15.	Institution/laboratory should have defined roles and responsibilities for biosafety and should also be commitment to safety as well as comprehensive biosafety documentation and should conduct biosafety drills or exercises

BSC, biological safety cabinet; PAPR, powered air-purifying respirator

giving protection to laboratory workers and reduce the suspicion of the staff to be getting infection in such emergency situation. Basic biosafety requirements for the laboratories that include important features of procedures and processes to be followed during processing the samples for COVID-19 laboratory diagnosis are provided in Table II.

Biorisk assessment carried out for individual laboratory personnel and to the community with regard to providing molecular diagnosis for COVID-19 is provided in the Figure. This biorisk assessment will also provide guidance for the future laboratories that are opting to provide laboratory diagnosis for this infection.

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Policy Guidelines

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Quick Response Code:

Guidance for building a dedicated health facility to contain the spread of the 2019 novel coronavirus outbreak

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Preparedness for the ongoing coronavirus disease 2019 (COVID-19) and its spread in India calls for setting up of adequately equipped and dedicated health facilities to manage sick patients while protecting healthcare workers and the environment. In the wake of other emerging dangerous pathogens in recent times, such as Ebola, Nipah and Zika, it is important that such facilities are kept ready during the inter-epidemic period for training of health professionals and for managing cases of multi-drug resistant and difficult-to-treat pathogens. While endemic potential of such critically ill patients is not yet known, the health system should have surge capacity for such critical care units and preferably each tertiary government hospital should have at least one such facility. This article describes elements of design of such unit (*e.g.*, space, infection control, waste disposal, safety of healthcare workers, partners to be involved in design and plan) which can be adapted to the context of either a new construction or makeshift construction on top of an existing structure. In view of a potential epidemic of COVID-19, specific requirements to handle it are also given.

Key words Biocontainment - biosafety - coronavirus - designated health facility - infection prevention and control

Introduction

Increasing clusters of people affected with novel severe acute respiratory syndrome coronavirus (SARS-CoV-2)-infected pneumonia (COVID-19) began to be reported from Wuhan, a metropolitan in People's Republic of China, in December 2019[1-4]. CoVs represent a major family of viruses, which have been implicated in several multi-country outbreaks of severe respiratory illnesses such as the Middle East Respiratory Syndrome CoV (MERS-CoV) and SARS. The World Health Organization (WHO) defines SARS-CoV-2 as 'a new strain of coronavirus that has not been previously identified in humans'[5]. The SARS-CoV-2 was identified through the ongoing surveillance

for 'pneumonia of unknown aetiology', which was initiated in 2003-2004, in the aftermath of the SARS outbreak[6]. The first four cases of COVID-19 were identified through this routine surveillance programme deployed through the local healthcare facilities and were epidemiologically linked to the Huanan (Southern China) Seafood Wholesale Market[7]. Since then, there has been a steady increase in the burden of COVID-19, with 60,347 confirmed cases and 1,369 deaths reported globally, as of 13th February 2020. With cases emerging from as many as 29 countries, and travel-related importations also being reported, the global health security implications of COVID-19 have come to the fore[4,8,9].

In response, susceptible countries have mounted public health measures to mitigate the threat of proliferation of COVID-19. In India, airport entry screening has been initiated, existing visas from China have been cancelled, and travellers returning from COVID-19-affected areas have been quarantined. Given the implications of importation of cases and the impact thereof on the local epidemiology of COVID-19 in secondary foci, it is essential to break ground on preparing health facilities to combat a local cluster of COVID-19 cases.

A well-equipped dedicated hospital facility (DHF) to deal with these cases with adequate protections for healthcare workers and other patients is the key to the standard of care. Critical care and humane approach to acutely ill patients are essential ingredients of an epidemic control. While the course of the CoV in India is uncertain, setting up of such units is important for future epidemics due to dangerous pathogens and antimicrobial resistant organisms including drug-resistant tuberculosis during the inter epidemic period. Such wards can be used for training of physicians during inter epidemic period.

Here, we focus on establishing a biosecurity ward *de novo* or in a makeshift manner in a tertiary healthcare facility to manage patients or suspected cases in an effort to combat the spread of COVID-19.

Key partners

A scientific and evidence-based design shall be the key to initiate the process. The designing of DHF should have inputs from the infectious disease department, critical care, engineering and nursing department, departments of microbiology and virology, hospital administration and waste disposal facilities, referral ambulance services, social workers or counsellors for patients' families and situation room with digital connection with national programme. Given the multi-disciplinary clinical needs and the specialized requirements for maintenance of the infrastructure related to DHF, it is vital that all the participating disciplines be brought together under a unified umbrella to identify the existing capacity and infrastructure, and needs, to make a streamlined plan for establishing the unit.

Pre-requisites for dedicated health facility

The DHF must be a self-contained establishment that can meet most of its daily needs with only essential but limited contact with the outside world. Basic requirements need to be accounted for continuous safe water supply; appropriate cleaning practices; adequate floor space for beds; appropriate handwashing facilities; adequate ventilation for isolation rooms and procedure rooms; adequate isolation facilities for airborne, droplet, contact isolation and protective environment; regulated and rational traffic flow to minimize exposure of high-risk patients and facilitate patient and clinical material transport; precautions to control rodents, pests and other vectors and appropriate waste management facilities/practices must be ensured. The unit can be a standalone facility or can be housed in a tertiary healthcare facility with the equipment and capacity to care for critically ill patients such as those with septic shock requiring vasopressors, bedside surgical procedures, acute respiratory distress syndrome (ARDS) requiring mechanical ventilation, acute kidney injury requiring dialysis and multi-organ failure requiring high degree of quality and multi-disciplinary care with organ support.

Space

An isolation room should be identified within the emergency room. This room will be used to isolate patients who raise any suspicion of COVID-19 infection based on a set of validated, screening questions[10]. This approach may be adapted for other outbreak-prone infectious diseases as well.

For mapping the patient transfer, the path of transport and specific elevators should be identified. The number of rooms with biocontainment facilities should be mobilized based on the magnitude of the emerging situation. In an epidemic situation, it is ideal to have separate rooms for suspected and confirmed cases[11,12].

Within the DHF, two units should be built. The first one will be an isolation space for laboratory confirmed

cases. Multiple patients can be kept in the same room. Barrier nursing practices and protective isolation facility will be presented to prevent nosocomial infections.

The second unit will be made for suspected cases which will include family and hospital contacts who are suspected to have potential contact with confirmed cases but await laboratory confirmation. This room will be built to include only one suspect per room. Figure 1 is a conceptual drawing of this unit. Figure 2 is a conceptual drawing of multi-bedded isolation room for suspects.

Staff

One full-time physician, one paediatrician and one resident should be identified for the management of the unit. Systems should be developed to ensure that an identified clinical rapid response team (RRT) is always available on call. Intensive care unit (ICU) nursing staff should be identified specifically for this DHF, and the ratio of nursing staff in this unit should be similar to ICUs[13]. Paramedical and housekeeping staff should also be identified. Precautions should be taken to minimize healthcare worker (HCW) exposure. Staff attached to this facility should undergo focused training in infection prevention and control. Mock drills should also be conducted to assure preparedness of the unit and as part of ongoing quality improvement measures.

Training and capacity building

Training will have to be carried out for all the healthcare providers who will participate in the care of the patients including doctors, nurses, paramedical staff, patient transporters, phlebotomists, laboratory technicians and housekeeping staff. This will require both technical training for the physicians and nurses and infection prevention and control training for all the cadres.

Systems

Screening systems will have to be identified in the triage area to isolate patients who present with: (*i*) Fever and cough with a history of travel to COVID-19 affected countries; (*ii*) HCWs with fever and cough caring for patients with pneumonia whose cause of pneumonia is unknown; (*iii*) Patients with fever and cough with contact history with someone with known

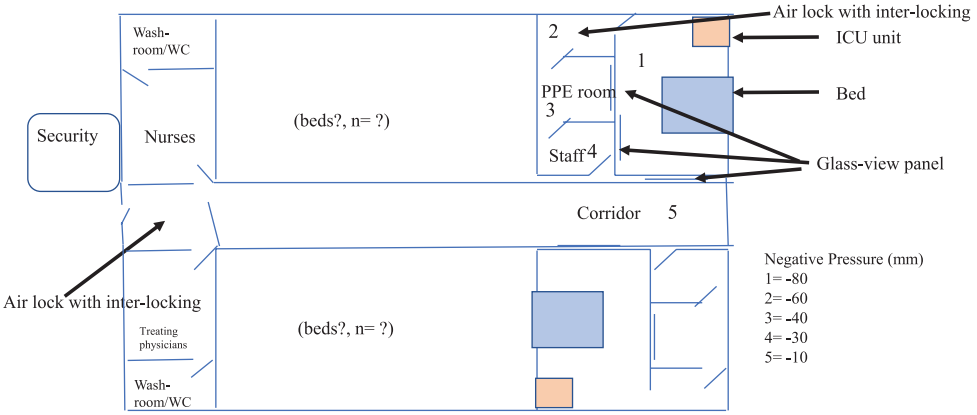

Fig. 1. Conceptual figure of an isolation unit. WC, water closet; PPE, personal protective equipment; ICU, intensive care unit.

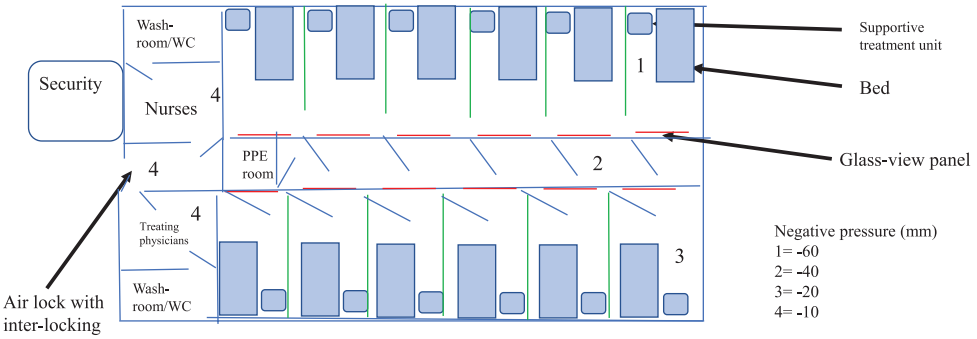

Fig. 2. Conceptual figure of multi-bedded isolation room for suspects.

SARS-CoV-2 infection; and (*iv*) Patients with fever or cough with a history of visit to a healthcare facility with a case of COVID-19 infection.

Once a patient has been isolated, RRT should be activated. Simultaneously, it should be reported to the nodal officers in the Ministry of Health and Family Welfare, State Department of Health, for notifying the WHO in accordance with International Health Regulations (2005)[14]. Specimens for transport must be placed in leak-proof specimen bags, which have a separate sealable pocket for the specimen (*i.e*., a plastic biohazard triple-layered specimen bag). The hospital which is receiving the patient should be informed ahead of time to initiate the necessary precautions. All specimens are to be delivered by hand if possible. Any shipment of specimens should be in accordance with the prevailing International Air Transport Association[15] and UN/WHO norms[16].

Food should be served on disposable crockery and should be consumed with disposable cutlery. Non-disposable crockery and cutlery should be washed using hot water (70°C) and detergent, rinsed and dried. Where possible, eating utensils should be cleaned in a dishwasher using a hot water cycle (reaching at least 70°C). A record of all patients or staff entering the room should be maintained.

Infection prevention and control

***Standard hygiene*:** Standard precautions must be followed by all HCWs, for all patients and at all times[17]. This comprises broadly of hand hygiene practices, proper donning, doffing and disposal of the personal protective equipment (PPE) and maintaining respiratory hygiene and cough etiquettes[18].

Hand hygiene is recommended with alcohol-based handrubs (ABHR) or soap and water. Printed posters of both the methods should be pasted near all hand hygiene units. Adequate supply of ABHR (60-80% ethanol is recommended) and antiseptic soap solution (chlorhexidine gluconate 2% and alcohol combination) has been seen to be synergistic[19].

***Personal protective equipment (PPE)*:** All HCWs involved should have the knowledge of the correct donning and doffing steps, along with appropriate disposal of PPE and be trained in this procedure. Non-powdered latex-free gloves should be used by all HCWs. Eye protection and face shield should be used[20]. Respiratory hygiene and cough etiquette must be followed.

Gowns should be long-sleeved and made of non-absorbable (fluid-resistant) materials. The same gown should not be worn for all patients. In case gowns are not available, waterproof aprons should be used.

The WHO recommends the use of medical masks (surgical or procedure masks which may be flat or pleated and are affixed with head straps) and particulate respirators (NIOSH-certified N95, EU standard FFP2 or equivalent) for contact and airborne precautions as well as aerosol-generating procedures (AGPs), respectively[18,21]. The mask has to be worn before entering the patient's room and has to be removed once outside the room. If the patient is under droplet precautions and requires to be moved or transported, he/she should wear a mask. Hand hygiene should be ensured after removing the masks. Each room should have dedicated equipment such as sphygmomanometer, thermometer and stethoscopes.

***Patient transport and stay*:** The movement of patients should be minimized within the hospital premises. If such transport is necessary, patients must don either medical masks or particulate respirators, whichever is available. The area to which they are being transported should be alerted about their arrival. A separate corridor should be preferred. In case the patients meet any surfaces, they must be disinfected. Complete inactivation of the virus is seen with 70 per cent ethanol and povidone-iodine with an exposure time of one minute or 2.5 per cent glutaraldehyde with an exposure time of five minutes. The Central Drugs Standard Control Organization registered disinfectant or 1:100 dilution of household bleach and water will suffice for disinfection of surface of non-critical patient care equipment. High-level disinfection and sterilization of semi-critical and critical devices, respectively, does not need to be altered for patients with known or suspected COVID-19.

When transmission-based precautions are being practiced, the ideal condition is to have single occupancy rooms for every patient. Since that may not be feasible in every scenario, the practice of cohort isolation can be followed with a spatial separation of ≥3 feet (or 1 m). There are certain environmental cleaning procedures one needs to keep in mind. Dusting should be avoided; floors should not be carpeted, and rooms should not have upholstery. For environmental cleaning of areas needing airborne precautions, high touch surfaces should be cleaned every day, and as needed, while low touch surfaces can be cleaned on a scheduled basis. Primary focus should remain in

adherence to required PPE and additional entry/exit procedures. For undertaking droplet and/or contact precautions, cleaning may be done twice a day, or as needed, for high touch surfaces, with a focus on all surfaces within the patient zone, non-critical patient care equipment, and any surface visibly soiled with blood or body fluids[22].

***What to do with the body of the deceased, when transmission-based precautions are being observed*:** Dead bodies are categorized into three categories based on the level of PPE being observed. All tubings attached to the bodies, such as nasogastric tubes, Foley's catheter, and any others, are to be removed, keeping biosafety in place. All orifices are to be cleaned and disinfected and plugged to prevent any fluid leakage. All wounds must be redressed after disinfection with impermeable dressings.

All PPE must be removed immediately, and hand hygiene must be performed immediately. Once the body reaches the mortuary, the following precautions must be taken: (*i*) All bodies must be categorized and identified correctly; (*ii*) Bodies found to be soiled with blood/body fluids should be placed in disposable plastic bags; (*iii*) Dead bodies must be kept in cold temperatures of −4°C; and (*iv*) All staff posted in the mortuary should follow strict PPE guidelines.

Funeral workers must also observe strict adherence to PPE and follow strict environmental cleaning precautions.

***Ventilation*:** The overall risk of infection in a room can be mitigated by ventilation through two principles: dilution and removal. Clean air, when added to a room, dilutes the airborne contaminants present in the room, thus reducing the chances of inhalation of infectious droplets. For outbreak-prone and epidemic-prone infectious diseases such as COVID-19, the WHO recommends the institution of contact and droplet precautions[18]. The Centers for Disease Control and Prevention and WHO guidelines provide the following recommendations for preventing airborne infections[18,23]: (*i*) To help prevent airborne infections, adequate ventilation in healthcare facilities in all patient-care areas is necessary; (*ii*) For natural ventilation, the following minimum hourly averaged ventilation rates should be provided: (a)160 l/s/patient (hourly average ventilation rate) for airborne precaution rooms (with a minimum of 80 l/sec/patient for new health care facilities and major renovations); and (b) 2.5 l/sec/m^3 for corridors and other transient spaces without a fixed number of patients; however, when patient care is undertaken in corridors during emergency or other situations, the same ventilation rate requirements for airborne precaution rooms or general wards will apply.

The design should consider fluctuations in ventilation rate.

For aerosol generating procedures (AGPs) (Supplementary Table I), adequate airflow is at least 160 l/sec/patient or in negative pressure rooms at least 12 air changes per hour (ACH) and controlled direction of air flow when using mechanical ventilation.

***Negative pressure rooms*:** Negative pressure rooms include mechanical ventilation systems which maintain the pressure of the room at a slightly lower level than the pressure of the entry area so that it allows air to flow into the isolation room but not escape from the room, as air naturally flows from areas with higher pressure to areas with lower pressure, thereby preventing contaminated air from the isolation room to escape outwards.

The negative pressure room should have a minimum of 12 ACH in a high-risk area for airborne transmission, compared to six ACH per hour in a low-risk area. Negative pressure differential between airflow from adjacent spaces to the patient room should be >2.5 Pascal. An airflow differential >125 cfm (56 l/sec) should be maintained between exhaust and supply. Sealing of the room (entry) should have provision of allowing approximately 0.5 square feet (0.046 m^2) leakage. Clean air entering the room should flow first to the area of the room where staff or visitors are likely to be present and then flow across the bed area to the exhaust. Direction of airflow and patient beds should not be in the same direction. The air from inside the room should be allowed to flow out *via* an exhaust fan or a high-efficiency particulate air (HEPA) filter. There should be an anteroom outside of the negative pressure room. The pressure of which should be negative to the hallway and positive to the patient room so that unidirectional airflow is maintained[24].

HEPA filter should be placed in each room, and if not available, adequate ventilation should be ensured. Exhaust fans must be properly installed closely fitting to the window. HEPA filter is useful in small volume settings such as bronchoscopy suites, laboratories or individual TB patient rooms. Careful attention should be given to the equivalent ACH the filter requires as most filters clean very little air per hour. Maintenance of

HEPA filters with timely replacement of the membrane is necessary to ensure its functionality.

The efficiency of filters and sustainability of negative pressure must be validated before DHF is open to patients. Subsequently, validation must be undertaken regularly and at pre-decided intervals through a certified agency.

***Renovating or converting a room*:** While establishing an isolation room or a Class N room (negative pressure room) in an existing facility, available infrastructure and financial implications for carrying out changes must be considered. It is rarely possible to create an ideal room. There is an extensive list of requirements given in 'Guidelines for the classification and design of isolation rooms in healthcare facilities' which are the basic requirements to be met before any conversion[25].

Some of the salient requirements are the presence of a clinical handwash basin with non-touch, fixed tap, wall mounted soap dispensers, handrub dispensers, disposable towel holders, glove dispensers, clean waste bins in accordance to 'the Bio-Medical Waste Management (Amendment) Rules, 2018'[26] and provision of two-way intercommunication system between the patient's room and the nurses' station. This document also gives insight into what considerations an institute needs to consider before converting a room into a class N room. The recirculating air system needs to be disconnected. It would be a preferred option to adjust the building ventilation system to create a permanent negative pressure room or to add a HEPA filtration unit as a supplement or install it permanently in the hospital ventilation system. One should be able to seal the room adequately; dampers should be adjusted.

Supply

Since clinical manifestations of SARS-CoV-2 infection range from septic shock, pneumonia, ARDS, acute kidney injury to multi-organ dysfunction, the unit should be equipped like an ICU. A detailed list of disease commodity package for novel CoV has been published by the WHO[27]. Additional material is shown in Supplementary Table II.

Conclusion

Developing the infrastructure and mobilizing the human resources needed to counter a rapidly emerging outbreak of a highly contagious or lethal disease needs responsive systems, mounting multi-disciplinary response, one critical part of which is enabling key healthcare facilities in providing high-quality clinical care. This not only helps in limiting the loss of lives from the onslaught of the contagions, but also interrupts the transmission by excluding the infective patients from the general population. As such, biocontainment provides value for money in the longer term, even if it is a resource-intensive affair in the short term. These actions will substantially contribute to global health security.

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Protocol

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Quick Response Code:

Lopinavir/ritonavir combination therapy amongst symptomatic coronavirus disease 2019 patients in India: Protocol for restricted public health emergency use

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As of February 29, 2020, more than 85,000 cases of coronavirus disease 2019 (COVID-19) have been reported from China and 53 other countries with 2,924 deaths. On January 30, 2020, the first laboratory-confirmed case of COVID was reported from Kerala, India. In view of the earlier evidence about effectiveness of repurposed lopinavir/ritonavir against severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronavirus (CoV), as well as preliminary docking studies conducted by the ICMR-National Institute of Virology, Pune, the Central Drugs Standard Control Organization approved the restricted public health use of lopinavir/ritonavir combination amongst symptomatic COVID-19 patients detected in the country. Hospitalized adult patients with laboratory-confirmed SARS-CoV-2 infection with any one of the following criteria will be eligible to receive lopinavir/ritonavir for 14 days after obtaining written informed consent: (*i*) respiratory distress with respiratory rate ≥22/min or SpO_2 of <94 per cent; (*ii*) lung parenchymal infiltrates on chest X-ray; (*iii*) hypotension defined as systolic blood pressure <90 mmHg or need for vasopressor/inotropic medication; (*iv*) new-onset organ dysfunction; and (*v*) high-risk groups - age >60 yr, diabetes mellitus, renal failure, chronic lung disease and immunocompromised persons. Patients will be monitored to document clinical (hospital length of stay and mortality at 14, 28 and 90 days), laboratory (presence of viral RNA in serial throat swab samples) and safety (adverse events and serious adverse events) outcomes. Treatment outcomes amongst initial cases would be useful in providing guidance about the clinical management of patients with COVID-19. If found useful in managing initial SARS-CoV-2-infected patients, further evaluation using a randomized control trial design is warranted to guide future therapeutic use of this combination.

Key words Coronavirus disease 2019 - COVID-19 - lopinavir/ritonavir - severe acute respiratory syndrome coronavirus 2 - treatment outcome

Coronaviruses (CoVs) are enveloped non-segmented positive-sense RNA viruses, belonging to the family *Coronaviridae* and the order *Nidovirales*, and are broadly distributed in humans and other mammals[1]. In December 2019, a series of cases of pneumonia of unknown aetiology emerged in

Wuhan, Hubei, China, with clinical presentations greatly resembling viral pneumonia. Deep-sequencing analysis from lower respiratory tract samples indicated a novel CoV (nCoV)[2], which was named severe acute respiratory syndrome (SARS)-CoV-2[3]. Since December 31, 2019 and as of February 29, 2020, a total of 85,403 cases of CoV disease 2019 (COVID-19) have been reported, including 2,924 deaths. Of the total deaths reported, 2,838 were in People's Republic of China (PRC). Other than China, confirmed cases have been reported from 53 countries[4]. As per the statement of the WHO Emergency Committee, COVID-19 had a case-fatality ratio (CFR) of four per cent; however, recent reports suggested it to be between 1 and 2 per cent[5-7].

The published studies from China indicated that most cases with SARS-CoV-2-infected pneumonia were aged above 50 yr (median age: 55-59 yr), predominantly men (54-68%) and had chronic medical conditions (46.4-51%). The common symptoms included fever, fatigue, dry cough, myalgia, dyspnoea, expectoration and diarrhoea[8-11]. The common laboratory abnormalities amongst 138 patients were lymphopenia [lymphocyte count, 0.8×10^9/l (interquartile range IQR, 0.6-1.1), 70.3%], prolonged prothrombin time [PT, 13.0 sec (IQR, 12.3-13.7), 58%] and elevated lactate dehydrogenase [261 U/l (IQR, 182-403), 39.9%][10]. Unilateral (25%) or bilateral (75%) pneumonia and multiple mottling and ground-glass opacities (14%) were the common findings on chest X-ray/computed tomography (CT) scan[9,10]. Patients were treated with antivirals including oseltamivir, ganciclovir and lopinavir and ritonavir, antibiotics and glucocorticosteroids[8,10].

No antiviral treatment for SARS-CoV-2 infection has been proven to be effective. A few historical control studies or case reports indicate the effectiveness of combination of lopinavir/ritonavir against SARS-CoV and MERS-CoV infections. Ritonavir-boosted lopinavir was approved for use amongst HIV-infected individuals in September 2000 by the U.S. Food and Drugs Administration[12]. The drug has been used for over 15 years in India. Heat stable version of the medicine that is based on malt-extrusion technology Meltrex™ Technology was launched in India in 2007[13]. Lopinavir is metabolized by cytochrome P4503A (CYP3A) isoenzyme in the liver. Lopinavir is always used with ritonavir to reduce the dose of lopinavir and increase the plasma levels of lopinavir as ritonavir inhibits CYP3A isoenzyme. Lopinavir and ritonavir are antiretroviral protease inhibitors used in combination as a second-line drug for the treatment of HIV-1 infection in children and adults and have limited side effects[14,15]. As per the NACO (National AIDS Control Organization) guidelines, lopinavir/ritonavir is used as a second-line drug in the treatment of HIV in combination with nucleoside reverse transcriptase inhibitors (NRTIs). It is also recommended by NACO in post-exposure prophylaxis in HIV for 28 days. It is also part of the first-line regimen for patients with HIV-2 infection[16]. The drug has been used extensively in the management of paediatric HIV infection, especially amongst infants. Thus, there is a long period of experience of its use in India. A systematic review suggested no safety or efficacy concerns for use of standard-dose lopinavir/ritonavir amongst pregnant women[17]. Boosted lopinavir has been used to prevent mother-to-child transmission of HIV-1 and HIV-2 infection[16]. The main viral protease has been regarded as a suitable target for drug design against CoV infection due to its vital role in polyproteins processing necessary for CoV reproduction. Lopinavir/ritonavir has been shown to have the highest inhibitory potency against CoV amongst several anti-HIV-1 protease inhibitors[18].

In a historical control study, lopinavir/ritonavir with ribavirin amongst SARS-CoV patients was associated with substantial clinical benefit. The adverse clinical outcome (ARDS or death) was significantly lower in the treatment group than in the controls who received only ribavirin (2.4 vs. 28.8%, $P<0.001$) at day 21 after the onset of symptoms. A reduction in steroid usage and nosocomial infections was seen in patients initially treated with lopinavir/ritonavir, and these patients had a decreasing viral load and rising peripheral lymphocyte count[19]. Findings from *in vitro* and clinical studies, together with the availability and safety profiles of lopinavir/ritonavir and interferon beta-1b (IFN-β1b) suggest that the combination of these agents has potential efficacy for the treatment of patients with MERS-CoV. Oral treatment with lopinavir/ritonavir in the marmoset model of MERS-CoV infection resulted in modest improvements in MERS disease signs, including decreased pulmonary infiltrates identified by chest X-ray, decreased interstitial pneumonia and decreased weight loss[20]. Studies on MERS patients with treatment regimens including lopinavir-ritonavir reported positive disease outcomes including defervescence, viral clearance from serum and sputum and survival[21-24]. Arabi *et al*[25] initiated a

placebo-controlled trial of IFN-β1b, lopinavir and ritonavir amongst patients with MERS-CoV infection in Saudi Arabia.

In India, the first laboratory-confirmed case of COVID-19 was reported from Kerala on January 30, 2020 (*https://pib.gov.in/PressReleseDetail.aspx?PRID=1601095*). Subsequently, two more cases were reported from Kerala. All cases had recently returned from Wuhan, PR China, had mild illness and were managed symptomatically. More such cases can be expected amongst individuals travelling from China, and Wuhan in particular, and amongst their close contacts. As COVID-19 is an emerging virus, an effective treatment has not been developed for disease resulting from this virus. In view of the earlier evidence about the effectiveness of lopinavir/ritonavir against SARS and MERS-CoV, the Indian Council of Medical Research (ICMR) has suggested off-label emergency use of lopinavir/ritonavir combination for symptomatic COVID-19 patients detected in the country. Use of IFN-β1b and ribavirin was not considered due to their reported toxicity, whereas oseltamivir was not considered due to its unproven efficacy against CoVs. This article describes the protocol for the administration of lopinavir/ritonavir to such patients and their clinical monitoring.

Proposed protocol

This protocol is to be implemented along with the WHO guidelines on clinical management of severe acute respiratory infection when nCoV infection is suspected: Interim Guidance[26].

Patient eligibility criteria

Inclusion criteria: Include (1) Adult over 18 yr of age; (2) Laboratory confirmation of COVID-19 infection by real-time reverse transcription-polymerase chain reaction (qRT-PCR) from the recommended sample (3) Symptomatic patients with any one of the following: (*i*) Respiratory distress with respiratory rate ≥22/min or SpO_2 of <94 per cent, (*ii*) Lung parenchymal infiltrates on chest X-ray or CT scan, (*iii*) Hypotension defined as systolic blood pressure <90 mmHg or need for vasopressor/inotropic medication, (*iv*) New-onset organ dysfunction (one or more of the following): (a) Increase in creatinine by 50 per cent from baseline, glomerular filtration rate (GFR) reduction by >25 per cent from baseline or urine output of <0.5 ml/kg for six hours, (b) Reduction of Glasgow Coma Scale (GCS) score by two or more, and (c) Any other organ dysfunction; (*v*) High-risk groups with age >60 yr, and those with hypertension, diabetes mellitus, renal failure, chronic lung disease and immunocompromised persons; (*vi*) Informed consent from patient and caretaker. Consent from legally authorized representative in case the patient is not able to provide the same due to his/her medical condition.

Exclusion criteria: (*i*) A patient with hepatic impairment [Child Pugh C or alanine aminotransferase (ALT) over 5X the upper limit of normal]; (*ii*) Use of medications that are contraindicated with lopinavir/ritonavir and that cannot be replaced or stopped, *e.g.*, rifampicin, benzodiazepines, simvastatin, voriconazole and sildenafil; and (*iii*) Known HIV-infected individual receiving other protease inhibitors containing regimens that cannot be replaced by lopinavir/ritonavir.

***Dosage of lopinavir/ritonavir*:** (*i*) Lopinavir/ritonavir 200 mg/50 mg - two tablets every 12 h for 14 days or for seven days after becoming asymptomatic, whichever is earlier; and (*ii*) For patients who are unable to take medications by mouth, 400 mg lopinavir /100 mg ritonavir 5 ml suspension every 12 h for 14 days or seven days after becoming asymptomatic whichever is earlier, *via* a nasogastric tube.

***Baseline laboratory investigations*:** (*i*) Haemogram; (*ii*) Liver function tests (LFTs); (*iii*) Renal function tests (RFTs); (*iv*) Haemoglobin A_{1c} and blood sugar, if required; (*v*) RT-PCR for SARS-CoV-2 (respiratory samples: nasopharyngeal swab, oropharyngeal swab, in addition, sputum, bronchoalveolar lavage (BAL), if available); (*vi*) PT/international normalized ratio, electrolytes, arterial blood gas; (*vii*) Lipid profile; (*viii*) Chest X-ray; (*ix*) Electrocardiogram (ECG); (*x*) Hepatitis B and C; and (*xi*) Other investigations as deemed appropriate by the treating physician.

***Laboratory sample collection (other than investigations for routine clinical monitoring)*:** (*i*) Oropharyngeal swabs (every third day) - for SARS-CoV-2 RT-PCR (samples to be transported to ICMR-National Institute of Virology, Pune, as per the guidelines); (*ii*) Blood sample (every week) - Haemogram, LFT (alternate days), RFT and electrolytes (to monitor drug-induced adverse events); (*iii*) ECG; and (*iv*) Other investigations as deemed appropriate by the treating physician.

All samples would be stored for future-related tests.

***Frequency and duration of monitoring*:** (*i*) Patients should be monitored daily until discharge from the hospital and followed up till 90 days; and

(*ii*) Patient should be discharged on clinical recovery and after obtaining two consecutive negative RT-PCR results at least 24 h apart from oropharyngeal swabs (to demonstrate viral clearance).

Outcome assessment

Clinical outcomes: (*i*) Hospital length of stay; (*ii*) Intensive care unit (ICU)-free days; (*iii*) Requiring use of ventilator; (*iv*) Mortality in the ICU; (*v*) Mortality in the hospital; and (*vi*) Mortality at 14, 28 and 90 days.

Safety outcomes: (*i*) Acute pancreatitis (defined as having: (a) abdominal pain radiating to the back; (b) serum amylase at least three times greater than the upper limit of normal; (c) radiological evidence, such as contrast CT/magnetic resonance imaging/ultrasonography, of acute pancreatitis); (*ii*) Elevation of ALT to more than five-fold upper normal limit; (*iii*) Anaphylaxis; and (*iv*) Adverse events and serious adverse events.

Laboratory outcomes: (*i*) Viral RNA loads and cycle threshold values in serial samples of nasopharyngeal and oropharyngeal swabs and blood, collected every third day (to document viral replication kinetics).

The schedule of key investigations is given in the Table.

Discussion

The complete clinical picture of COVID-19 is not fully understood. The clinical manifestations in infected patients could range from mild illness to severe disease requiring ICU admission and ventilatory support. The CFR of COVID-19 is lower than that of SARS (CFR: 14-15%) and MERS (34%)[27,28]. Till now, no effective treatment has been recommended for COVID-19, except meticulous supportive care[26]. The ICMR has suggested lopinavir/ritonavir combination therapy for laboratory-confirmed COVID-19 patients based on the observational studies of clinical benefit amongst patients with SARS-CoV and MERS-CoV[19-21], as well as the docking studies conducted by the National Institute of Virology, Pune[29]. The Indian Regulatory Authority, Central Drugs Standard Control Organization, has accorded approval for restricted public health emergency use of this treatment protocol.

The initial treatment protocol was for administering the combination treatment to all laboratory-confirmed patients. However, the first three laboratory-confirmed patients from Kerala had mild symptoms on diagnosis and had a stable course of illness. Hence, lopinavir/ritonavir treatment was not administered in these patients. It is however, crucial to initiate the treatment before patient develops features of severe

Table. Schedule of investigations for the administration of lopinavir/ritonavir combination

Parameters	Days during admission period														
	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
Haemogram[@]	✓		✓		✓		✓		✓		✓		✓		✓
Liver function test[*]	✓		✓		✓		✓		✓		✓		✓		✓
Renal function test[#]	✓		✓		✓		✓		✓		✓		✓		✓
HbA_{1C} and blood sugar	✓														
qRT-PCR for SARS-CoV-2	✓			✓			✓			✓			✓		
Electrolytes	✓		✓		✓		✓		✓		✓		✓		✓
PT/INR, arterial blood gas	✓														
Lipid profile	✓														
Chest X-ray	✓							✓							✓
ECG	✓		✓		✓		✓		✓		✓		✓		✓
HBV and HCV ELISA	✓														

[@]Hb%, total leucocyte count and differential WBC - neutrophils, lymphocytes, eosinophils, monocytes and basophils, RBC count, platelet count; [#]Renal function test - BUN, Creatinine; [*]Liver function test - albumin, bilirubin, ALT, AST, alkaline phosphatase. AST, aspartate transaminase; ALT, alanine aminotransferase; RBC, red blood cell; WBC, white blood cell; BUN, blood urea nitrogen; HBV, hepatitis B virus; HCV, hepatitis C virus; ECG, electrocardiogram; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; qRT-PCR, real-time reverse transcription-polymerase chain reaction; PT, prothrombin time; INR, international normalized ratio; HbA_{1c}, haemoglobin A_{1c}; Hb, haemoglobin

illness. In view of this, the treatment protocol was subsequently amended to include additional criteria of severity as well as organ damage for initiating the combination treatment. The inclusion criteria also include high-risk group patients associated with higher risk of mortality (age >60 yr, hypertension, diabetes mellitus, renal failure, chronic lung disease and immunocompromised persons) for initiating the combination therapy. The treatment protocol also emphasizes the need for obtaining written informed consent and patients to be enrolled into this protocol on case-to-case basis. It is also equally important to monitor COVID-19 patients closely to generate reliable data about clinical, laboratory, as well as safety outcomes.

This treatment protocol has a limitation. The combination treatment is approved for emergency public health use, only amongst laboratory-confirmed patients with moderate degree of severity and not designed as a controlled clinical trial. However, the treatment outcomes amongst the first few cases would be useful in providing guidance about clinical management of COVID-19 cases in future. If found useful in managing initial COVID-19 infected patients, further evaluation using a randomized control trial design is warranted to guide future therapeutic use of this combination.

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Quick Response Code:

Prudent public health intervention strategies to control the coronavirus disease 2019 transmission in India: A mathematical model-based approach

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***Background & objectives*: Coronavirus disease 2019 (COVID-19) has raised urgent questions about containment and mitigation, particularly in countries where the virus has not yet established human-to-human transmission. The objectives of this study were to find out if it was possible to prevent, or delay, the local outbreaks of COVID-19 through restrictions on travel from abroad and if the virus has already established in-country transmission, to what extent would its impact be mitigated through quarantine of symptomatic patients?**

***Methods*: These questions were addressed in the context of India, using simple mathematical models of infectious disease transmission. While there remained important uncertainties in the natural history of COVID-19, using hypothetical epidemic curves, some key findings were illustrated that appeared insensitive to model assumptions, as well as highlighting critical data gaps.**

***Results*: It was assumed that symptomatic quarantine would identify and quarantine 50 per cent of symptomatic individuals within three days of developing symptoms. In an optimistic scenario of the basic reproduction number (R_0) being 1.5, and asymptomatic infections lacking any infectiousness, such measures would reduce the cumulative incidence by 62 per cent. In the pessimistic scenario of R_0=4, and asymptomatic infections being half as infectious as symptomatic, this projected impact falls to two per cent.**

***Interpretation & conclusions*: Port-of-entry-based entry screening of travellers with suggestive clinical features and from COVID-19-affected countries, would achieve modest delays in the introduction of the virus into the community. Acting alone, however, such measures would be insufficient to delay the outbreak by weeks or longer. Once the virus establishes transmission within the community, quarantine of symptomatics may have a meaningful impact on disease burden. Model projections are subject to substantial uncertainty and can be further refined as more is understood about the natural history of infection of this novel virus. As a public health measure, health system and community preparedness would be critical to control any impending spread of COVID-19 in the country.**

Key words Airport screening - COVID-19 - deterministic model - mathematical model - mitigation - quarantine - transmission

As per the World Health Organization (WHO), 85,403 cases of coronavirus disease 2019 (COVID-19) were reported globally, as of February 29, 2020, including 79,394 cases (2838 deaths) from China and 6009 cases (86 deaths) from 53 other countries/territories/areas[1]. Initially, all of the cases detected in countries other than China were linked to infected cases from China, with subsequent generation of cases in some of the countries, the latest being Japan, South Korea and Italy. Considering the high population mobility through air travel and the documented person-to-person transmission, the WHO provided an advisory on exit screening in countries with the ongoing transmission of COVID-19 and entry screening in countries without transmission, including screening for the signs and symptoms of respiratory infection with focus on temperature screening to detect potential suspects who would require further laboratory tests for the confirmation of infection[2]. As per a stochastic, worldwide, air transportation network dynamic model, India ranks 17th among the countries at the highest risk of importation of COVID-19 through air travel[3]. The probability of an infected air traveller to come to India as the final destination was 0.209 per cent, with the highest relative import risk in Delhi (0.064%) followed by Mumbai, Kolkata, Bengaluru, Chennai, Hyderabad and Kochi[3]. This in the context of an epidemic that has already set in and travel from infected areas continues.

The Ministry of Health and Family Welfare (MoHFW) of India had initially advised to refrain from travelling to China and quarantine of those coming from China[4]. Those returning from Wuhan, China, after January 15, 2020 were to be tested for COVID-19. Those feeling sick within a month of return from China were advised to report to the nearest health facility in addition to maintaining self-isolation at home[5]. Initially, thermal entry screening of passengers from China was established at 21 airports across the country with universal screening for all flights from China, Hong Kong, Singapore, Thailand, Japan, South Korea, Iran and Italy. Symptomatic passengers were advised to volunteer for screening. Similar screening was in place at international seaports[6]. Till February 29, 2020, three cases were reported from India[7].

In the absence of a licensed vaccine or effective therapeutics for COVID-19, in addition to the non-pharmaceutical measures of hand hygiene and cough etiquettes, quarantine becomes a critical strategic containment and mitigation intervention towards the early detection and isolation of cases to break the chain of transmission and slow down the spread of the outbreak. This analysis was done with the following objectives: (*i*) is it feasible to prevent, or delay, the local outbreaks in India through restrictions on travel from countries with COVID-19 transmission; and (*ii*) in the event that COVID-19 transmission becomes established in India, the extent to which its impact could be mitigated through quarantine.

Material & Methods

This analysis was based on a simple Susceptible-Exposed-Infectious-Recovered (SEIR) model to capture the natural history of COVID-19 and its transmission dynamics. The model structure is summarised in Fig. 1, with the following governing equations:

$$\frac{dS}{dt} = -\lambda S$$

$$\frac{dE}{dt} = \lambda S - rE$$

$$\frac{dI}{dt} = rE - \gamma I - \mu I$$

$$\lambda = \frac{\beta I}{N} + \frac{k\beta E}{N}$$

where the compartments are as follows: susceptible (S); exposed and infectious but not yet symptomatic (E); infected and symptomatic (I) and recovered (R). Model parameters are as follows: among those

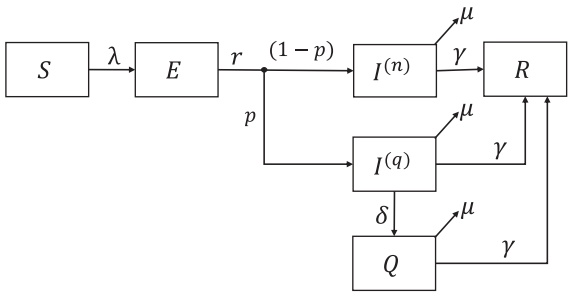

Fig. 1. Summary of the model structure used to represent coronavirus disease 2019 transmission and control in Indian cities. The population in each metropolitan area is divided into different compartments, representing states of disease, with flows between compartments given by the rates shown in the diagram. Thus, susceptible individuals (S), upon acquiring infection, enter a state of asymptomatic infection (E) and with some delay develop symptomatic disease (I). It is assumed that a proportion p of symptomatic cases is subject to quarantine [$I^{(q)}$] and the remainder [$I^{(n)}$] is not. The relative size of these two populations (p) reflects the coverage of quarantine efforts. Individuals in $I^{(q)}$ are quarantined with an average quarantine delay ($1/\delta$). Finally, individuals may be cured (R) or die as per recovery rate (γ) or mortality rate (μ), respectively. Those people who are successfully quarantined (Q) do not contribute to onward infection.

Table I. Model parameters for optimistic and pessimistic scenarios of coronavirus disease-19 transmission in India

Parameters	Optimistic scenario	Pessimistic scenario
Basic reproduction number (R_0)	1.5	4
Infectiousness of asymptomatic cases, relative to symptomatic case (k)	0	0.5

exposed, per-capita rate of developing symptoms (r); among symptomatics, per-capita rates of recovery and death (γ and μ, respectively) and the average number of infections caused per day per symptomatic case (β) and the infectiousness of exposed/asymptomatic cases, relative to symptomatic (k).

With the evolving understanding of the natural history of COVID-19 infection, it was assumed that all infections would go through an asymptomatic stage lasting three days on an average, followed by a symptomatic stage, also lasting three days on an average. Previous work has shown that the extent of transmission that occurs before symptoms develop can be an important factor in the feasibility of control[8]. The estimates for the basic reproduction number (R_0) range between 1.5 and 4.9[8-16]. In the current study, we sought to capture a wide range of possible scenarios by adopting two contrasting scenarios, as listed in Table I.

Containment: Port-of-entry screening model: First, a deterministic epidemic was simulated in Wuhan, China, governed by the equations above, to inform projections for the daily introductions of COVID-19 that would arrive on Indian airports. This simulation provided estimates for the prevalence of infection in China, denoted by $E^{(source)}(t)$ and $I^{(source)}(t)$, for the proportion of the population having asymptomatic and symptomatic infection, respectively, at time t.

Then the following stochastic process was simulated for transmission in India: (*i*) A transmission process governed by the equations above, using a simple Gillespie algorithm[17] to translate these to stochastic dynamics, assuming that infection events are independent of one another; and (*ii*) Initial conditions being zero prevalence and universal susceptibility, but with a time series of $M_E(\tau)$, $M_I(\tau)$, introductions of cases of E and I on day τ into the community, for all $\tau > 0$ (these being arrivals from China who have not been stopped at the airport).

To calculate the latter, it was assumed that each day, there were a total of A arrivals from the source region into Indian airports, ignoring seasonality or secular temporal trends. Recalling that $E^{(source)}(t)$ and $I^{(source)}(t)$ are proportions, then on any given day, the proportion of airport arrivals that is infected and asymptomatic is $E^{(source)}(t)$. If we assume that symptomatic cases are m times less likely to travel than those without symptoms, then the proportion of arrivals being infected and symptomatic is $I^{(source)}(t)/m$. Further, assuming that as a result of airport screening, a proportion pE of infected and asymptomatic cases is stopped at the airport before entering the community, and likewise for a proportion pI of infected and symptomatic cases.

Putting these factors together, the number of cases of E being introduced into the community in India, per day would be calculated as:

Introductions of E on day $\tau \sim Bin\ (A, q[\tau])$

where 'Bin' denotes a binomial distribution, and $q(\tau) = \int_{\tau} E^{(d)}(t)\,dt$

We modelled similarly for the number of introductions of I on day τ, but with the adjustment m described above.

For traveller demographics, we assume conservatively that A=500, meaning that on an average, 500 passengers are arriving per day in Indian airports, from areas in China where COVID-19 transmission is established; the prevalence of asymptomatic infection in international arrivals is the same as in their city of origin and the prevalence of symptomatic infection is half as much (m=½), assuming that symptomatics are half as likely to travel. Airline transportation data suggested that, on an average, there were 3565 passengers arriving from the entire China per day, in Indian airports, during the period from October 2018 to March 2019[18]. We expect this number to have been reduced substantially following recent travel restrictions, but the relevant data are not yet publicly available. Thus, we expect our assumption to be an underestimate.

Under the given scenarios for the proportion of asymptomatic and symptomatic cases that would go undetected by screening, we simulated the stochastic epidemic that would occur in India as a result of the daily introductions and estimated the average 'time to epidemic' as the number of days to reach a prevalence of 1000 cases. This threshold, although arbitrary,

represents a level at which it is clear that transmission has been established in India.

Mitigation: Within-country model: In the event that COVID-19 started spreading in India, we developed a mathematical model to simulate the transmission dynamics in the four most populated metropolitan areas (Delhi, Mumbai, Kolkata and Bengaluru metropolitan areas) in India, as well as their population connectivity. We chose to focus on these population centres on the assumption that the introduction of COVID-19 was most likely to occur in international transportation hubs, and thus that these cities were most likely to be the focal points of initial COVID-19 transmission in the country.

As an intervention, we modelled a 'quarantine of symptomatics' scenario wherein a proportion p of symptomatic cases was quarantined within an average of d days of developing symptoms. To incorporate this intervention, we adapted the model equations above, as follows:

$$\frac{dS_i}{dt} = -\lambda_i S_i$$

$$\frac{dE_i}{dt} = \lambda_i S_i - rE_i$$

$$\frac{dI_i^{(q)}}{dt} = rpE_i - \gamma I_i^{(q)} - \mu I_i^{(q)} - \delta I_i^{(q)}$$

$$\frac{dI_i^{(n)}}{dt} = r(1-p)E_i - \gamma I_i^{(n)} - \mu I_i^{(n)}$$

$$\frac{dQ_i}{dt} = \delta I_i^{(q)} - \gamma Q_i - \mu Q_i$$

$$\frac{dR_i}{dt} = \gamma I_i^{(q)} + \gamma I_i^{(n)} + \gamma Q_i$$

$$\lambda_i = \frac{\beta I}{N} + \frac{k\beta E}{N}$$

$$\lambda_i = \sum_{ij} \beta c_{ij} \left[\left(I_j^{(q)} + I_j^{(n)} \right) + kE_j \right] / N_j$$

where the subscript i represents city i; $I^{(q)}$ is the number with symptomatic infection who will self-quarantine after an average delay of d days; $I^{(n)}$ is the number who are symptomatic yet do not quarantine and the rate parameter δ is the inverse of the average quarantine delay, d. The infectiousness of exposed/asymptomatic cases, relative to symptomatic cases, is termed as relative infectiousness (k).

Finally, c_{ij} is the connectivity between cities i and j. We used domestic airline transportation data[18] as a proxy for c_{ij}, while also conducting a sensitivity analysis to address intercity travel through other means, including rail and road. These coefficients (c_{ij}) were estimated as a proxy for the frequency of daily population movement between cities as a proportion of the population of those cities. In sensitivity analysis, we assumed ten times the rates shown in Table II, to address the potential contributions from the lack of rail and road travel data.

Table II. Model coefficient for connectivity between cities

C_{ij}	Delhi	Mumbai	Kolkata	Bengaluru
Delhi	1	0.00045	0.00029	0.00058
Mumbai	0.00048	1	0.00019	0.00052
Kolkata	0.00032	0.00018	1	0.00025
Bengaluru	0.00058	0.00052	0.00025	1

C_{ij}, connectivity between cities i and j

Using this deterministic model, as summarized in Fig. 1, we simulated the introduction of COVID-19 and the resulting epidemic in one of the metropolitan areas. We simulated the epidemic in various scenarios for the proportion of symptomatics being quarantined; the delay to quarantine and the natural history scenarios are shown in Table I.

We present the hypothetical scenario for COVID-19 transmission and interventional effects in Delhi metropolitan area, as an illustration. We estimated the time to hypothetical peak epidemic in days. As an intervention, we modelled a scenario where a given proportion of symptomatic cases (50% at most) could self-quarantine, within a given delay after developing symptoms (at least two days). The indicators for the impact of intervention on the hypothetical epidemic scenario were reduction in cumulative incidence, peak prevalence mitigation (proportional reduction in the highest number of prevalent cases) and attack rate mitigation (proportional reduction in cumulative incidence).

Results

Containment: Airport screening: Fig. 2 shows the delays that could be achieved in the introduction of infection within India, as a result of screening airport arrivals. If symptomatic arrivals alone were screened (blue curve), the model projections for the time to epidemic ranged from 45 to 47.7 days. For illustration, we also examined the impact of screening among asymptomatic individuals (red curve). Results showed

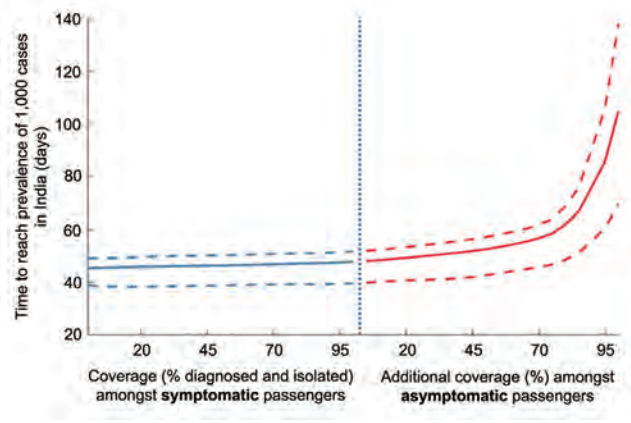

Fig. 2. Model projections for the time to epidemic in India (the time to reach a prevalence of 1000 cases), under different scenarios for the intensity of port-of-entry screening. The left half of the figure illustrates the effect, on epidemic timing, of screening symptomatic passengers alone; the right half illustrates the additional effect of diagnosing coronavirus disease-19 amongst asymptomatic passengers, assuming full screening of symptomatic passengers (infeasible, but illustrative). Solid lines show central estimates, whereas dashed lines span 95 per cent of simulated uncertainty intervals.

that identifying at least 75 per cent of the asymptomatic individuals was needed, in order to delay the within-country outbreak by an appreciable amount. Additional detection of 90 per cent asymptomatic individuals would delay the average time to epidemic by 20 days (Table III). These levels of coverage among asymptomatic cases are practically infeasible, requiring almost all passengers from the identified flights to be screened. However, this hypothetical scenario offers a helpful approach for explaining the lack of impact from addressing symptomatic cases alone (Fig. 2, blue curve). Any containment strategy focused on symptomatic infections, no matter how comprehensively tends to be negated by the asymptomatic infections that escape detection and can go on to cause onward transmission in the community.

Mitigation: Within-country interventions: Fig. 3 illustrates the hypothetical epidemic dynamics that would result in the four metropolitan areas, from an outbreak beginning in Delhi metropolitan area, and under an 'optimistic' scenario for transmission. The Figure illustrates the seeding of transmission in other cities that could arise, as a result of air transportation between these populations. The Figure also illustrates the impact of a hypothetical intervention, wherein 50 per cent of symptomatic cases are quarantined (whether voluntarily or through screening and testing), within an average of three days of developing symptoms. Such measures could reduce the peak prevalence substantially, thus minimizing the pressure on public health services. As a consequence, the intervention has the effect of 'flattening' the epidemic curve, distributing cases over a longer duration than in the absence of intervention. The intervention could reduce the cumulative incidence by 62 per cent. We next illustrate how these impacts may vary, under different transmission and intervention scenarios.

Impact of quarantine of symptomatics: In the 'optimistic' scenario, quarantining 50 per cent of symptomatic cases within three days of developing symptoms would reduce the cumulative incidence by 62 per cent and the peak prevalence by 89 per cent. By contrast in a 'pessimistic' scenario, the projected impact on the cumulative incidence falls to two per cent and the peak prevalence by eight per cent. The corresponding impact on peak prevalence is similarly low, as shown in Fig. 4.

Fig. 5 shows that the duration of the outbreak would be much lower in the scenario of 'no intervention' compared to 'intervention'. As illustrated in Fig. 3, the overall effect of symptomatic quarantine is to flatten the outbreak and increase the duration of the outbreak.

Table III. Alternate scenarios for the effect of airport entry screening of symptomatic and asymptomatic passengers on the delay in average time to epidemic (days to reach a prevalence of 1000 cases) in India by R_0 and relative infectiousness of asymptomatics

Parameters		Delay in average time to epidemic (days)		
R_0	Relative infectiousness, asymptomatic versus symptomatic	All symptomatic COVID-19 identified, but zero diagnosis in asymptomatics	All symptomatic COVID-19 identified, with 50 per cent diagnosis in asymptomatics	All symptomatic COVID-19 identified, with 90 per cent diagnosis in asymptomatics
2	0.5	1.2	5.7	16
2	0.1	2.9	7.4	20
4	0.5	0.5	1.9	5.7
4	0.1	0.8	2.9	7.9

COVID-19, coronavirus disease 19

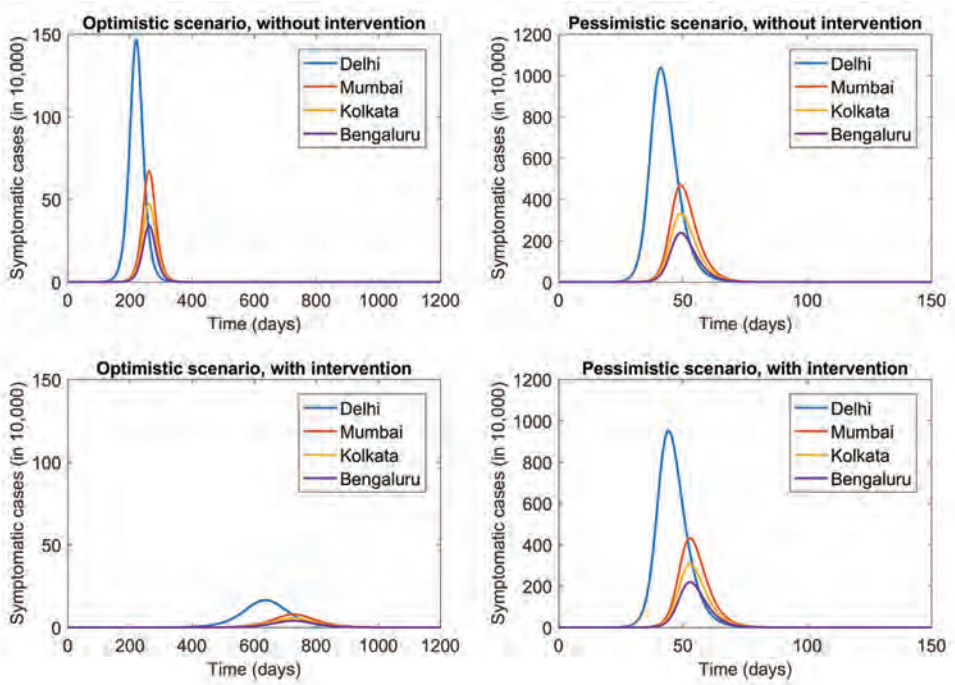

Fig. 3. Model projections for the hypothetical epidemic dynamics (symptomatic prevalence over time) with and without intervention under different scenarios for epidemiologic parameters considering an intervention, in which 50 per cent of the symptomatic cases are isolated within three days of developing symptoms.

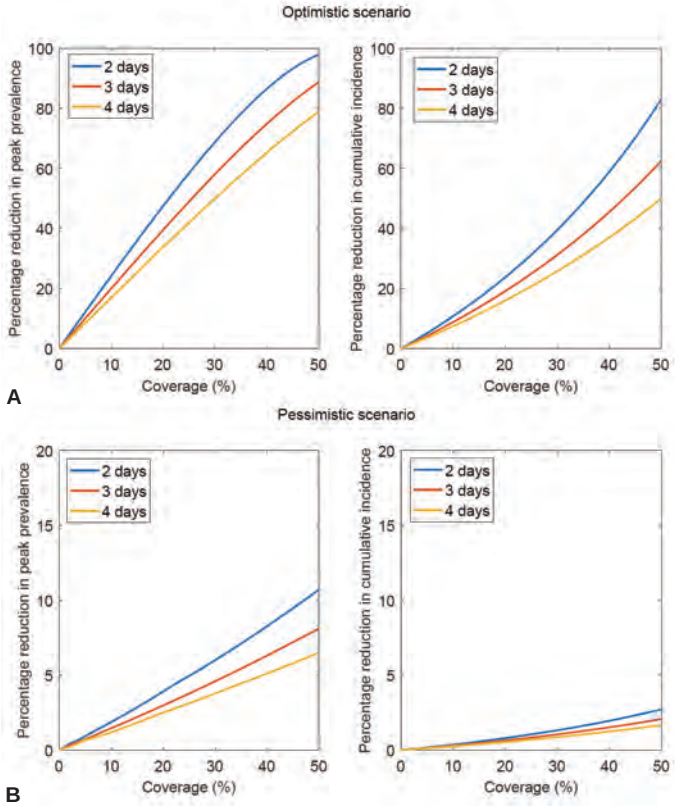

Fig. 4. Model projections for the per cent reduction in hypothetical peak prevalence and per cent reduction in hypothetical cumulative incidence by initiation of quarantine of symptomatics within two, three and four days under the 'optimistic' (**A**) and 'pessimistic' (**B**) scenarios described in the main text.

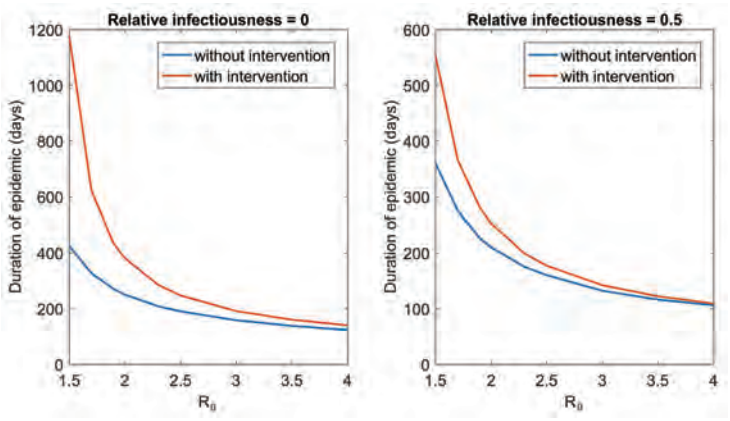

Fig. 5. Projected duration of epidemic (days) for the scenarios with and without symptomatic quarantine at 50 per cent coverage in three days by R_0 and relative infectiousness of asymptomatic cases. Here, the 'epidemic duration' is measured as the duration (in days) over which the prevalence of symptomatic infection is >1 case.

Discussion

The focus of our analysis was not towards predicting the burden of COVID-19 cases but to identify rational intervention strategies that might work towards control of the outbreak in India. We modelled the potential impact of containment strategy of point-of-entry screening and a mitigation response through symptomatic screening on hypothetical COVID-19 transmission scenario in India. Our results suggest that in order to have an appreciable effect on delaying the establishment of transmission of COVID-19 in India, airport arrival screening will need to have near-complete capture of incoming COVID-19 cases, including asymptomatic cases. Although not practically feasible using the currently available tools, our results provide a hypothetical illustration of the additional benefit of identifying asymptomatic cases: if they escape any containment effort, they would tend to negate the effects of that effort, by the onward transmission that they can cause. Presently, there is no accurate, rapid test for COVID-19 that could be deployed in this setting, to reach the required levels of detection among asymptomatic cases; the only way to reach 90 per cent diagnosis among asymptomatic arrivals may be through isolation and quarantine of all arrivals from specified origin airports. Resources may be better spent on the mitigation of infection in the community.

Recent studies indicate that airport screening may not be able to sufficiently detect COVID-19-infected travellers. Quilty *et al*[19] estimated that 46 per cent (95% confidence interval: 36 to 58) of infected travellers would not be detected by thermal screening at airport exit and entry, depending on incubation period, sensitivity of exit and entry screening and proportion of asymptomatic cases. Gostic *et al*[20] estimated that travel screening would miss more than half of the infected travellers on account of being asymptomatic and being unaware of exposure, emphasizing the need for post-travel symptom tracking among them. Our study adds to this by considering the population implications of such leakages in arrival screening. Our analysis shows that, even if symptomatic cases are comprehensively identified and quarantined, the delay in epidemic timing within India would be in days and not weeks. According to the data shared by the Delhi Health Department[21], till February 13, 2020, 17 of 5700 (0.3%) passengers, who had arrived from China and other COVID-19-affected countries prior to the beginning of airport screening from January 15, 2020, were found symptomatic and hospitalized, while the rest were advised for home isolation. The status of another 885 passengers remains unknown[21]. Entry screening or travel restrictions may be beneficial in reducing the risk of outbreak in countries with relatively low connectivity to China, and our study illustrates the critical importance of community-based measures to detect potential cases and prevent transmission.

We also examined the potential impact of quarantine of symptomatics, in controlling transmission within India, with a focus on four major metropolitan areas. Our results suggest that it may be possible to interrupt the transmission of COVID-19 in India, but only in the most optimistic scenarios (for R_0 and for coverage). Even with high R_0 and suboptimal coverage, symptomatic quarantine can still achieve meaningful reductions in peak prevalence, resulting in 'spreading out' of the outbreak. This would make it easier to cope

with the peak demand on health services. However, such measures would have very little effect on the overall epidemic size. The actual numerical impact will be highly sensitive to the natural history of COVID-19, the parameters for which are very uncertain at present.

The WHO Scientific and Technical Advisory Group for Infectious Hazards has recommended continuation of the containment strategy and monitoring for the community transmission of COVID-19[22]. It recommends close monitoring of the effectiveness and social acceptance of public health strategies to control COVID-19 transmission in the light of its evolving epidemiological understanding, including engagement of vulnerable populations, and intensified active surveillance[22].

Continuous follow up of passengers returning from COVID-19-affected countries and their contact tracing for the emergence of suggestive symptoms would put a high strain on the healthcare system, more so in the eventuality of the introduction of community transmission. The increasing numbers would make it impractical to use laboratory testing to confirm each case, and therefore, use of symptomatic surveillance should become the primary public health strategy to detect and respond in the most effective and timely manner. We could draw examples from the syndromic surveillance approach for influenza-like illness in the context of H1N1[23]. In practice, this could be achieved either through public advisories for sick individuals to self-quarantine, along with active engagement with the community, or through intensive surveillance for symptoms, followed by testing and quarantine. A combination of both approaches is likely to be needed, although promoting self-quarantine is likely to be more sustainable in the event that transmission becomes widespread. Engagement of local volunteers and community-based organizations can provide the much-needed boost to the efforts of the public health system. Considering the widespread use of mobile phones in the country, mobile applications can be used to self-monitoring and sharing of symptom information on a real-time basis. The same was done for monitoring the passengers on the cruise ship off the Japanese coast[24].

With the evolving understanding of COVID-19 epidemiology, especially the proportion of asymptomatic infected cases, it is difficult to predict the number of beds required or ventilators necessary for COVID-19 cases at this stage. As per reports from other affected countries, we may expect eight to ten severe and 40-50 non-severe COVID-19 cases for every death[25,26]. In a closed setting of similar nature as that on the cruise ship 'Diamond Princess,' we may expect 26 per cent of the entire population to get infected and one in 450 infected individuals to die[27]. We deduce that around five per cent of the infected patients will require intensive care and half of those admitted in the intensive care unit will require mechanical ventilation. Over time, once the model is validated, appropriate numbers can be generated for healthcare planning.

It is pertinent that frontline healthcare workers are identified and trained before the outbreak sets in. Health and life insurance should be announced for healthcare workers if they contract COVID-19. Considering the reports of a high number of infected healthcare workers, measures should be taken to build biosecurity wards and prepare for the outbreak in earnest. Resources should be earmarked; adequate supplies should be procured before the outbreak gains momentum. Healthcare workers should be trained in the use of personal protective equipment, screening of asymptomatic contacts, isolation measures and management of COVID-19 cases. Public health measures should be initiated at multiple levels, including but not limited to public messaging, and community health worker-based education.

Limitations of the model: As with any modelling study, our analysis has some limitations to note. The mean duration of asymptomatic and symptomatic stages is very much uncertain. Some infections may be subclinical and never develop symptoms. In the port-of-entry screening model, we adopted simple assumptions on the number of daily arrivals from non-coronavirus-affected areas due to lack of data. However, considering that we have only used data for airport arrivals and in particular from China, these assumptions are likely to be underestimates in the current situation where people are travelling from many other countries that are now reporting COVID-19 cases, and are thus conservative with respect to our conclusions; higher numbers of daily arrivals would tend to narrow the gap in epidemic timing, between baseline and interventional scenarios. Other important uncertainties include natural history parameters, for example, the average duration of infection; the incubation period and the case fatality rate. Though we have tried to address some of these uncertainties through examining different scenarios for transmission, yet we caution that our model findings may also be sensitive to these other

parameters. As more data become available about this new virus, subsequent modelling work can be refined accordingly.

For the country-level model, for simplicity, we created hypothetical scenarios only in four metropolitan areas that have the highest population density. These areas cover only about seven per cent of the total population of India. We ignored the rural population surrounded by these areas and their connectivity. Future work to address this gap will benefit from more systematic information on the rates of population flow between these different settings, data that were not available for our current study. We have simplified our meta-population model by considering constant connectivity between different cities, ignoring age-dependent mobility among the population. How seasonality will change the endemicity of COVID-19 is still unknown and hence not considered in the model. Although there appear to be differences in the immune responses of children compared to adults, for simplicity, this model has not accounted the disease prevalence with age structure.

Comparison of our projected figures with data from countries such as Japan, the Republic of Korea and Iran can help to validate our model, assuming similar transmission dynamics in India. It may be noted that our analysis is based on the available global epidemiological parameters from the initial phase of the outbreak. However, we believe that the predicted direction of the model-based impact of the proposed interventions would remain unaffected, although the onset, magnitude and timing of the simulated epidemic may change, even with the use of updated parameter values from the evolving global situation of COVID-19 epidemic. Validation of mathematical models using real-time data is important to gauge the accuracy of predicted transmission dynamics of infectious diseases. While some models for Ebola virus disease[27] provided fairly reasonable estimates, recent COVID-19 models[28] were inconsistent in their prediction.

Public health implications: At present, it is not clear to what extent the COVID-19 epidemic would establish itself in India. As the introduction of cases may take anywhere from a minimum of 20 days to a few months to be visible, we need to enhance surveillance and prepare the community in a proportionate way that is neither alarmist nor complacent. The critical concerns are the efficiency and timeliness of quarantine and isolation and the challenges of detection of COVID-19 with symptoms similar to many other lower respiratory tract infections. There is a need to engage community-based organizations that can take up the work of symptomatic surveillance, as well as raising awareness of the need for self-quarantine where possible, and referral to hospital where necessary, till infection is confirmed. Till that time, assurance of food and supplies should be given following examples of such practices in Kerala[29]. It is pertinent to engage with the media on a proactive basis with the provision of facts promptly such that reporting of these events does not create a picture of the overwhelming burden of COVID-19 in the country and lead to undue anxiety among the population that may negatively influence self-quarantine. Health authorities need to be on alert and be prepared to closely monitor the situation with the establishment of an intensified surveillance. We advocate for a rational, flexible and resilient approach that is sensitive to the outbreak stage as the health system prepares for the control of COVID-19 transmission in India.

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***Conflicts of Interest*:** None.

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Quick Response Code:

Full-genome sequences of the first two SARS-CoV-2 viruses from India

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***Background & objectives*: Since December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has globally affected 195 countries. In India, suspected cases were screened for SARS-CoV-2 as per the advisory of the Ministry of Health and Family Welfare. The objective of this study was to characterize SARS-CoV-2 sequences from three identified positive cases as on February 29, 2020.**

***Methods*: Throat swab/nasal swab specimens for a total of 881 suspected cases were screened by *E* gene and confirmed by *RdRp* (1), *RdRp* (2) and *N* gene real-time reverse transcription-polymerase chain reactions and next-generation sequencing. Phylogenetic analysis, molecular characterization and prediction of B- and T-cell epitopes for Indian SARS-CoV-2 sequences were undertaken.**

***Results*: Three cases with a travel history from Wuhan, China, were confirmed positive for SARS-CoV-2. Almost complete (29,851 nucleotides) genomes of case 1, case 3 and a fragmented genome for case 2 were obtained. The sequences of Indian SARS-CoV-2 though not identical showed high (~99.98%) identity with Wuhan seafood market pneumonia virus (accession number: NC 045512). Phylogenetic analysis showed that the Indian sequences belonged to different clusters. Predicted linear B-cell epitopes were found to be concentrated in the S1 domain of spike protein, and a conformational epitope was identified in the receptor-binding domain. The predicted T-cell epitopes showed broad human leucocyte antigen allele coverage of A and B supertypes predominant in the Indian population.**

***Interpretation & conclusions*: The two SARS-CoV-2 sequences obtained from India represent two different introductions into the country. The genetic heterogeneity is as noted globally. The identified B- and T-cell epitopes may be considered suitable for future experiments towards the design of vaccines and diagnostics. Continuous monitoring and analysis of the sequences of new cases from India and the other affected countries would be vital to understand the genetic evolution and rates of substitution of the SARS-CoV-2.**

Key words Epitope - genomes - India - Kerala - next-generation sequencing - phylogeny - real-time reverse transcription-polymerase chain reaction - severe acute respiratory syndrome coronavirus 2

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The *Coronaviridae* family encompasses viruses with a single-stranded, positive-sense RNA genome of size approximately 26-32 kb. Initially, the virus was associated with human and animal infections that caused intestinal as well as respiratory infections[1,2]. In 2002, the severe acute respiratory syndrome (SARS) coronavirus (CoV) outbreak that claimed the lives of many people in China raised the alarm towards these viruses[2]. Further, after a decade, another human pathogenic virus emerged, Middle East respiratory syndrome CoV (MERS-CoV) that affected the Middle Eastern countries[2]. Current knowledge identifies six virus groups that can infect humans[3] in the *Coronaviridae* family, which includes SARS-CoV (now termed as SARS-CoV-1) and MERS-CoV.

Recently in December 2019, China reported cases with pneumonia of unknown aetiology in the Hubei province, Wuhan city[4]. Further analysis of these cases was carried out to identify the causative agent of pneumonia[5]. Virus isolation and genomic characterization of the complete sequence of the virus through next-generation sequencing (NGS), identified it as a novel CoV, named 2019-nCoV[3]. The virus characterization revealed that it is an enveloped RNA virus with a genome size of 29,903 bp. The phylogenetic analysis of the sequence showed that it belonged to the *Sarbecovirus* subgenus of genus *Betacoronavirus* and the family *Coronaviridae*. The sequence was closely related (~87.5% sequence similarity) to two bat-derived SARS-like CoV strains (bat-SL-CoVZC45 and bat-SL-CoVZXC21) that are known to infect humans, including the virus which led to the 2003 SARS-CoV-1 outbreak[6]. The 2019-nCoV is now named as SARS-CoV-2[7]. Further, based on SimPlot analyses, it was demonstrated that SARS-CoV-2 was more closely related to the BatCoV RaTG13 sequence (~96.3% similarity) throughout the genome. The bat-SL-CoVZC45 and bat-SL-CoVZXC21 strains clustered differently from the group formed by SARS-CoV-2 and BatCoV RaTG13 in the region spanning the 3′-end of open reading frame (ORF)1a, the ORF1b and almost half of the spike region[8].

The receptor-binding domain (RBD) of the spike protein mediates interaction with the host cell receptor[9], and the angiotensin-converting enzyme 2 (ACE2) has been identified as the receptor for the SARS-CoVs[10]. Specific mutations in the RBD of the SARS-CoV-2 spike glycoprotein were found to have enhanced binding to the ACE2[11].

The human-to-human transmission of the SARS-CoV-2 created an alert with the increasing number of cases[12]. The WHO report dated February 28, 2020 confirmed 83,652 cases of SARS-CoV-2, with a total of 2,858 deaths from 52 countries[12]. After the first report of SARS-CoV-2 from Wuhan, China, the Government of India reviewed and initiated multisectoral measures for the mitigation of this emerging public health crisis. These include point-of-entry surveillance at 21 international airports, enhanced State-level surveillance programmes and preparedness for handling clinical cases in designated hospitals. Till date, the Integrated Disease Surveillance Programme (IDSP), a national health programme, Government of India, has collected samples from symptomatic travellers in liaison with the State-level Viral Research and Diagnostic Laboratories (VRDLs), Department of Health Research. These VRDLs respond for timely diagnosis during outbreaks.

The suspected samples were collected and transported to the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune, for the diagnosis of SARS-CoV-2. The specimens of the positive cases were diagnosed with real-time reverse transcription-polymerase chain reaction (RT-PCR)-specific for SARS-CoV-2 using the protocol published by the WHO[13] and characterized by complete genome sequencing and epitope prediction analyses. These sequences were also compared with the available GenBank sequences to monitor the mutations and understand their relation with other known SARS-CoV-2 available in the public database. Here, we report molecular characterization of SARS-CoV-2 sequences from three positive cases.

Material & Methods

The clinical samples were referred by the hospital authorities through the Kerala State Health Services for diagnostic purposes. Further samples were received from different parts of India for establishing the presence of SARS-CoV-2.

Detection of SARS-CoV-2 in suspected samples: Blood and throat swab (TS) specimens were collected from the suspected cases that complied with the case definition of SARS-CoV-2 infection as per the guidelines of the Ministry of Health and Family Welfare[14]. The TS was collected in viral transport medium. These samples were referred to the ICMR-NIV, Pune, India (which is the national reference laboratory for India, also referred as the government's apex laboratory). As

of February 29, 2020, 881 samples of suspected cases referred from different States, with a travel history to Wuhan, China, and other SARS-CoV-2-affected countries, were screened.

The viral RNA was extracted from the TS sample using the Magmax RNA extraction kit (Applied Biosystems, USA) as per the manufacturer's instructions. The extracted RNA was immediately used for testing the presence of SARS-CoV-2 using the real-time RT-PCR protocol published by the WHO[12] for the detection of *RdRp* (1), *RdRp* (2), *E* gene and *N* gene. *RNase P* gene was used as the internal control for the analysis. Confirmatory laboratory tests were performed as per the WHO-recommended test protocols[13]. These samples were also sequenced using the NGS approach to retrieve the complete genome of the virus.

NGS of SARS-CoV-2 from India - Phylogenetic analysis and molecular characterization: The total RNA of three positive TS specimens from Kerala, was extracted from 250-300 µl of the SARS-CoV-2 real-time RT-PCR positive samples. QIAamp Viral RNA extraction kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions. The extracted RNA was further quantified using a Qubit RNA High-Sensitivity kit (Invitrogen, USA). RNA libraries were prepared as per the earlier-defined protocol and quantified using KAPA Library Quantification Kit (Kapa Biosystems, Roche Diagnostics Corporation, USA) as per the manufacturer's protocol. Further, individual libraries were neutralized and loaded on the Miniseq platform (Illumina, USA). The detailed protocols for the steps undertaken have been published earlier[15,16]. The data generated from the machine were analyzed using CLC genomics workbench version 11.0 (CLC, QIAGEN, Germany). Reference-based mapping was performed to retrieve the sequence of the SARS-CoV-2.

Full-length genome sequences of SARS-CoV-2 were downloaded from the GISAID database[17] (Supplementary Table I). Multiple sequence alignment was performed using the MEGA software version 7.0[18] with retrieved sequences from two of the three positive cases and the available GISAID sequences. A phylogenetic tree was generated using the neighbour joining method and the Kimura-2-parameter as the nucleotide (nt) substitution model with 1000 bootstrap replications as implemented in MEGA software[18]. Per cent nucleotide divergence and amino acid (aa) divergence were calculated using the p-distance method[18]. Mutations specific to the Indian SARS-CoV-2 viruses were identified by comparing the coding regions with respect to the SARS-CoV-2, Wuhan, China (Wuhan hu-1).

Three-dimensional (3D) model of the spike protein and epitope prediction: The pre-fusion structure of the Indian case 1 SARS-CoV-2 spike (S) glycoprotein was modelled using the Swiss-Model server (*https://swissmodel.expasy.org/interactive*) and the corresponding S protein of Wuhan-Hu-1 (6VSB.PDB) as the template (99.97% identity). Sequential (linear) B-cell epitopes were predicted using BepiPred-2.0 server (*http://www.cbs.dtu.dk/services/BepiPred/*). The ABCpred prediction tool (*http://crdd.osdd.net/raghava/abcpred/*) was also used to identify the B-cell epitopes in the Indian SARS-CoV-2 sequence. The epitope prediction probability of >0.8 was set to increase the specificity of the peptide stretch. The overlapping epitopes predicted by BepiPred-2.0 online server and the ABCpred prediction tool were identified. The antigenicity of the shortlisted peptide sequences was further predicted using the Vaxijen online server (*http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html*) with a default threshold of 0.4.

Discontinuous epitopes on the modelled structure of the Indian case 1 SARS-CoV-2 spike protein were predicted using the online servers, Ellipro (*http://tools.iedb.org/ellipro/*) and DiscoTope 2.0 (*http://tools.iedb.org/discotope/*), integrated in the Immune Epitope Database. Ellipro predicts epitopes based on the protusion index (PI), wherein the protein shape is approximated as an ellipsoid (Ref for Ellipro and DiscoTope). An ellipsoid with the PI value of 0.8 indicates that 80 per cent of the residues are within the ellipsoid and 20 per cent are outside. All residues that are outside the 80 per cent ellipsoid will have a score of 0.8. Residues with larger scores are associated with greater solvent accessibility. The PI value was set to a score of 0.8. DiscoTope predicts epitopes using 3D structure and half-sphere exposure as a surface measure in a novel spatial neighbourhood definition method. Default values were set for sensitivity (0.47) and specificity (0.75) for selecting the amino acids forming discontinuous epitopes. A sensitivity of 0.47 means that 47 per cent of the epitope residues are predicted as part of the epitopes, while a specificity of 0.75 means that 25 per cent of the non-epitope residues are predicted as part of the epitopes. Outputs from both the methods were combined, and the final regions

were mapped on the modelled 3D-structure as the most probable conformational epitopes. In addition, we also predicted N-linked glycosylation sites in the S protein using NetNGlyc 1.0 Server (*http://www.cbs.dtu.dk/services/NetNGlyc/)*. The spike proteins were also screened for the presence of potential epitopes presented by major histocompatibility complex (MHC) class I molecules to cytotoxic T lymphocytes (CTLs). The online NetCTL1.2 server (*http://www.cbs.dtu.dk/services/NetCTL/)* based on machine learning techniques such as artificial neural network (ANN) and support vector machine (SVM) was used to predict the T-cell epitopes. The prediction was made for all the human leucocyte antigen (HLA) supertypes and the available human alleles. The C terminal cleavage, weight of transport-associated protein (TAP) efficiency and threshold for identification were kept as default. VaxiJen v2.0 tool was used to predict the antigenicity of the predicted epitopes (*http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html)*. The sequences were further screened to be potential epitopes using the CTLPred online server (*http://crdd.osdd.net/raghava/ctlpred/*).

The ability of the predicted linear B-cell and the T-cell epitopes to mount interferon-gamma (IFN-γ) response was assessed using the IFNepitope (*http://crdd.osdd.net/raghava/ifnepitope/index.php*).

Results

Detection of SARS-CoV-2 in suspected samples: Three of the 881 TS/nasal swab (NS) specimens from the suspected cases, tested positive for the SARS-CoV-2 using the real-time RT-PCR specific to *E* gene, *RdRp* (1), *RdRp* (2) and *N* gene. The Ct value of the *E* gene ranged from 19.8 to 34.5 for the TS/NS specimens. Detailed Ct values for the real-time RT-PCRs specific to the above-mentioned genes of the positive specimens are given in Table I. Blood samples were found to be negative for the SARS-CoV-2.

Case 1 travelled from Wuhan, China, reached India on January 23, 2020 and further travelled to the final destination of Kerala on January 24. This individual developed cough on January 25 and further experienced a sore throat and mild fever and was admitted to the General Hospital, Thrissur, Kerala. The second case travelled from Wuhan and had close contact with case 1 during the travel to the final destination in India. Case 2 developed similar symptoms along with fever and diarrhoea on January 26, and the collected TS specimens were referred to the ICMR-NIV on January 28. The second case was hospitalized on January 30, in a medical college, Alappuzha, Kerala. The clinical sample (TS) was collected on January 31, 2020. Case 3 travelled from China to India, developed a runny nose on January 30 and was admitted to the General Hospital, Kasaragod, Kerala, on January 31, 2020. TS specimens were collected on January 31, 2020.

NGS of SARS-CoV-2 from India - Phylogenetic analysis and molecular characterization: NGS analysis from the TS specimens retrieved two complete genome sequences from case 1 and case 3. The complete genomic sequence data for case 2 could not be recovered due to the lower kappa concentration of the sample and hence not included in the study for analysis. The FastQ files were reference mapped with the available Wuhan seafood pneumonia virus (Wuhan Hu-1) complete SARS-CoV-2 genome (accession number: NC 045512.2). The total reads which were mapped and the percentage of the genome recovered for the two cases are summarized in Table I.

Analysis of the complete genome sequences of SARS-CoV-2 from the positive cases in India revealed that the percentage nt and aa differences between case 1 and case 3 were 0.038 and 0.10 per cent, respectively. The sequences of case 1 and case 3 diverged from the Wuhan-Hu1 sequence by 0.017 per cent nt and 0.041 per cent aa respectively. Indian SARS-CoV-2 clustered with the *Sarbecovirus* subgenus of the *Betacoronavirus* genus and was closest to the BatCoV RaTG13 sequence (96.09% nt)[8]. The phylogenetic comparison showed the clustering of the genome sequences of case 1 and case 3 with the existing sequences of the SARS-CoV-2 sequences (Fig. 1). The phylogeny revealed emerging heterogeneity within the SARS-CoV-2 sequences globally. The Indian SARS-CoV-2 viruses were positioned in different clusters.

Indian SARS-CoV-2 sequences showed two changes 408 Arg→Ile and 930 Ala→Val in the spike protein compared to the Wuhan Hu-1 sequence. The mutations were further mapped on the spike protein model of the Indian sequence (Supplementary Fig. 1). Deletion of a three-nucleotide stretch, encoding tyrosine residue at position 144, of the spike gene was also observed in the Indian SARS-CoV-2 from case 1 when compared to the other SARS-CoV-2 sequences. As noted in the earlier SARS-CoV-2 sequences, both the Indian sequences possessed the polybasic cleavage site (RRAR) in the spike protein at the junction of S1 and S2, the two subunits of the spike protein[19].

Table I. Real-time reverse transcription-polymerase chain reaction (RT-PCR) values for *RdRp* (1), *RdRp* (2), *E* gene and *N* gene, per cent genome coverage recovered and reads mapped for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive cases

Positive cases	Ct values for real-time RT-PCR for the confirmation of SARS-CoV-2					Relevant reads	Total reads	Genome length recovered (bp)	Per cent genome coverage
	RdRp (1)	*RdRp* (2)	*E* gene	*N* gene	*Rnase P* internal control				
Case 1	33.33	27.93	34.5	33.90	Positive	20,096	5,615,846	29,854	99.83
Case 2	24.6	29	19.8	38	Positive	610	8,587,146	16,047	53.66
Case 3	34.17	32.64	28.98	36.35	Positive	11,296	1,405,038	29,851	99.83

Epitope predictions: Thirty one linear B-cell epitopes were predicted by Bepipred in the Indian SARS-CoV-2, of which three were found to have a length of <6 amino acids and hence not considered. Linear epitopes were also predicted using the ABCpred prediction tool, which predicted 47 epitopes based on the threshold of 0.8. Regions common to both the prediction methods (n=17) were identified manually. The 17 epitopes were screened for their antigenicity using the VaxiJen v2.0 tool (*http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html*), and nine of these epitopes were shortlisted. These epitopes were further screened for their ability to elicit an IFN-γ response, which was predicted using the IFNepitope tool. Finally, five epitopes, four in the S1 domain and one in the S2 domain, were predicted, which could possibly generate an immune response and suppress the IFN-γ response (Table II). N-linked glycosylation site prediction revealed that two putative glycosylation sites (with a low value for jury agreement) were present within the epitope stretch 328-344.

The discontinuous epitopes in the spike protein of the Indian SARS-CoV-2 were further identified using multiple methods, Ellipro and DiscoTope. Conformational epitopes based on these methods were mapped on the pre-fusion structure of the modelled Indian SARS-CoV-2 spike protein. The newly released structure of the SARS-CoV-2 spike protein was used as the template for modelling the Indian spike protein. Ramachandran plot statistics revealed 83.7 per cent of the residues to be in the core region, 14.4 per cent in the additionally allowed region and 0.5 per cent in the disallowed region. Four epitopes were predicted by Ellipro based on the PI threshold of 0.8 (Supplementary Table II). The result from the DiscoTope is presented in Supplementary Table III. The mapped conformational epitopes are depicted in Figure 2. For the purpose of comparison, the Indian S protein sequence was also modelled using the pre-fusion structure of SARS-CoV-1 (6ACC.PDB; 87.29% identity), and the results for the conformational epitopes predicted are in Supplementary Table IV and Supplementary Figure 2.

T-cell epitope prediction revealed 105 strong binding epitopes capable of binding to different HLA types using the NetCTL1.2 software based on the threshold of 0.4. Twelve of these were shortlisted, considering a binding efficiency of >0.5 nM and capable of eliciting IFN-γ response (Table III).

Discussion

Till February 29, 2020, three positive cases of SARS-CoV-2 were reported from India from 881 suspected cases tested at ICMR-NIV, Pune. All the three cases had a travel history from Wuhan, China, during January 2020. Although NGS was performed on the specimens for all the three positive cases, the complete genome sequence could be retrieved only from case 1 and case 3. The three cases were recovered after hospitalization and were home quarantined as per the guidelines of the Ministry of Health and Family Welfare, Government of India[14].

The low viral copy number of the TS specimen from case 2 could be the possible reason for lesser viral reads being retrieved during the NGS run, leading to a fragmented genome. The recent study from China on serial samples (TSs, sputum, urine and stool) from two patients followed days 3-12 and days 4-15 post onset[20]. *N* gene-specific real-time RT-PCR assay showed that the viral loads in TS and sputum samples peaked at around 5-6 days after symptom onset, ranging from around 10^4-10^7 copies per ml during this time[20]. In another study, the virus was detected in the saliva specimens of 11 of the 12 patients, and serial saliva testing showed declines of viral RNA levels[21].

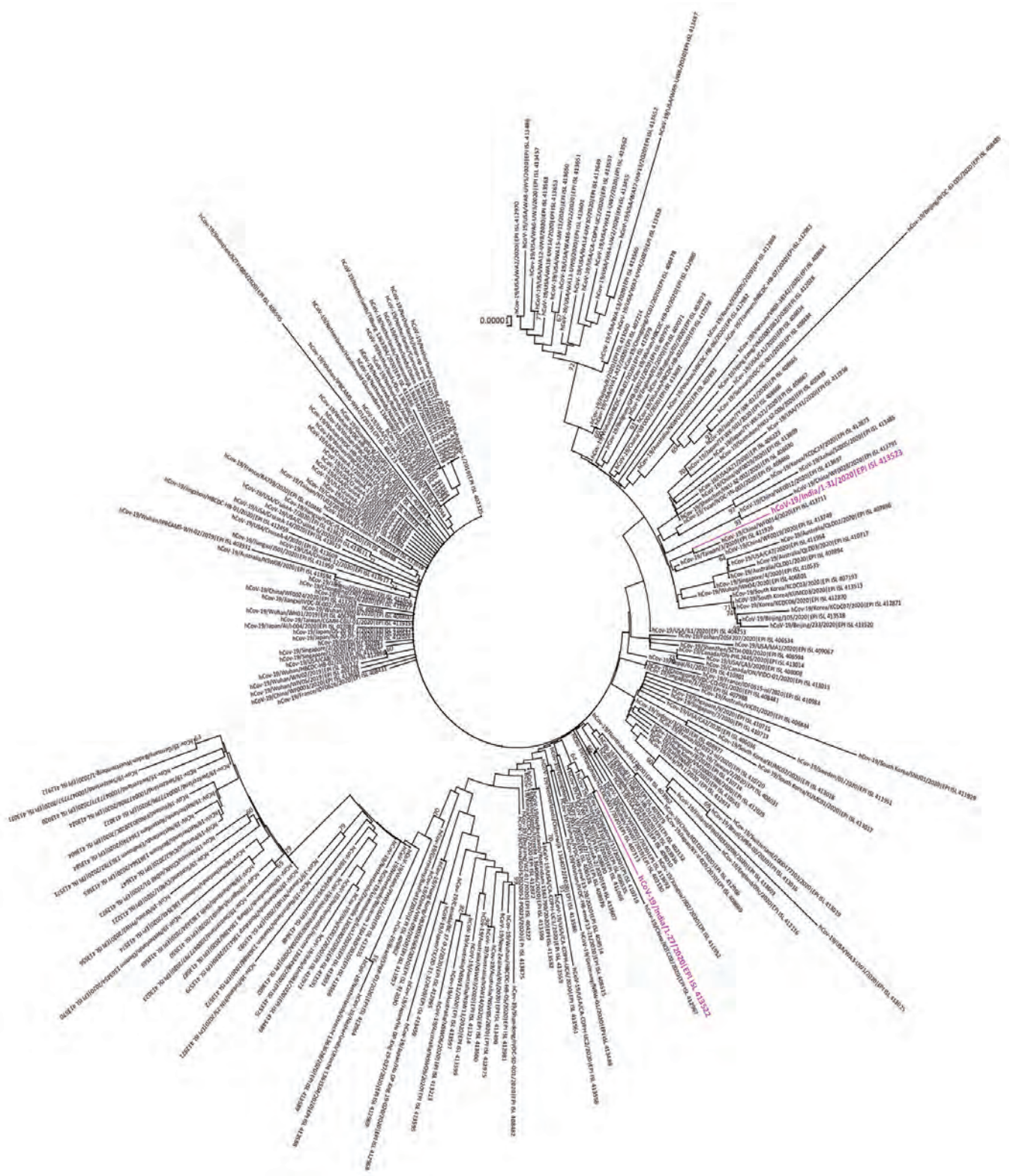

Fig. 1. Phylogenetic tree of the complete genomes of severe acute respiratory syndrome coronavirus 2 viruses. Indian viruses are shown in magenta font colour.

Table II. Linear B-cell epitopes predicted on the spike protein of the Indian severe acute respiratory syndrome coronavirus 2

Peptide	Epitope probability	Vaxigen score	Interferon (IFN)-γ response[#]
243-HRSYLTPGDSSSGWTA-258	0.92	Antigen (0.602)	Negative (1)
327-FPNITNLCPFGEVFNA-342	0.82	Antigen (0.606)	Negative (−0.132)
404-EVIQIAPGQTGKIADY-419	0.86	Antigen (1.231)	Negative (1)
413-TGKIADYNYKLPDDFT-428	0.84	Antigen (0.9642)	Negative (−0.334)
1204-YEQYIKWPWYIWLGFI-1219	0.89	Antigen (0.951)	Negative (1)

Epitopes were predicted using a combination of the Bepipred server and the ABCpred prediction server. The antigenicity was predicted using the VaxiJen v2.0 tool. IFN-γ response was predicted using the INFepitope server. [#]Values in bracket show prediction score given by the software

Table III. Spike protein peptides capable of binding to major histocompatibility complex (MHC) class I predicted using NetCTL server

Peptide	Vaxijen	Interferon (IFN)-γ response	CTLPred Score (ANN/SVM)	MHC restriction
89-GVYFASTEK-97	0.711	Positive (1)	0.58/0.986	HLA-A*1101, HLA-A3, HLA-A*3101, HLA-A68.1, HLA-B*2705
166-FEYVSQPFL-174	0.632	Positive (0.087)	0.65/0.184	HLA-A2, HLA-A*0201, HLA-A*0205, HLA-A2.1, HLA-B*2702, HLA-B*2705, HLA-B*3701, HLA-B40, HLA-B*4403, HLA-B*5301, HLA-B*5401, HLA-B*51, HLA-B60, HLA-B61, HLA-Cw*0301, H2-Kb, H2-Kk,
256-WTAGAAAYY-264	0.630	Positive (0.576)	0.82/0.544	HLA-A1, HLA-B*2702, HLA-B*3501, HLA-B*4403, HLA-B*5301, HLA-B*5401, HLA-B*51, HLA-B*5801, HLA-B62, HLA-Cw*0702
348-VYAWNRKRI-356	0.500	Positive (0.499)	0.93/0.497	HLA-A24, HLA-B*5101, HLA-B*5102, HLA-B*5103, HLA-B*51, HLA-Cw*0401, H2-Db, H2-Kd, H2-Kk
503-YQPYRVVVL-511	0.596	Positive (0.292)	0.40/0.596	HLA-A*0201, HLA-A*0205, HLA-A24, HLA-B14, HLA-B*2702, HLA-B*2705, HLA-B*3902, HLA-B*5201, HLA-B*5301, HLA-B*5401, HLA-B*51, HLA-B60, HLA-B62, HLA-B7, HLA-B8, HLA-Cw*0401, HLA-Cw*0602, H2-Dd, H2-Kb, H2-Ld
510-VLSFELLHA-518	1.077	Positive (0.268)	0.86/0.276	HLA-A*0201, HLA-A*0205, HLA-A3, HLA-B*5301, HLA-B*51, HLA-B62
825-TLADAGFIK-833	0.578	Positive (0.014)	0.75/0.992	HLA-A1, HLA-A*1101, HLA-A3, HLA-A*3101, HLA-A68.1, HLA-A20, HLA-B*2705
1058-VVFLHVTYV-1066	1.512	Positive (1)	0.77/0.779	HLA-A2, HLA-A*0201, HLA-A*0205, HLA-A68.1, HLA-A2.1, HLA-B14, HLA-B*5101, HLA-B*5102, HLA-B*5103, HLA-B*5201, HLA-B*5301, HLA-B*5401, HLA-B*51
1210-WPWYIWLGF-1218	1.495	Positive (0.221)	0.68/0.0695	HLA-B*2702, HLA-B*2705, HLA-B*3501, HLA-B*3801, HLA-B*5101, HLA-B*5102, HLA-B*5201, HLA-B*5301, HLA-B*5401, HLA-B*51, HLA-B*5801, HLA-B62, HLA-B*0702, HLA-Cw*0401, HLA-Cw*0702. H2-Ld

Threshold of >0.7 nM was used for increased specificity of the prediction. The peptides were reconfirmed using CTLPred server using default parameters. The peptides that were classified as epitopes were further checked for their antigenicity score using the VaxiJen v2.0 tool

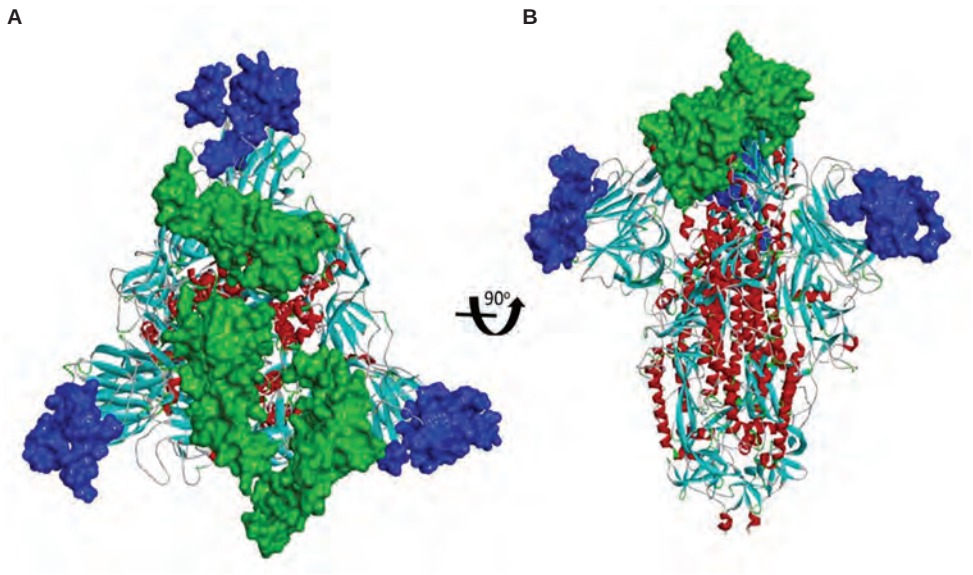

Fig. 2. Predicted conformational B-cell epitopes mapped on the pre-fusion structure of the modelled Indian severe acute respiratory syndrome coronavirus 2 spike protein using the pre-fusion structure of severe acute respiratory syndrome-coronavirus-2 (6VSB.PDB) (colour key: blue - epitopes 67-261; green - epitopes 341-507 based on the predicted epitopes as shown in Supplementary Table II). (**A**) Top view (**B**) Side view.

The two Indian SARS-CoV-2 sequences were found to be non-identical (0.04% nt divergence), and the result of phylogenetic analysis indicated that there were two different introductions into the country. A recent study using 52 published GenBank sequences showed evidence of substantial genetic heterogeneity and estimated the time to the most recent common ancestor to be December 5, 2019 (95% confidence interval: November 6 - December 13, 2019)[22]. Continuous monitoring and analysis of the sequences from the affected countries would be vital to understand the genetic evolution and rates of substitution of the SARS-CoV-2.

The comparison of the amino acid sequences of the non-structural (nsp1-nsp16) and structural polyproteins was undertaken with reference to the Wuhan-Hu1 strain for molecular characterization. Some human *Betacoronaviruses*, including HCoV-HKU1 (lineage A), have a polybasic cleavage site as well as predicted O-linked glycans near the S1/S2 cleavage site of the spike protein. As published recently, the polybasic cleavage site that has not been previously observed in related lineage B *Betacoronaviruses* and is a unique feature of SARS-CoV-2 was noted in the Indian SARS-CoV-2. The mutation Arg408Ile in the spike protein of one of the Indian sequences is noted to be in the RBD and Ala930Val, is located in the S2 domain. However, both are away from the ACE2 receptor-binding interface[19,23]. Mutations in the spike protein sequences of SARS-CoV-2 observed currently are localized over the S1 and S2 domains and, so far have not been found in the ACE2-binding interface.

From the alignment of the spike protein sequences of SARS CoV-1 and SARS-CoV-2 (Wuhan-Hu1 and India), it can be observed that the three nucleotide-deletion in the case 1 SARS-CoV-2 from India, is located close to the insert 1 region of the SARS CoV-1 (Supplementary Fig. 3). Notably, case 1 and case 2 were in close contact while travelling to India, but due to the absence of the complete genome of case 2, the genetic relatedness and source of infection could not be pinpointed.

Among the SARS-CoV structural proteins, the spike protein has been found to elicit neutralizing antibodies[24]. In this study, it was observed that of the five B-cell linear epitopes, which were predicted, four epitopes were present in the S1 domain and one in the S2 domain. Prediction of conformational B-cell epitopes revealed that one of these (residue positions 341-505) in the spike protein incorporates two of the predicted linear epitopes (327-342 and 404-419) having good antigenicity along with a favourable IFN-γ response that enables differentiation and proliferation of the B-cells[25]. Notably, an equivalent epitope (347-499) is predicted for the model generated using the SARS-CoV-1 S protein as a template. In both cases, this epitope lies within the RBD[6]. Although the epitope has two putative N-linked glycosylation sites within it at positions 330 and 332, the probability of these sites being actually glycosylated is very low. A major immuno-

dominant epitope has been reported from SARS-CoV between residues 441 and 700[26]. Hence, the predicted B-cell conformational epitope identified in the present study may play an important role in initiating a B-cell response. Among the five linear epitopes predicted in this study, epitopes 327-342 and 1204-1219 are conserved between SARS-CoV-2 and SARS-CoV-1. Epitopes 243-258, 404-419 and 413-428 are found to have variations.

The spike protein of SARS-CoV has also been reported to be immunogenic and elicit high IFN-γ-specific T-cell response[26]. The prediction results in this study revealed that nine possible CTL epitopes possessing good antigenicity and inducing IFN-γ response were present in the S protein. A recent report[27] also predicted T-cell epitopes in the S protein based on a similar ANN/SVM method and antigenicity score. Although the IFN-γ response was not considered by these authors, it was noted that two of the predictions were found to be common. Among the T-cell epitopes predicted in the present study, four epitopes 89-97 and 256-264 in the S1 domain and 825-833 and 1058-1066 in the S2 domain were found to have good CTL prediction scores with a broad HLA allele coverage of A and B supertypes. These HLA supertypes being predominant in the Indian population, the predicted epitopes may be considered suitable for future experiments towards vaccine design.

To conclude, the prompt intervention by the Government of India and the health authorities of the State of Kerala, ensured that the said cases did not become secondary foci of transmission. Further, the timely identification of SARS-CoV-2 in these suspected cases by the ICMR-NIV, Pune, has helped in the isolation of the patients, containment and enhanced surveillances for the virus and its restricted movement. The availability of the genomic sequences of the identified cases will contribute to the public repositories and help towards the development of diagnostics, vaccines and antivirals. The sequence data would also help in tracking the virus from its origin and evolution with its transmission in time.

Availability of data: Sequences are deposited in GISAID database, with accession numbers EPI ISL 413522 and EPI ISL 413523.

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Quick Response Code:

Strategic planning to augment the testing capacity for COVID-19 in India

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***Background & objectives*: Nearly 5,500 tests for coronavirus disease 2019 (COVID-19) had been conducted on March 31, 2020 across the Indian Council of Medical Research (ICMR)-approved public and private laboratories in India. Given the need to rapidly increase testing coverage, we undertook an exercise to explore and quantify interventions to increase the daily real-time reverse transcription-polymerase chain reaction (qRT-PCR)-based testing capacity over the next few months. The objective of this exercise was to prepare a potential plan to scale-up COVID-19 testing in India in the public sector.**

***Methods*: Potential increase in daily testing capacity of the existing public laboratories was calculated across the three base scenarios of shifts (9, 16 and 24 h). Additional testing capacity was added for each shift scenario based on interventions ranging from procurement of additional qRT-PCR machines, leveraging spare capacity on available qRT-PCR machines not drafted into COVID-19 testing, to in-laboratory process optimization efforts.**

***Results*: Moving to a 24 h working model in the existing approved laboratories can enhance the daily testing capacity to 40,464 tests/day. The capacity can be further bolstered by leveraging qRT-PCR and nucleic acid amplification test (NAAT)-based machines available with the Multidisciplinary Research Units (MRUs), National AIDS Control Organisation (NACO) and National Tuberculosis Elimination Programme (NTEP). Using combination/multiplex kits, and provision of automated RNA extraction platforms at all laboratories could also optimize run time and contribute to capacity increase by 1.5-2 times.**

***Interpretation & conclusions*: Adopting these interventions could help increase public sector's daily testing capacity to nearly 100,000-120,000 tests/day. It is important to note that utilization of the scaled-up testing capacity will require deployment of additional workforce, procurement of corresponding commodities for testing and scale-up of sample collection and transportation efforts.**

Key words Capacity - laboratory - real-time reverse transcription-polymerase chain reaction test

Coronavirus disease 2019 (COVID-19), first detected in China, has spread to more than 200 countries across the world[1]. The WHO declared the disease a pandemic on March 11, 2020[2]. Since January 2020, India has undertaken several measures to contain and manage the spread of the disease including international and domestic travel restrictions, rational screening and mandatory quarantines[3]. One of the key strategies for containing the disease across the world, is to undertake widespread testing for COVID-19 followed by isolation and treatment of confirmed cases and containment measures for clusters of confirmed cases[4]. The WHO recommends the real-time reverse transcription-polymerase chain reaction (qRT-PCR) diagnostic panel for the detection of 2019 novel coronavirus, a strategy that India has also adopted[5]. As on March 31, 2020, the daily testing was close to approximately 5,500 tests across public and private laboratories[6]. Delays in testing can lead to large disease cluster forming, unchecked progression of severe cases and overburdening of the health system with critically ill patients. The present study explores and quantifies interventions to scale-up qRT-PCR-based testing capacity per day across public laboratories in India. The interventions range from optimizing the existing capacity of manual qRT-PCR instruments through multiple shifts and reduction in laboratory-level manual RNA extraction effort; deploying additional manual and automated machines from other public institutes and research organizations and procuring automated high-throughput instruments. The objective of this exercise was to prepare a potential plan to scale-up COVID-19 testing in India in the public sector.

Material & Methods

The various options for scaling up testing facilities were discussed. The potential daily testing capacity calculated for various scenarios, as follows:

(*i*) Batch size = 36 of 45 possible samples (20% of slots blocked for confirmatory tests); run time/batch with manual extraction = 5 h/batch; run time/batch with automated extraction = 3 h/batch (1/4th of the public laboratories already use automated RNA extraction); no down time on machines.

(*ii*) Batch size = 36 of 45 possible samples (20% of slots blocked for confirmatory tests); run time/batch with automated extraction = 3 h/batch (all public laboratories use automated RNA extraction); no down time on machines.

(*iii*) Batch size = 45 of 45 possible samples (no slots blocked for confirmatory tests); run time/batch with manual extraction = 5 h/batch; run time/batch with automated extraction = 3 h/batch (1/4th of the public laboratories already use automated RNA extraction); no down time on machines.

(*iv*) A total of 42 additional manual qRT-PCR machines deployed from medical and research units; batch size = 36 of 45 possible samples (20% of slots blocked for confirmatory tests); run time/batch with manual extraction = 5 h/batch (all additional machines to run on manual RNA extraction); no down time on machines.

(*v*) About 10-20 per cent of available nucleic acid amplification test (NAAT)-based point-of-care (POC) testing platforms under the National Tuberculosis Elimination Programme (NTEP)[7] can be considered for COVID-19 testing; batch size = 4/run; run time/batch = 2 h/batch; 25 per cent of load reduction in the existing tuberculosis (TB) case load due to reduced footfall; no down time on machines.

(*vi*) Seventy to hundred per cent of the available automated qRT-PCR-based platforms under the National AIDS Control Organisation (NACO)[8] can be considered for COVID-19 testing; batch size = 90/run; run time/batch = 8 h/batch; 90 per cent of spare capacity available due to reduced patient footfall and potential deferral of non-diagnostic HIV viral monitoring; no down time on machines.

(*vii*) Twelve high-throughput automated qRT-PCR-based platforms to be deployed by the Indian Council of Medical Research (ICMR) (two already in country); daily capacity = 1400 samples in 24 h; 80 per cent of utilization/day; no down time on machines.

An item-wise projection was also made for commodities required to activate the scaled-up testing capacity under various assumptions on the start dates of different interventions (Table I).

Results

Several options have been proposed to be enacted upon in the short and medium term. Short-term measures can be implemented with the existing diagnostic equipment already present in the public sector. These include:

(*i*) Optimization of starting capacity: The existing 216 manual qRT-PCR machines in approved laboratories can be enhanced from one shift (9 h scenario) to two shifts (16 h scenario) and further to three shifts (24 h scenario), thereby increasing the capacity to 40,464 tests per day (Figs 1-3).

Table I. Assumptions on start dates of different interventions

Intervention	Intervention subtype	Number of machines (approx.)	Start date
Increase in working hours	Move to 16 h shifts	All	April 10
	Move to 24 h shifts	All	April 20
Redeploy qRT-PCR machines in MRUs	qRT-PCR machines in co-located MRUs	60 per cent	April 10
	qRT-PCR machines in the remaining MRUs	40 per cent	May 3
Leverage qRT-PCR machines under NACO	Co-located machines	65 per cent	May 15
	Remaining functional machines	35 per cent	June 1
Leverage NAAT POC machines under NTEP	30 per cent of ~100 machines	30	May 15
	30 per cent of ~100 machines	30	May 21
	40 per cent of ~100 machines	40	May 31
	Additional machines installed with BSL-2	150	June-December
Automated high-throughput platform to be deployed or procured by the ICMR	Delhi 1 machine	1	April 5
	Bhubaneswar 1 machine	1	April 20
	Accenture 1 machine	1	May 15
	4-10 newly procured machines installed in tranches	4-10	June 1
Automated RNA extraction machines	30 laboratories per week till all laboratories	33 per cent	May 8
		67 per cent	May 15
		100 per cent	May 23
Combination kits	Some laboratories	50 per cent	May 1
	All laboratories	100 per cent	June 1

qRT-PCR, real-time reverse transcription-polymerase chain reaction; ICMR, Indian Council of Medical Research; TB, tuberculosis; NTEP, National Tuberculosis Elimination Programme; POC, point-of-care; NAAT, nucleic acid amplification testing; NACO, National AIDS Control Organisation; MRUs, Multidisciplinary Research Units; BSL-2, biosafety level 2

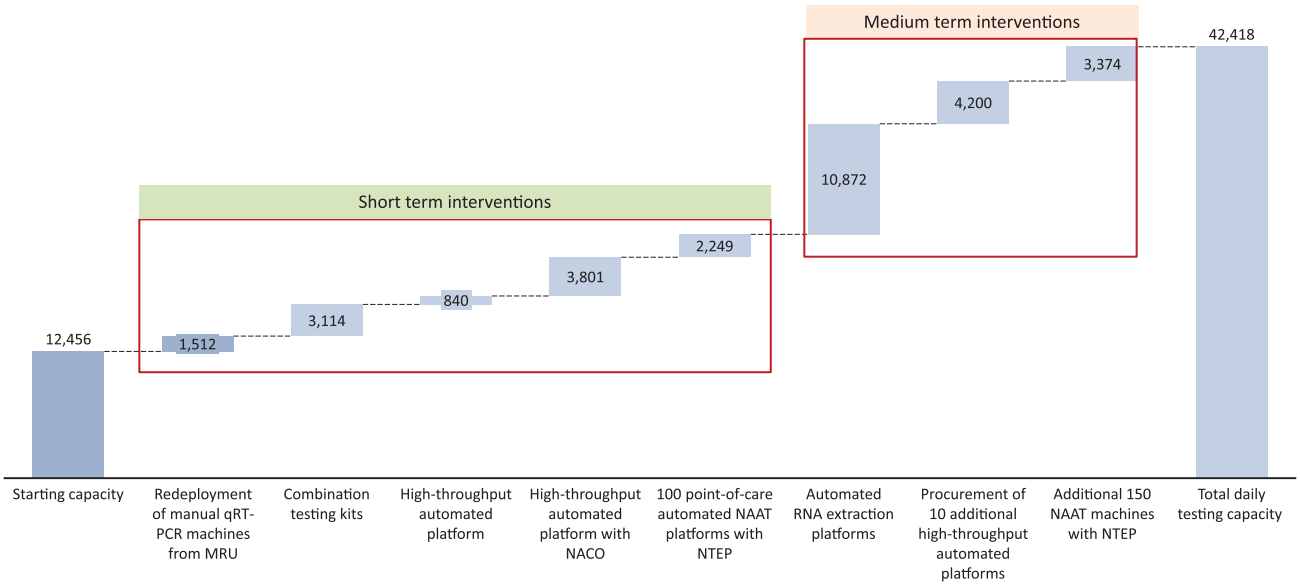

Fig. 1. Impact on overall daily testing capacity by intervention type in nine working hours (conservative). qRT-PCR, real-time reverse transcription-polymerase chain reaction; NAAT, nucleic acid amplification test; MRU, Multidisciplinary Research Unit; POC, point-of-care; NACO, National AIDS Control Organisation; NTEP, National Tuberculosis Elimination Programme.

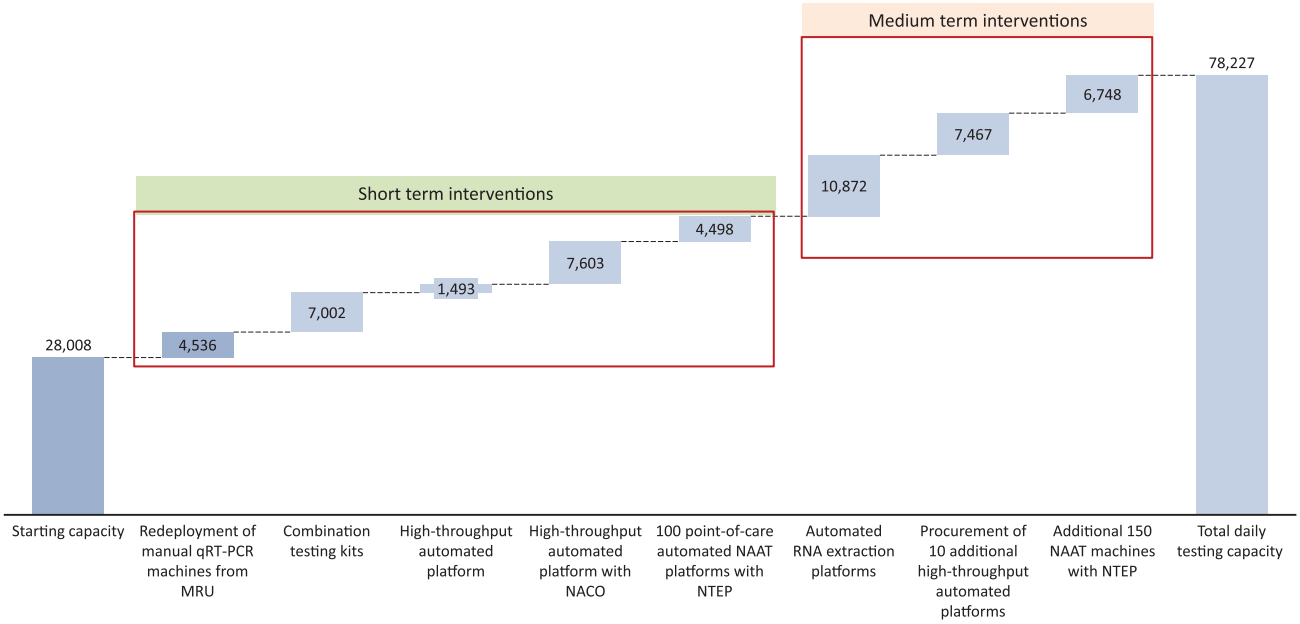

Fig. 2. Impact on overall daily testing capacity by intervention type in 16 working hours (moderate).

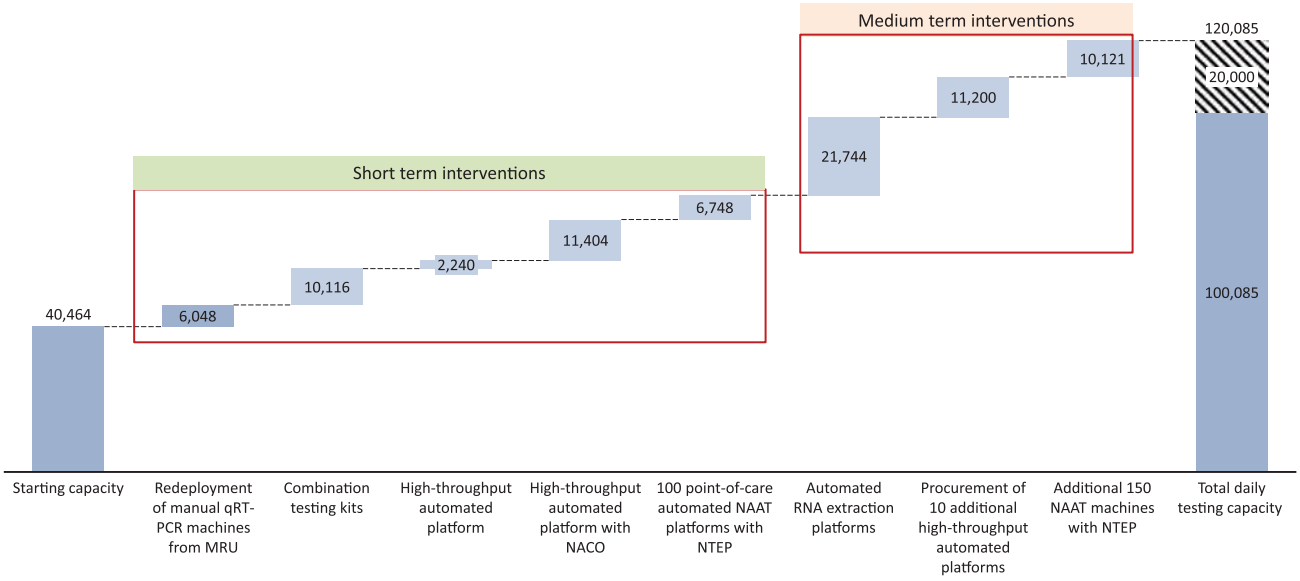

Fig. 3. Impact on overall daily testing capacity by intervention type in 24 working hours (aggressive). Grey area is the expected additional tests (20,000) that can be conducted if all the laboratory resources operate at full efficiency.

(*ii*) Redeployment of manual qRT-PCR machines: There are 42 manual qRT-PCR machines in Multidisciplinary Research Units (MRUs). These can be redeployed and allocated for COVID-19 testing.

(*iii*) Procurement of combination testing kits that can do both screening and confirmatory tests in one machine run.

(*iv*) High-throughput automated platform: Operationalizing high-throughput platforms capable of conducting up to 1400 tests/day/machine. Two machines have already been installed in the country at National Institute of Biologicals (NIB), Noida, Uttar Pradesh and Regional Medical Research Center (RMRC), Bhubaneshwar, Odisha.

(*v*) High-throughput automated platform with NACO: Leveraging spare capacity available due to reduced footfall in lockdown situation on functional automated high-throughput qRT-PCR latforms under NACO; these platforms have U.S. Food and Drug Administration (FDA) Emergency Use Authorization

(EUA) for COVID-19 testing. Currently, these are used for early infant diagnosis and HIV viral load monitoring. Two third of the functional machines have been co-located in the existing ICMR-approved laboratories.

(*vi*) Point-of-care NAAT-based automated platform with NTEP: The existing public sector laboratories approved by the ICMR cover only 114 of the 736 districts in the country; the laboratory network needs to be decentralized to increase coverage and ease sample transportation concerns. About 100 of the operational POC NAAT-based machines across the 725 districts in the country, used for TB diagnosis, are biosafety level 2 (BSL-2) approved and can be considered for capacity sharing.

For platforms under the NACO and NTEP, supply of corresponding reagents and cartridges from international suppliers will have to be secured. The ICMR has already approved some of the closed platforms with these programmes for COVID-19 diagnosis. Medium-term measures involve a lag time depending on supply-side contingencies. These include:

(*i*) Procurement and installation of automated RNA extraction platforms along with the procurement of requisite extraction kits: Currently, only about 25 per cent of the laboratories (29 laboratories) have automated RNA extraction capability, while the remaining conduct time-consuming and cumbersome manual extraction. Installation and/or operationalization of automated RNA extraction platforms supported by requisite extraction kits at the remaining 75 per cent of the laboratories could increase testing capacity by 1.5-2 times within the same operating hours.

(*ii*) High-throughput automated platform: Ten additional high-throughput automated machines may be procured and deployed in the country.

(*iii*) Point-of-care automated NAAT platform with NTEP: An additional 150 machines under the NTEP can be considered for capacity sharing after getting the required BSL-2 approvals.

Table II outlines the total commodities that would be required to utilize the scaled-up testing capacity.

Discussion

A potential plan to scale-up COVID-19 testing in the public sector was prepared in India. By implementing all the above mentioned interventions, public sector's daily testing capacity could be scaled-up to nearly 100,000-120,000 tests/day (depending on conservative, moderate or aggressive operating scenarios), which can help enhance readiness for worst-case scenario. In order to utilize the scaled-up testing capacity, increased workforce, adequate testing commodities and collection of enough samples/day would be critical. Commodities required to operationalize the plan include viral transport medium (with two swabs) commercially available, RNA extraction kits, testing kits for manual qRT-PCR (combination kits), automated qRT-PCR kits and platforms and testing kits (probe, primer and Master

Table II. Total commodities required in order to activate scaled-up testing capacity as per timelines in Table I

Items Commodities	Projected requirement						Total
	April 1-15	April 16-30	May 1-15	May 16-30	June 1-15	June 16-30	April 1 - June 30
VTM (with two swabs) (commercially available)	5,50,000	6,50,000	11,15,000	14,60,000	19,75,000	19,75,000	77,25,000
RNA extraction kits[a,b]	5,00,000	5,00,000	8,80,000	12,25,000	14,00,000	14,00,000	59,05,000
Testing kits (complete)							
For manual qRT-PCR (combination kits)[c]	0	0	2,80,000	4,20,000	14,00,000	14,00,000	35,00,000
For automated qRT-PCR (ICMR)	50,000	1,50,000	1,00,000	1,00,000	2,00,000	2,00,000	8,00,000
For automated qRT-PCR (NACO)	0	0	75,000	75,000	1,75,000	1,75,000	5,00,000
For POC automated NAAT (NTEP)	0	0	60,000	60,000	2,00,000	2,00,000	5,20,000
Testing kit (probe, primer and MasterMix separately)							
For manual qRT-PCR	5,00,000	5,00,000	6,00,000	8,05,000	0	0	24,05,000

[a]Only 1/4[th] of the laboratories out of the existing approved laboratories have automated RNA extraction capability. Additional procurement of automated RNA extraction equipment is planned. Prospectively, only automated RNA extraction kits should be procured, which are compatible with the RNA extraction machines procured, [b]The RNA extraction kits required are one per manual qRT-PCR test, [c]Projection for requirement of combination kits (screening + confirmatory). VTM, viral transport medium

Mix separately) for manual qRT-PCR. Additionally, all private and government medical colleges may be urged to eventually create the state-of-the-art virology laboratories and support the country's fight against COVID-19. Furthermore, sample collection and transportation efforts will have to keep pace with the increased available testing capacity at the laboratories.

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***Conflicts of Interest*:** None.

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Laboratory preparedness for SARS-CoV-2 testing in India: Harnessing a network of Virus Research & Diagnostic Laboratories

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***Background & objectives*: An outbreak of respiratory illness of unknown aetiology was reported from Hubei province of Wuhan, People's Republic of China, in December 2019. The outbreak was attributed to a novel coronavirus (CoV), named as severe acute respiratory syndrome (SARS)-CoV-2 and the disease as COVID-19. Within one month, cases were reported from 25 countries. In view of the novel viral strain with reported high morbidity, establishing early countrywide diagnosis to detect imported cases became critical. Here we describe the role of a countrywide network of VRDLs in early diagnosis of COVID-19.**

***Methods*: The Indian Council of Medical Research (ICMR)-National Institute of Virology (NIV), Pune, established screening as well as confirmatory assays for SARS-CoV-2. A total of 13 VRDLs were provided with the *E* gene screening real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay. VRDLs were selected on the basis of their presence near an international airport/seaport**

[†]Equal contribution

and their past performance. The case definition for testing included all individuals with travel history to Wuhan and symptomatic individuals with travel history to other parts of China. This was later expanded to include symptomatic individuals returning from Singapore, Japan, Hong Kong, Thailand and South Korea.

***Results*: Within a week of standardization of the test at NIV, all VRDLs could initiate testing for SARS-CoV-2. Till February 29, 2020, a total of 2,913 samples were tested. This included both 654 individuals quarantined in the two camps and others fitting within the case definition. The quarantined individuals were tested twice - at days 0 and 14. All tested negative on both occasions. Only three individuals belonging to different districts in Kerala were found to be positive.**

***Interpretation & conclusions*: Sudden emergence of SARS-CoV-2 and its potential to cause a pandemic posed an unsurmountable challenge to the public health system of India. However, concerted efforts of various arms of the Government of India resulted in a well-coordinated action at each level. India has successfully demonstrated its ability to establish quick diagnosis of SARS-CoV-2 at NIV, Pune, and the testing VRDLs.**

Key words COVID-19 - diagnosis - preparedness - quality control - quarantine - severe acute respiratory syndrome-CoV-2 - Virus Research and Diagnostic Laboratory

Coronaviruses (CoVs) are a group of enveloped viruses with non-segmented positive sense RNA belonging to the family Coronaviridae and the order Nidovirales. On the basis of phylogenetic clustering, they are classified into three different genera: alpha, beta and gamma. While alpha and beta types have mammalian hosts, gamma type CoVs have avian hosts. Alpha- and beta-CoVs are widely distributed in humans and other mammals including bats and cause mild respiratory infections[1]. However, two beta coronaviruses causing severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) were responsible for widespread epidemics with a case fatality rate of 10 per cent for SARS[2] and 35 per cent for MERS CoVs[3].

The World Health Organization (WHO) reported cases of pneumonia of unknown aetiology in Wuhan city, Hubei province of People's Republic of China, on December 31, 2019[4]. On January 7, 2020, Chinese authorities officially announced that the illness was caused by a novel CoV. The WHO has named the disease as COVID-19[5], and based on its similarity to SARS-CoV (2002-2003), the CoV Study Group of the International Committee on Taxonomy of Viruses (ICTV) has named the virus as SARS-CoV-2[6]. A viral genome sequence was released in public domain on January 10, 2020 (Wuhan-Hu-1, GenBank accession number MN908947[7]), followed by four other genomes deposited on January 12, in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data (GISAID). The novel beta coronavirus shows 89 and 82 per cent nucleotide identity to bat CoV, CoVZXC21, and SARS-CoV (2002-2003), respectively[8]. Since its emergence, the disease has rapidly spread to neighbouring provinces of China as well 53 other countries through international travel[9]. Infection is spread through droplets or prolonged contact with infected patients[10].

Virus isolate is the gold standard for establishment and standardization of assay performance. Since SARS-CoV-2 virus isolate was not available earlier, based on the genetic sequence of SARS-CoV-2 and closely related SARS-CoV (2002-2003), the WHO shared protocols (*E, N, RdRp* and *S* genes) for screening and confirmation of probable cases[11].

Here, we briefly describe the efforts made by the Government of India (GoI) towards reducing the risk of emergence of COVID-19 in India. We also provide a detailed description of the role of a well-established countrywide network of Virus Research and Diagnostic Laboratories (VRDLs) which could be rapidly enabled to scale up testing capacity for SARS-CoV-2 in different parts of India.

Material & Methods

Identifying suspected cases and contacts: Screening of passengers returning from China was initiated on January 18, 2020, at seven different airports throughout India and subsequently extended to 21 different airports. Universal thermal screening of all passengers has been made mandatory for all flights from Singapore, Japan and South Korea besides China and Hong Kong. Screening of passengers was also initiated at 12 major

seaports and all minor ports in the country to identify crew and passengers travelling back from China and to undertake required measures for isolation if found symptomatic. In addition, the GoI also initiated screening at all integrated checkposts from Nepal in the States of Uttar Pradesh (UP), Uttarakhand, West Bengal, Sikkim and Bihar.

Individuals evacuated from China and Japan: The GoI evacuated Indian citizens residing in Wuhan and neighbouring cities in the Hubei province of China. Two subsequent evacuations were undertaken on January 31 and February 1, 2020. A total of 654 individuals (including 645 Indians residing in Wuhan, 7 Maldivian nationals and 2 members from the crew) were brought back in dedicated aircrafts. Similar operations were conducted to evacuate 112 individuals from Wuhan (including 76 Indians and 36 people from Madagascar, Maldives, Myanmar, South Africa and the USA) and 124 from Diamond Princess ship, Japan (including 119 Indians and 5 people from Nepal, Peru, South Africa and Sri Lanka) on February 27, 2020. All the individuals were quarantined in two separate facilities close to New Delhi, India. The facilities were managed by the Army and Indo-Tibetan Border Police.

Inclusion criteria for testing for COVID-19: The following inclusion criteria were followed for considering samples for COVID-19 testing: (*i*) Symptomatic (fever, sore throat, running nose, dyspnoea, *etc*.)/asymptomatic individuals returned from Wuhan, China, after 15 January 2020; (*ii*) Only symptomatic individuals returned from rest of China, which was subsequently expanded to include Hong Kong, Japan, South Korea, Singapore, Iran and Italy, were also included; (*iii*) Close contacts of confirmed positive cases of COVID-19 infection; and (*iv*) All individuals evacuated from Wuhan, China, and Diamond Princess ship, Japan, in four different operations.

Inclusion criteria for laboratory testing are evolving in India, given the increased number of cases of COVID-19 being reported from different countries[10].

Sample collection and transport: For all suspected cases with symptoms and/or travel history, nasopharyngeal and/or oropharyngeal swabs were collected in viral transport medium. Samples were sent to testing laboratories at 4ºC with ice packs within 24 h of collection. Special provision was made with the national and international courier agencies to facilitate uninterrupted sample transport from field to laboratories and for quality control purpose.

For a subset of the individuals, blood samples were collected in serum separator and EDTA (ethylenediaminetetraacetic acid) tubes for serum and plasma, respectively. Stool and urine samples were also collected from the laboratory-confirmed positive cases. Since SARS-CoV-2 is a new virus and diagnostic tests are evolving rapidly, it was considered critical to have various types of clinical samples for standardization and validation of new tests. However, owing to very low positivity rates of SARS-CoV-2 infection, it was subsequently decided to collect blood samples from only laboratory-confirmed positive cases and review the strategy later.

Diagnostic assays for SARS-CoV-2: Following release of the first sequence results of the SARS-CoV-2 virus by China[7], candidate diagnostic real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays were designed and made available in the public domain for researchers[12]. The first-line screening assay targeted the SARS-CoV-2-specific *E* gene. Confirmatory assays targeted the '*RdRp* gene', '*N* gene' and '*ORF-1b*'. Positive control materials for these assays were obtained from Charité, Berlin, via EVAg[13]. Known copy numbers of *in vitro* transcribed RNA standard were used as the positive controls for the rRT-PCR assays. Detection of RNAse *P* gene was used as an internal positive control to monitor sample quality, RNA extraction and detection of PCR inhibitors. The Indian Council of Medical Research (ICMR)-National Institute of Virology (NIV), Pune, which is the apex laboratory for viral diagnosis and research in India, optimized the conventional and real-time PCR assays targeting different genomic regions of SARS-CoV-2 and initiated testing of suspected cases.

Laboratory organization for SARS-CoV-2 testing in India: The Department of Health Research (DHR)/ICMR initiated establishment of a network of public health laboratories to enhance capacity for diagnosis and detection of viruses of public health importance in the Indian setting in 2013. Over the years, this network has been expanded, and currently, a total of 106 VRDLs have been established throughout the country (Fig. 1). Detection of viral pathogens using serological methods and molecular diagnostic tools is the major focus of the VRDLs. In addition, a subset of these also have capabilities to perform

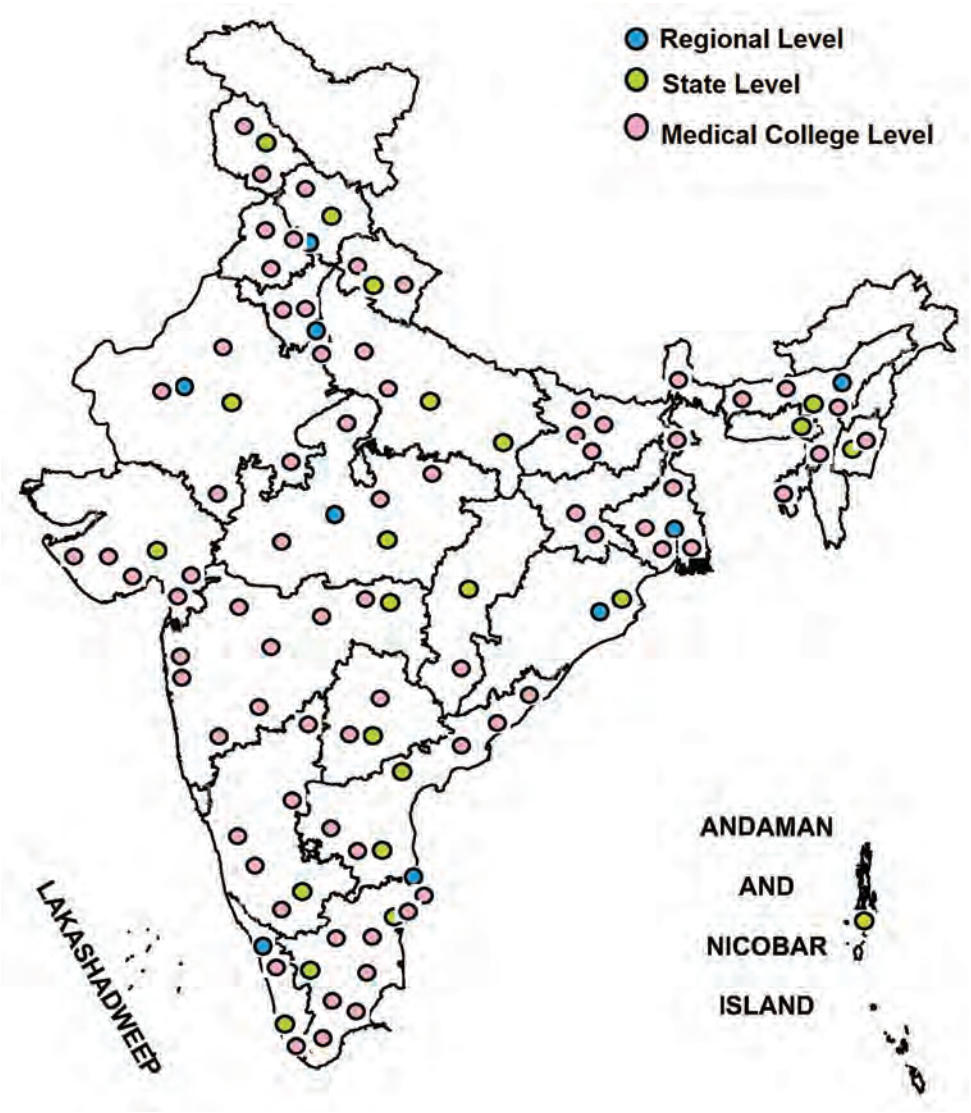

Fig. 1. Virus Research and Diagnostic Laboratory Network of 106 laboratories in India. *Source*: Map outline reproduced with permission from Survey of India, Department of Science & Technology.

cell culture and virus cultivation. All established VRDLs are equipped to perform testing for viral aetiologies at least under Biosafety Level 2 (BSL-2) conditions. Further, 10 VRDLs are in various stages of operationalization of a BSL-3 facility for detection of high-risk pathogens[14].

The NIV, Pune, functions as the resource centre for the VRDL network and is responsible for providing technical training for performing molecular and serological assays for virological diagnosis. It also performs the important task of standardizing assay procedures for the network as well as quality control and quality assurance activities.

VRDLs as State nodal centres for coordination of sample collection and shipment: In the last week of January 2020, the DHR/ICMR provided directives on sample collection and transport to all the 106 VRDLs currently under the network. Video conferences were held with VRDLs where the directives were explained and issues regarding sample collection and shipment were discussed. From each State or Union Territory, one VRDL was designated as the nodal centre for coordination of collection and shipment of samples to testing laboratories. On collection of sample(s) from any suspected case, the hospital or nearest VRDL was expected to contact the designated nodal centre for that State. The role of the nodal centre was to enable timely

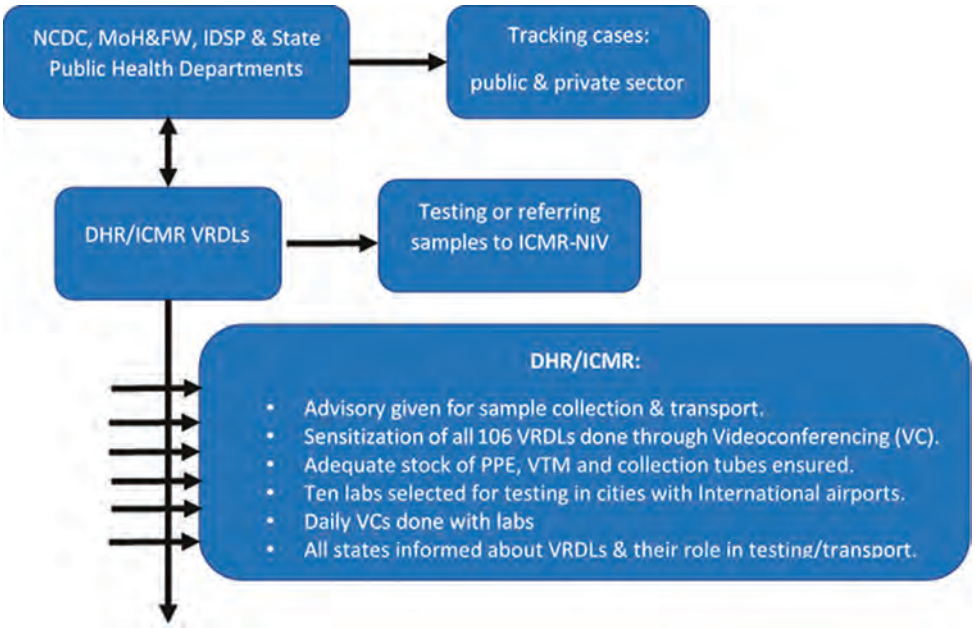

Fig. 2. Process from sample collection in the field, transportation, testing and reporting of results. NCDC, National Centre for Disease Control; MoH&FW, Ministry of Health & Family Welfare; IDSP, Integrated Disease Surveillance Programme; DHR, Department of Health Research; ICMR, Indian Council of Medical Research; VRDLs, Virus Research and Diagnostic Laboratories; ICMR-NIV, ICMR-National Institute of Virology.

and proper transport of the collected samples from the VRDLs to the designated testing laboratory.

The National Centre for Disease Control (NCDC), Ministry of Health and Family Welfare, Integrated Disease Surveillance Programme (IDSP) and State public health departments were informed regarding the nodal centres and assigned testing laboratories for the respective States. The VRDLs complemented the IDSP in an effective way. Flow diagram depicting the complete process from sample collection in the field, transportation, testing and reporting of results is shown in Figure 2.

All the laboratories were supplied with the primers, probes, PCR reagents, positive and negative controls and standard operating procedure (SOP) for the real-time RT-PCR (rRT-PCR) assay by NIV, Pune. A stringent inventory control was maintained at NIV and VRDLs based on the upsurge in number of cases and evolution of the disease.

Expansion of testing capabilities and selection of testing laboratories for SARS-CoV-2: Following the increase in the load of screening samples from suspected cases with symptoms and travel history to China or asymptomatic persons with travel history to Wuhan after January 15, 2020, it was decided that strategically located VRDLs needed to start testing for SARS-CoV-2 in addition to the apex laboratory at NIV, Pune. VRDLs were chosen based on their location in cities with international airports receiving travellers from China, the capability of the VRDLs to perform real-time PCR assays and their involvement in the ongoing testing for influenza viruses within the network. A total of 13 VRDLs across 11 States were selected for SARS-CoV-2 testing. After February 29, 2020, based on the upsurge in the number of suspect cases, primarily due to outbreaks reported from countries other than China (Iran, South Korea, Italy and Japan), the number of testing laboratories was scaled up to a total of 31 laboratories. It is eventually proposed to involve all 106 VRDLs in COVID-19 testing or sample collection and transportation. The distribution of COVID-19 testing (operational and new) laboratories in India is depicted in Figure 3.

Before initiating testing of clinical specimens from suspected cases of SARS-CoV-2, each VRDL shared results from the rRT-PCR runs performed with positive and negative controls with the apex laboratory (NIV, Pune). After satisfactory performance of the laboratories, independent testing was initiated in the designated catchment areas. Frontline screening rRT-PCR test targeting SARS-CoV-2-specific *E* gene was performed. Initial support was also provided to NCDC, New Delhi, for initiation of SARS-CoV-2 testing.

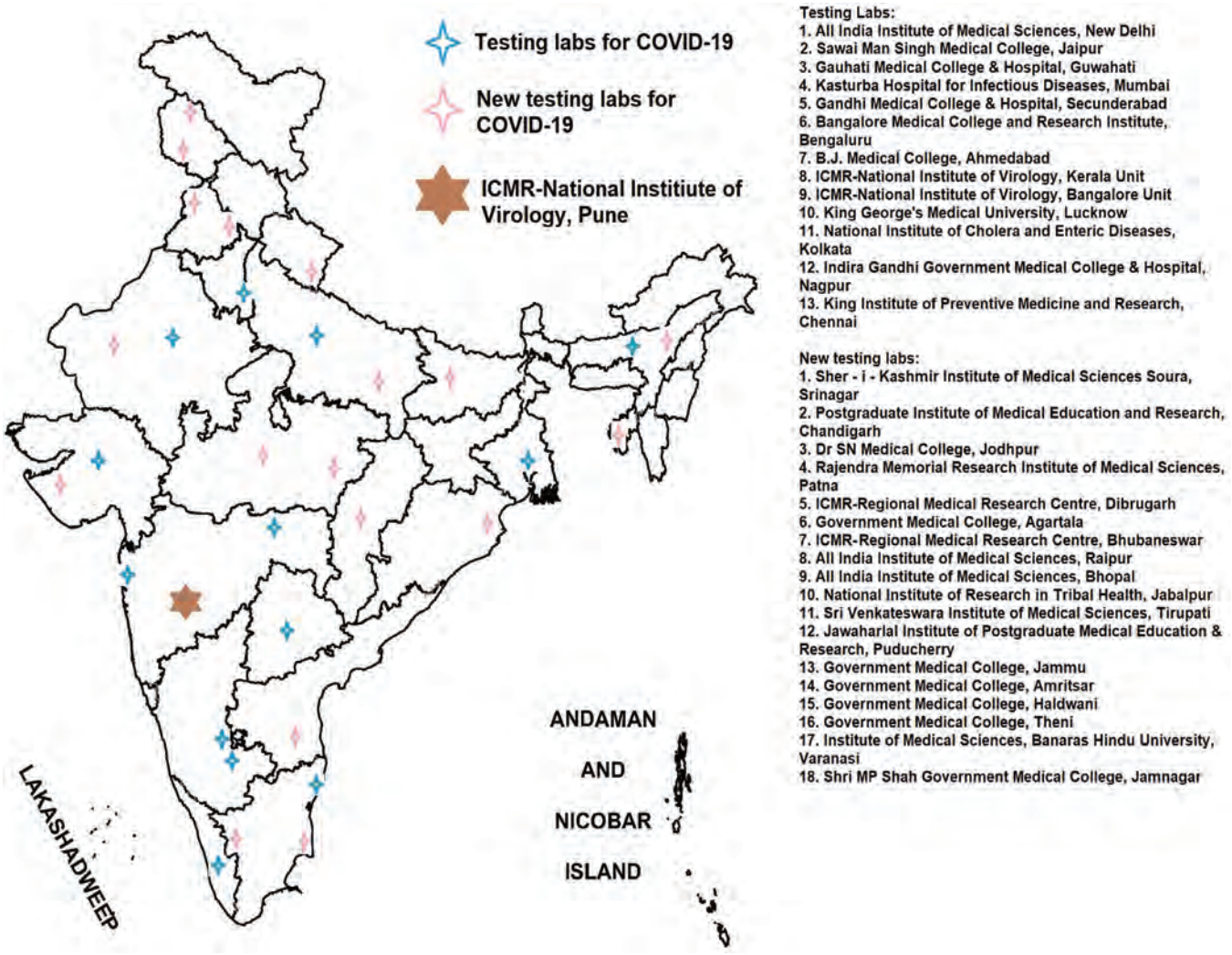

Fig. 3. Testing laboratories for severe acute respiratory syndrome coronavirus 2. *Source*: Map outline reproduced with permission from Survey of India, Department of Science & Technology.

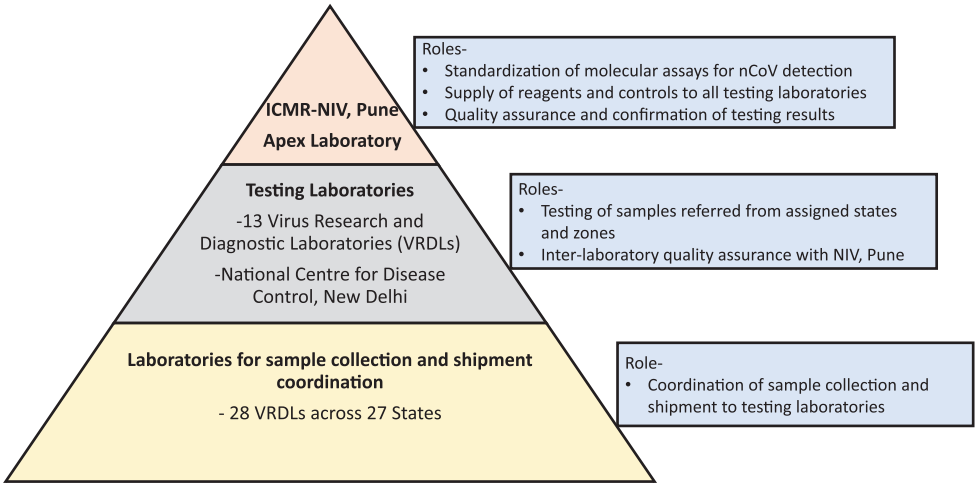

Fig. 4. Organization of laboratories for severe acute respiratory syndrome coronavirus 2 diagnosis in India and their respective roles.

Thereafter, NCDC, Delhi, initiated independent testing; however, results were shared with ICMR on a daily basis.

Figure 4 depicts the organization of laboratories for SARS-CoV-2 diagnosis and their respective roles.

Quality control for SARS-CoV-2 testing at VRDLs: NIV, Pune, coordinated the quality control activities for SARS-CoV-2 testing VRDLs. The laboratories shared first 10 negatives, all positive and equivocal samples for SARS-CoV-2 to NIV, Pune, for confirmation. Samples tested positive at VRDLs were subjected to confirmatory tests at NIV, Pune. The final result for any SARS-CoV-2-positive samples was released only after confirmation at the apex laboratory. Ten negative samples from VRDLs were also randomly picked up and subjected to next-generation sequencing (NGS).

Results

Testing of suspected cases and contacts: Based on the inclusion criteria, as on February 29, 2020, a total of 1,369 individuals including suspected cases and contacts were tested at the designated laboratories. The testing VRDLs as well as NIV, Pune, had evaluated a varying number of samples from suspected cases/contacts as follows: NIV, Pune (n=343); All India Institute of Medical Sciences (AIIMS), New Delhi (n=16); NIV Field Unit, Bangaluru (n=152); NIV Field Unit, Kerala (n=435); National Institute of Cholera and Enteric Diseases, Kolkata (n=42); Kasturba Hospital for Infectious Diseases, Mumbai (n=49); Sawai Man Singh Hospital, Jaipur (n=68); King George's Medical University, Lucknow (n=54); Gandhi Medical College, Secunderabad (n=93); King Institute of Preventive Medicine and Research, Chennai (n=52); Indira Gandhi Government Medical College & Hospital, Nagpur (n=21); Bangalore Medical College and Research Institute, Bengaluru (n=26); Gauhati Medical College, Guwahati (n=6); and B.J. Medical College, Ahmedabad (n=12). Repeat testing of suspect cases was also done at VRDLs (numbers excluded). Eleven VRDLs initiated independent testing between February 1 and 3, 2020. Two VRDLs at Ahmedabad and Guwahati were added from February 8, 2020 as per need. Time taken by all VRDLs from receipt of reagents to initiation of independent testing ranged between 24-36 h. The turnaround time for testing was 12-24 h depending on the number of samples tested. Results were communicated to the State IDSP and/or the referring contact point. Initial numbers referred to the testing network were high due to increased number of travellers returning from affected countries. However, this number declined after travel restrictions were imposed. As the VRDLs established testing for SARS-CoV-2, the number of samples referred to NIV dropped.

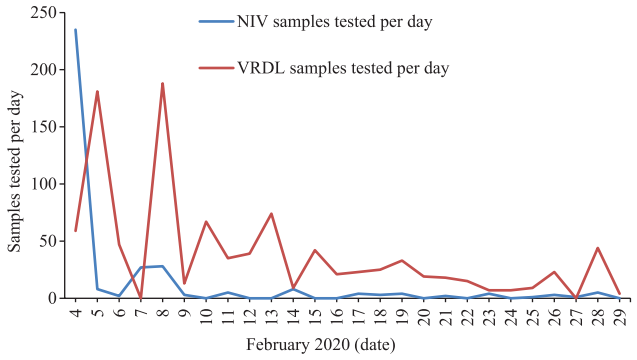

Fig. 5. The incremental decline in number of samples referred to the National Institute of Virology after testing at the Virus Research and Diagnostic Laboratories was established.

Figure 5 depicts the incremental decline in number of samples referred to NIV after testing at VRDLs was established.

Testing of evacuees from quarantine camps: On January 31 and February 1, 2020, the GoI evacuated 654 individuals from Wuhan. All these evacuees were quarantined for 14 days at two centres (Armed Forces Medical Services camp at Manesar and Indo-Tibetan Border Police Chhawla camp)[15]. On the day of arrival (day 0), nasopharyngeal and/or oropharyngeal samples were collected from all these individuals. The samples were tested for SARS-CoV-2 at ICMR-NIV, Pune, and DHR/ICMR Regional VRDL at AIIMS, New Delhi. All samples were tested negative. Between days 0 and 14, symptomatic quarantined individuals were subjected to repeat testing of SARS-CoV-2. At the end of two weeks, repeat samples were collected from all the 654 individuals and retested at the two sites. All samples were again tested negative. Subsequently, samples were tested from the second set of evacuees from Wuhan (112) and Diamond Princess ship, Japan (124), on day 0 at the same sites. A total of 1,544 samples were tested, and all were negative.

Demographic profile of individuals screened for COVID-19

Evacuees from China and Japan quarantined at army and border police camps: Of the 654 evacuees from

Wuhan, 29.2 per cent (191/654) were female and 70.5 per cent (461/654) were male. Gender information was not available for two evacuees. Manesar camp had 248 males, whereas the remaining 406 including 191 females were quarantined at ITBP Chhawla camp. The mean age of the evacuees was 23.5 yr (median: 22 yr; range: 1-72 yr); 77.2 per cent (492/637) of the evacuees for whom information about age was available were ≤25 yr of age.

The second batch of quarantined individuals at these two camps arrived on February 27, 2020 which included a total of 236 evacuees from China and from the cruise ship Diamond Princess off the coast of Japan. The second batch of quarantined individuals included a total of 41 females and 195 males. The mean age was 29.4 yr (median: 29 yr; range: one year nine months-56 yr); 76.3 per cent (180/236) were between 25 and 50 yr of age. All these were also tested negative.

Individuals tested for SARS-CoV-2 other than evacuees who were quarantined: Of the 1,369 individuals other than evacuees who were tested, all demographic details were available for a total of 1,263 (843 male, 420 female) individuals. The mean age of the individuals who underwent testing was 25.9 yr (median: 27 yr; range: eight months-76 yr). A little more than half of these individuals were in the 25-50 yr age group (54.2%).

Samples from three suspected cases with symptoms of acute respiratory illness were tested positive for SARS-CoV-2 at NIV, Pune. The first positive case was confirmed on January 30, 2020, followed by two other cases on February 2 and 3, 2020. All the three positive cases had travel history to Wuhan, China, and belonged to the State of Kerala. A total of 67 samples from 64 individuals with contact history with the three confirmed cases who developed respiratory symptoms were also tested for SARS-CoV-2. All the samples from the 64 contacts were negative for SARS-CoV-2.

Symptomatology and travel history of individuals screened for COVID-19 infection outside the quarantine camps: Of the total 1263 individuals, 19 per cent (n=240) did not report any specific symptoms for inclusion for COVID-19 testing. Among those reported as symptomatic, complete details of clinical history were accessed for 292 individuals. The presence of cough was the most common symptom reported in 49.7 per cent (n=145). Sore throat was reported by 21.2 per cent (n=62), whereas 34.6 per cent of the individuals (n=101) were febrile at the time of presentation; 17.1 per cent (n=50) of all symptomatic individuals presented with nasal discharge and rhinitis. Diarrhoea was a presenting symptom for only 1.4 per cent (4/292) of all symptomatic patients with available clinical history. Of the 1,263 individuals for whom travel-related data were available, any history of foreign travel was documented for 1,081 individuals (85.6%); 19.32 per cent (n=244) had a history of travel to Wuhan. A further 38.1 per cent (481/1263) had a history of travel to areas in China other than Wuhan, whereas 15.7 per cent (198/1263) had a history of travel to South-East Asian countries such as Thailand, Singapore, Malaysia, Indonesia and Vietnam. The remaining 158 (12.5%) individuals did not have any pertinent overseas travel history.

Quality control for ongoing testing: For quality control purposes, all testing VRDLs performing the *E* gene screening assay shared the first 10 negative samples and all positive or equivocal/borderline testing samples with ICMR-NIV, Pune, for reconfirmation. Eleven negative samples further subjected to NGS revealed negative results for SARS-CoV-2 but tested positive for other viruses: influenza A (2) and rhinovirus (3).

A total of 126 samples which tested negative by the *E* gene rRT-PCR screening assay at the 13 testing VRDLs were shared with NIV, Pune, till February 29, 2020, and these were all confirmed negative . The concordance for the negative samples was, therefore, 100 per cent.

The VRDLs also shared 13 samples which were borderline positives and showed amplification at late Ct values (range: 33-37 cycles) for confirmation. These included seven follow up samples collected from the three laboratory-confirmed positive cases which were also tested positive for the *E* gene at the testing VRDL. Testing results were concordant for five of seven follow up samples. The remaining six samples which showed borderline positivity with the screening qPCR at the testing VRDLs were found to be negative with the confirmatory qPCR assays performed at NIV, Pune.

Inventory control: Inventory control posed several challenges. Primers and probes were ordered by the NIV, Pune, once the laboratory protocols were shared by the WHO on January 15, 2020. Since nature of spread of the disease was unknown, testing reagents were stockpiled for only 5000 tests initially. NIV initiated testing from January 21, 2020. However, in

view of upsurge in global cases and deaths, the testing capacity was upscaled to another 13 laboratories and inventory at NIV was scaled up to 25,000 tests by February 1, 2020. Following further upsurge in global cases and deaths by February 25, 2020, the inventory was upscaled to 70,000 tests. Daily situational analysis of inventory at the apex and testing laboratories was undertaken to ensure optimum supplies and avoid exhaustion of reagents at the apex and testing laboratories.

Discussion

Global epidemics due to emerging/re-emerging infectious diseases have become common over the past two decades, with the major reason being increased trade and travel, leading to movement of people across borders. A recent example is the epidemic of SARS-CoV-2 in Hubei province of China which has spread to more than 70 countries within a two-month time. During the initial period, virus sequences were unknown, there were no known sources for acquiring positive controls and the virus isolates were not available. In view of this, the challenge to develop diagnostics for a new disease with a pandemic potential initially seemed unsurmountable. However, once the virus sequences were made available in public domain and source of positive controls and probes could be identified, India immediately established a network of testing laboratories for the new SARS-CoV-2 virus very swiftly. Starting with availability of validated diagnosis at the ICMR-NIV, Pune, testing capacity was further upscaled to another 13 DHR/ICMR VRDLs. The apex laboratory standardized the testing protocols within one week, and the VRDLs initiated testing within two weeks of release of laboratory protocols by the WHO. In addition, 35 laboratories fully equipped in terms of availability of adequate infrastructure and trained staff for SARS-CoV-2 testing were kept on a standby. An impressive model wherein the VRDLs have closely worked with the field surveillance programme of GoI (IDSP) has been operationalized during this public health crisis. However, this model worked well only in some States, but there were gaps in other states. Kerala, Karnataka, Telangana, Rajasthan and Uttar Pradesh clearly leveraged the capacity of existing VRDLs. The sample transport mechanisms from IDSP to VRDLs at these places were also good. The number of tests conducted by VRDLs in these States depicted the effective screening of suspect cases implemented at nearby airports, IDSP and State health programmes. However, in cities such as Kolkata, Chennai and Mumbai, despite the presence of a testing facility, the number of suspect cases referred was low. Liasoning between VRDLs and IDSP needs to be further strengthened in such areas.

Quality control programme implemented by NIV, Pune, for the VRDLs revealed 100 per cent concordance between the testing at VRDLs and NIV for negative samples. For borderline positive samples (Ct values between 33 and 35 cycles), concordance was low. This emphasized the need to continue reconfirmation of all positive samples at NIV, Pune. This has also indicated that *E* gene screening assay may not be sufficient to declare positivity. Further confirmatory tests as currently done by NIV, Pune, are required for confirming true positivity. To enhance the robustness of testing, it is essential to equip the SARS-CoV-2 testing VRDLs with additional confirmatory assays.

Inventory control posed challenges at every step. It was imperative to maintain optimum stocks of reagents, but simultaneously, it was important not to overestimate and waste the limited resources. Thoughtful optimization of inventory was undertaken. Planning of availability of tests at a given point was done in such a way that the excess tests could be used for routine surveillance in case the situation of COVID-19 does not worsen in India.

The threat of a potential pandemic due to SARS-CoV-2 has brought out the strength of the judiciously established network of VRDLs in India and also the capability of this network to rapidly adapt in times of any public health emergency, with appropriate quality checks. This already operational platform was able to switch gears to provide countrywide diagnosis for SARS-CoV-2 within a month after discovery of this novel virus. VRDLs offer a robust platform for early detection of emerging/re-emerging viral infections in all parts of India, facilitating early containment and prevention of larger epidemics.

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***Conflicts of Interest*:** None.

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Detection of coronaviruses in *Pteropus* & *Rousettus* species of bats from different States of India

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***Background & objectives*: Bats are considered to be the natural reservoir for many viruses, of which some are potential human pathogens. In India, an association of *Pteropus medius* bats with the Nipah virus was reported in the past. It is suspected that the recently emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) also has its association with bats. To assess the presence of CoVs in bats, we performed identification and characterization of bat CoV (BtCoV) in *P. medius* and *Rousettus* species from representative States in India, collected during 2018 and 2019.**

***Methods*: Representative rectal swab (RS) and throat swab specimens of *Pteropus* and *Rousettus* spp. bats were screened for CoVs using a pan-CoV reverse transcription-polymerase chain reaction (RT-PCR) targeting the RNA-dependent RNA polymerase (*RdRp*) gene. A single-step RT-PCR was performed on the RNA extracted from the bat specimens. Next-generation sequencing (NGS) was performed on a few representative bat specimens that were tested positive. Phylogenetic analysis was carried out on the partial sequences of *RdRp* gene sequences retrieved from both the bat species and complete viral genomes recovered from *Rousettus* spp.**

***Results*: Bat samples from the seven States were screened, and the RS specimens of eight *Rousettus* spp. and 21 *Pteropus* spp. were found positive for CoV *RdRp* gene. Among these, by Sanger sequencing, partial *RdRp* sequences could be retrieved from three *Rousettus* and eight *Pteropus* bat specimens. Phylogenetic analysis of the partial *RdRp* region demonstrated distinct subclustering of the BtCoV sequences retrieved from these *Rousettus* and *Pteropus* spp. bats. NGS led to the recovery of four sequences covering approximately 94.3 per cent of the whole genome of the BtCoVs from *Rousettus* bats. Three BtCoV sequences had 93.69 per cent identity to CoV BtRt-BetaCoV/GX2018. The fourth BtCoV sequence was 96.8 per cent identical to BtCoV HKU9-1.**

***Interpretation & conclusions*: This study was a step towards understanding the CoV circulation in Indian bats. Detection of potentially pathogenic CoVs in Indian bats stresses the need for enhanced screening for novel viruses in them. One Health approach with collaborative activities by the animal health and human health sectors in these surveillance activities shall be of use to public health. This would help in**

the development of diagnostic assays for novel viruses with outbreak potential and be useful in disease interventions. Proactive surveillance remains crucial for identifying the emerging novel viruses with epidemic potential and measures for risk mitigation.

Key words Bats - coronavirus - India - next-generation sequencing - phylogenetic - reverse transcription-polymerase chain reaction

A large number of emerging infectious diseases are known to be zoonotic in origin. In the last two decades, many viruses have been identified from bat species[1]. Bats have been recognized as the natural reservoirs of a variety of pathogenic viruses such as Rabies, Hendra, Marburg, Nipah and Ebola virus[2]. Bats are known to harbour coronaviruses (CoVs) and serve as their reservoirs. Alpha-CoV (α-CoV) and beta-CoV (β-CoV) have been detected in bats in Asia, Europe, Africa, North and South America and Australasia[3]. In the last two decades, bat CoVs (BtCoVs) garnered considerable attention as potential human pathogens[4,5]. Severe acute respiratory syndrome (SARS)-CoV-2 causing the current pandemic [CoV disease 2019 (COVID-19)] is also a member of the same genus and found to be similar to bat-derived CoV strain RATG13[6]. SARS-CoV-2 is reported to be 96 per cent identical to BtCoV at the whole genome level, and related viruses were identified in the previously sampled bat population in China[7].

CoVs are enveloped, single-stranded, positive-sense RNA viruses with a comparatively large genome size of 26 to 32 kb, classified under the family *Coronaviridae* in the order Nidovirales[8]. According to the International Committee on Taxonomy of Viruses (ICTV), they are classified into four genera, namely, α-CoV, β-CoV, γ-CoV and δ-CoV)[9]. β-CoVs are further classified into four different lineages [lineage A (L_A), lineage B (L_B), lineage C (L_C) and lineage D (L_D)][10]. Most of the human CoVs are either zoonotic in origin or circulate in animals[11]. CoVs can cause a wide range of infections, including respiratory tract infections, gastroenteritis, hepatitis and encephalomyelitis in their respective hosts. It is believed that many of the currently circulating α-CoVs and β-CoVs of mammals have evolutionary links to CoVs from bats[1].

India has a diverse population of bats; around 117 species of bats have been recorded, with around 100 subspecies coming under 39 genera belonging to eight families (*Pteropodidae*, *Rhinolophidae*, *Hipposideridae*, *Megadermatidae*, *Rhinopomatidae*, *Emballonuridae*, *Molossidae* and *Vespertilionidae*)[12]. The Indian Council of Medical Research-National Institute of Virology (ICMR-NIV) at Pune, India, has detected several viruses in bats, including the Nipah virus in *Pteropus medius*, Malsoor virus, Tioman virus and a novel adenovirus in *Rousettus leschenaultii*[13-15]. Nipah viral RNA antibodies could be detected in *Pteropus* bats from many States of India, and the possible link of transmission from bats could be established during the Nipah outbreak which occurred in Kerala in 2018 and 2019[16,17]. The use of conventional polymerase chain reaction/reverse transcription-polymerase chain reaction (PCR/RT-PCR), as well as metagenomics and next-generation sequencing (NGS) technologies, has led to the discovery of many novel viruses in bats. The identification of new CoVs in bats in several neighbouring Asian countries such as China[3], Sri Lanka[18] and Singapore[19,20] and the growing threats of novel CoV diseases such as COVID-19 led us to investigate *Pteropus* and *Rousettus* bats commonly found in India, for identification and characterization of BtCoVs.

Material & Methods

This study was approved by the Institutional Animal Ethics Committee (IAEC) of ICMR-NIV, Pune (IAEC/2019/MEZ/04). Permissions were also obtained from the Principal Chief Conservators of Forests (PCCF)/wildlife wardens of different States/ Union Territories (UT) (Kerala, Karnataka, Tamil Nadu, Himachal Pradesh, Punjab, Gujarat, Odisha, Telangana, Chandigarh and Puducherry).

Study sites and sample collection: Upon obtaining permission from the respective State authorities, bat-roosting sites in each State/UT were identified with the help of the forest officials. Bats were trapped using mist nets and were chemically restrained using isoflurane anaesthesia. Throat swabs (TS) and rectal swabs (RSs) were collected in virus transport medium (VTM) and were transported to ICMR-NIV, Pune, on dry ice. The specimens were collected from *Pteropus* spp. bats from

Kerala, Karnataka, Chandigarh, Gujarat, Himachal Pradesh, Odisha, Puducherry, Punjab, Tamil Nadu and Telangana and *Rousettus* spp. bats from Kerala, Karnataka, Chandigarh, Gujarat, Odisha, Punjab and Telangana States during 2018-2019. These bats were monitored and released after recovery. Twelve bats that died during the trapping process were transported to ICMR-NIV on dry ice. Necropsy of these bats was carried out in the Biosafety Level 4 (BSL-4) containment facility, and tissue specimens (intestine and kidney) collected were tested.

Detection of bat coronavirus using RT-PCR: RNA was extracted from the bat specimens using the MagMAX pathogen RNA/DNA isolation kit (Invitrogen, USA). RT-PCR was performed using Superscript III one-step RT-PCR (Invitrogen, USA) with Platinum High-Fidelity *Taq* polymerase (Invitrogen, USA) using the published BtCoV-specific primers targeting the conserved region of RNA-dependent RNA polymerase (*RdRp*) gene[21]. The amplicon of 440 bp was separated on 1.5 per cent agarose gel and visualized under VersaDoc MP 4000 ultraviolet transilluminator (Bio-Rad, USA).

Sequencing of the positive coronavirus specimens

Sanger sequencing of bat coronavirus: The RT-PCR products were separated on 1.5 per cent agarose gel, and 440 bp bands were excised. The excised gels were extracted and purified using a QIAQuick gel extraction kit (Qiagen, Hilden, Germany). The purified products were quantified, and chain-terminated PCR reactions were performed using pathogen-specific forward and reverse primers[21] with the BigDye Terminator 3.1 sequencing kit (Applied Biosystems, USA). BigDye reactions were purified using the DyeEx 2.0 spin kit (Qiagen, Germany). The purified chain-terminated reactions were sequenced using the ABI PRISM® 3100 Automated DNA Sequencer (Thermo Fisher Scientific, USA). The sequence data generated were assembled using the Sequencer 5.1 software (Accelrys Inc., USA).

Next-generation sequencing (NGS) of bat coronavirus: Selected bat specimens were used for RNA extraction[22,23]. RNA libraries were prepared and quantified by Qubit® 2.0 Fluorometer (Invitrogen, USA). NEB NextrRNA depletion kit (New England Biolabs, USA) was used to remove host ribosomal RNA and re-quantified using Qubit® 2.0 Fluorometer (Invitrogen, USA). In brief, the RNA library preparation involved fragmentation, adenylation, adapter ligation and amplification. The amplified libraries were quantified using KAPA Library Quantification Kit (KapaBiosystems, Roche, Switzerland) as per the manufacturer's protocol and further loaded onto the Illumina Miniseq NGS platform (Illumina, USA)[22,23].

The FASTQ files generated after the completion of the run were analyzed using CLC Genomics Workbench software version 11 (CLC, Qiagen, Germany). *De novo* assembly programme was used to assemble contiguous sequences (contigs). The contigs generated were analyzed using BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) to identify matching sequences. The closest matching sequence from GenBank (*https://www.ncbi.nlm.nih.gov/genbank/*) was used for reference mapping.

Phylogenetic analysis of partial and complete genome sequences of bat coronavirus: The CoV sequences retrieved from RS specimens of *Rousettus* spp. bats (n=4) were aligned with whole-genome sequences from GenBank using the create alignment function of the CLC genomics workbench (*https://digitalinsights.qiagen.com*). Partial *RdRp* gene sequences (~419 bp) retrieved by Sanger sequencing, for both the bat species specimens (genomic location: 14,701-15,120) were used to construct a phylogenetic tree along with the available *RdRp* sequences in GenBank. Phylogenetic analysis was carried out using the neighbour-joining method available in MEGA v7 software[24] using the Kimura 2-parameter nucleotide (nt) substitution model with 1000 bootstrap replicates. The nt divergence for the open reading frame (*ORF*) 1a polyprotein (*ORF* 1a), *ORF* 1b polyprotein (*ORF* 1b), spike protein (*S*), nucleocapsid phospoprotein (*N*), envelope protein (*E*) and membrane glycoprotein (*M*) genes was estimated using the Kimura 2-parameter model as implemented in the MEGA software. The sequences retrieved in the current study, along with those downloaded from GenBank, were grouped into the genus.

The viruses from the β-CoV genus were further grouped into lineages, L_A, L_B, L_C and L_D, to estimate the evolutionary divergence over the respective gene sequence pairs between groups using the MEGA software[24]. The distance was estimated using a Kimura 2-parameter model with uniform rates among the sites. The bootstrap of 500 replicates was used to estimate the variation in the model.

Results

The TS and RS specimens for 78 *Rousettus* spp. bats were collected in VTM from seven States (Kerala, Karnataka, Chandigarh, Gujarat, Odisha, Punjab and Telangana). The TS and RS specimens of 508 *Pteropus* spp. bats were also collected in VTM from 10 States/UTs in India (Kerala, Karnataka, Chandigarh, Gujarat, Himachal Pradesh, Odisha, Puducherry, Punjab, Tamil Nadu and Telangana). During the trapping process, 12 (8 *Rousettus* and 4 *Pteropus* spp.) bats died. Organ specimens (intestine and kidney) were collected from these bats (TS and RS specimens of these 12 bats were included in the total number of samples).

Detection of bat coronavirus using RdRp gene RT-PCR: Four of the 78 RS of *Rousettus* spp. bats screened for the BtCoV were found positive. All the positive RS samples belonged to Kerala State. Intestinal specimens of two bats were also found to be positive for the BtCoV. One bat (MCL-19-Bat-606), from Kerala, was tested positive in both the intestinal specimen and the RS. The second bat (MCL-20-Bat-76), from Karnataka, was tested positive only in the intestinal specimen. Altogether, five *Rousettus* spp. bats were positive for the BtCoV. All TS specimens from *Rousettus* spp. were found negative for BtCoV (Table I).

Twenty one of the 508 RSs from *Pteropus* spp. bats screened were tested positive for the BtCoV (Table I). These positive bats belonged to Kerala (n=12), Himachal Pradesh (n=2), Puducherry (n=6) and Tamil Nadu (n=1). The TS specimens of the same bats were tested negative for BtCoV. The TS specimens of RS-negative (n=42) bats were also screened and found to be negative (Table I). A total of 25 bats from both the species were found positive.

Sequencing of the positive coronavirus specimens

Sanger sequencing of bat coronavirus: Using the Sanger sequencing protocol, partial *RdRp* sequences of BtCoV were retrieved from two (out of 4 amplicons) specimens of *Rousettus* spp. One of the sequences (MCL-19-bat-588/2) showed close identity to BtCoV HKU9-5-2 (AN): HM211099.1; sequence identity (SI): 99.2 per cent, whereas the second *RdRp* sequence (MCL-20-bat-76/10) had an SI of 98.8 per cent with BtCoV HKU9-1 (AN: EF065513.1), both from China.

Sanger's sequencing protocol led to retrieval of eight partial *RdRp* sequences which belonged to *Pteropus* spp. These bats were collected from Kerala (n=5) and Tamil Nadu (n=3) States. One of the three partial *RdRp* sequences from Tamil Nadu had 97.93 per cent SI with BtCoV/B55951/Pte_lyl/CB2-THA (AN: MG256459.1, Thailand). The other two sequences had a minimum of 99.48 per cent SI with the CoV PREDICT_CoV-17/PB072 (AN: KX284942.1, Nepal). One of the five partial *RdRp* sequences from Kerala had 98.88 per cent SI with BtCoV/B55951/Pte_lyl/CB2-THA (AN: MG256459.1, Thailand). The remaining

Table I. Bat coronavirus positivity in bat specimens screened using RNA-dependent RNA polymerase (*RdRp*) gene-specific reverse transcription-polymerase chain reaction (RT-PCR) in different States

Place of collection	Number of positive/number tested (%) for different bat species for BtCoV *RdRp* gene-specific RT-PCR			
	Pteropus bats (%)		*Rousettus* bats (%)	
	Rectal swabs	Throat swabs	Rectal swabs	Throat swabs
Kerala	12/217 (5.53)	0/21 (0.00)	4/42 (9.52)	0/4 (0.00)
Karnataka	0/78 (0.00)	NT	0/4 (0.00)	0/4 (0.00)
Chandigarh	0/27 (0.00)	NT	0/6 (0.00)	0/6 (0.00)
Gujarat	0/30 (0.00)	NT	0/18 (0.00)	0/18 (0.00)
Odisha	0/30 (0.00)	NT	0/2 (0.00)	0/2 (0.00)
Punjab	0/14 (0.00)	NT	0/2 (0.00)	0/2 (0.00)
Telangana	0/30 (0.00)	NT	0/4 (0.00)	0/4 (0.00)
Himachal Pradesh	2/29 (6.89)	0/6 (0.00)	NA	NA
Puducherry	6/23 (26.09)	0/10 (0.00)	NA	NA
Tamil Nadu	1/30 (3.33)	0/5 (0.00)	NA	NA
	21/508 (4.13)	0/42 (0.00)	4/78 (5.13)	0/40 (0.00)

NT, not tested; NA, not available; BtCoV, bat coronavirus

Table II. Details of the genome recovered reads mapped and the per cent of reads mapped from the *Rousettus* bat samples

Sample details	Sample type	Virus retrieved	Relevant reads	Per cent of reads	Per cent of genome recovered
MCL-20-Bat-76	Kidney	Coronavirus BtRt-BetaCoV/GX2018	1632	0.015	94.39
	Intestine	BtCoV HKU9-1	4499	0.056	95.75
MCL-19-Bat-606	Rectal swab	Coronavirus BtRt-BetaCoV/GX2018	13,973	0.114	99.53
	Intestine	Coronavirus BtRt-BetaCoV/GX2018	10,214,492	93.476	99.87

four partial *RdRp* sequences had >97 per cent SI with CoV PREDICT_CoV-17/PB072 (AN: KX284942.1, Nepal).

Next-generation sequencing of bat coronavirus: NGS was performed on 10 specimens [4 RS, 2 kidney and 4 intestinal tissue) of the five *Rousettus* bats to retrieve the complete genome of the BtCoV. Kidney and intestine tissues of the bats from Karnataka State (MCL-20-Bat-76) and RS along with intestine tissue of bats from Kerala State (MCL-19-Bat-606) were used for sequencing and analysis.

Two different viruses were retrieved based on the BLAST analysis of the sequences from the kidney and intestine tissues of the bats from Karnataka. Kidney specimen of MCL-20-Bat-76 had an SI of 94 per cent and query coverage (QC) of 94 per cent with CoV BtRt-BetaCoV/GX2018 (AN: MK211379.1), whereas the intestine tissue of the MCL-20-Bat-76 had an SI of 96.8 and 95 per cent QC with the BtCoV HKU9-1 (AN: EF065513.1). The sequences from RS and intestine tissue of the MCL-19-Bat-606 from Kerala, had 93.69 and 93.99 per cent SI to CoV BtRt-BetaCoV/GX2018 (AN: MK211379.1), respectively, with 100 per cent QC. Further, 99.8 per cent of the CoV BtRt-BetaCoV/GX2018 sequences were retrieved from the intestine specimen of the MCL-19-Bat-606. The details of the genome recovered reads mapped and the per cent of reads mapped are summarized in Table II.

Phylogenetic analysis of partial and complete genome sequences of bat coronavirus: A neighbour-joining tree was generated using the partial *RdRp* region sequences derived from *Pteropus* and *Rousettus* spp. bat specimens. It was observed that all the BtCoV sequences were clustered within the L_D sequences of beta CoVs. A distinct subclustering of the sequences retrieved from *Pteropus* and *Rousettus* spp. bats is shown in Figure 1. The sequences in the light pink colour are retrieved from the *Pteropus* spp., whereas those in the dark pink region belong to *Rousettus* spp. The sequence divergence of 0.35 was observed between *Pteropus* spp. and *Rousettus* spp., which was obtained by averaging over all the sequence pairs between the two species, determining those to be distinct sequences to each species.

The complete genome sequences of four BtCoV obtained from *Rousettus* spp. specimens were used for generating a neighbour-joining tree (Fig. 2). These sequences were also clustered within L_D of β-CoVs as observed for partial *RdRp* sequence tree. These complete genome sequences were grouped into gene pairs to identify the gene with higher and lower divergence. The complete genomes of the Indian BtCoV sequences were grouped under L_D. The evolutionary divergence of *ORF* 1b was <0.54 between the different β-CoV lineages with a maximum score of 0.7 between different BtCoV sequences used in this study (Table III). *E* gene sequences had larger divergence within the β-CoV genus ranging from 2.18 to 0.94. Lineages L_A and L_C had the maximum divergence of 2.18, whereas the L_B and L_C were the least (0.94). *N* gene has an overall higher divergence among different lineages (ranging: 2.08-0.75). Overall, evolutionary divergence for the sequences of each gene pair demonstrated that *S*, *N*, *E* and *M* genes from the α- and δ-CoV highly diverged across the different genus. In contrast, the *ORF* 1b was less divergent across the genera (Table III).

Discussion

As per the available information, the BtCoV causing human infection belongs to α- and β-CoV genera of the *Coronaviridae* family. β-CoV genus has five strains known to infect humans[25]. The two human-infecting strains (NL63 and 229E) from α-CoV genus which cause mild-to-moderate respiratory infections are believed to have originated in bats[25]. Two members of the β-CoV genus (HCoV-OC43 and HCoV-HKU1) are known to cause the common cold and lower respiratory tract infections[26]. The other three

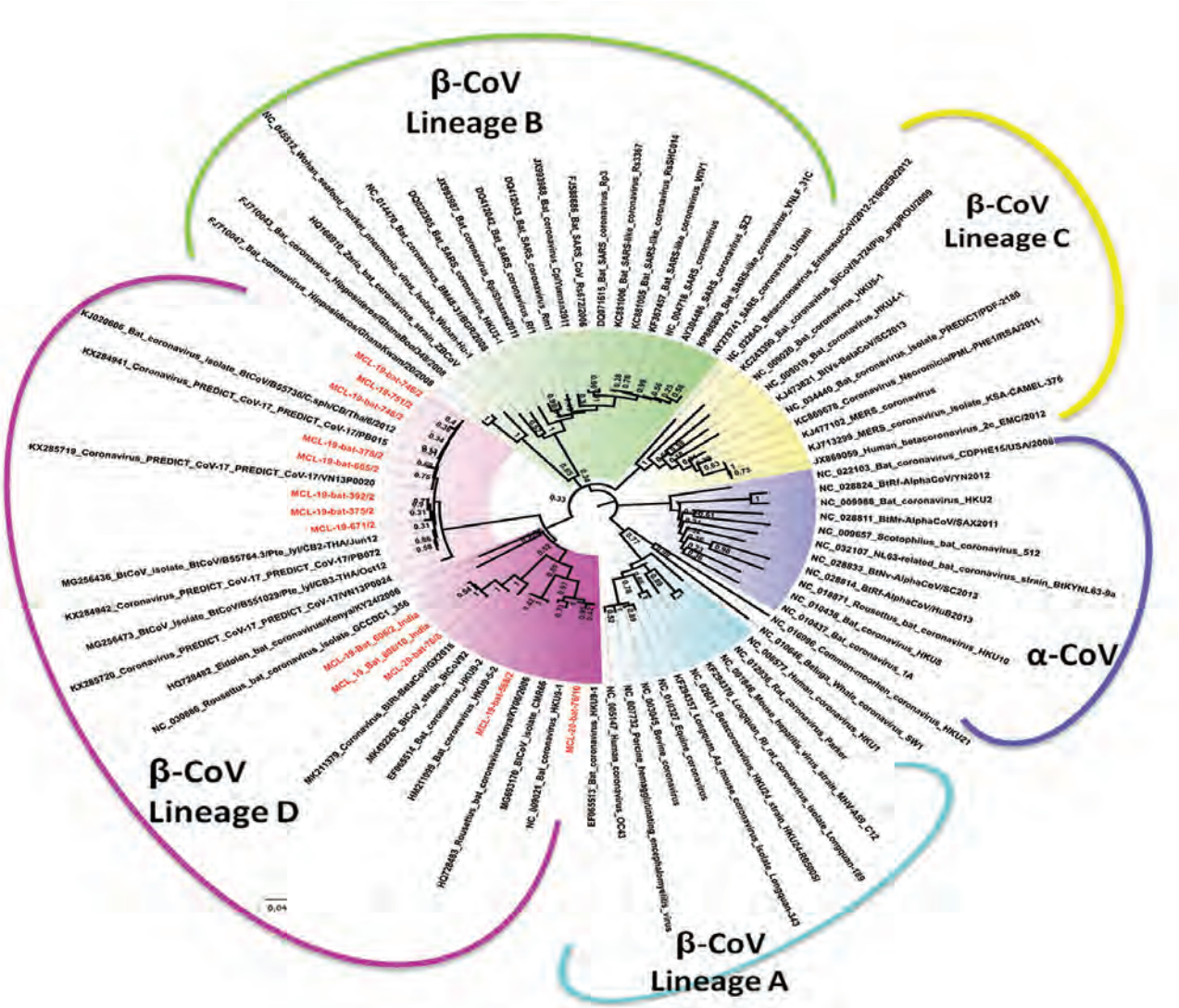

Fig. 1. Neighbour-joining tree for the RNA-dependent RNA polymerase (*RdRp*) partial sequence (genomic location: 14,701-15,120) generated from Sanger sequencing. The tree was constructed using the *RdRp* sequence available in the GenBank sequences. Kimura 2-parameter model was used as the substitution model to generate the tree. A bootstrap replication of 1000 cycles was performed to generate the tree to assess the statistical robustness.

are now shown to be pathogenic to humans (SARS-CoV-1, MERS-CoV and SARS-CoV-2). The SARS-CoV-1 and SARS-CoV-2 belong to L_B and MERS CoV belongs to L_C of β-CoV genus[27].

The phylogenetic analysis for the partial *RdRp* region revealed the presence of distinct BtCoVs in both the bats. The genomic sequences retrieved from the Indian sequences form a distinct cluster. The three CoV_BtRtBetaCoV/GX2018 sequences retrieved from the Indian *Rousettus* bats were 5.8-6.7 per cent different from the reference sequence, which was retrieved from *Rhinolophus affinis*. The two CoV_BtRtBetaCoV/GX2018 sequences retrieved from different bats were 1.2 per cent different from each other. The effect of host influence on the nt usage of the virus cannot be denied; however, it needs to be explored further in detail.

Bats are reservoirs for viruses with human pathogenic potential[28,29], and are known to harbour a broad range of CoVs[1]. The global distribution of bats, along with the different types of cell receptors present within them, favours virus replication, and is a possible link to their intraspecies transmission. The interspecies spill-over of a BtCoV to humans

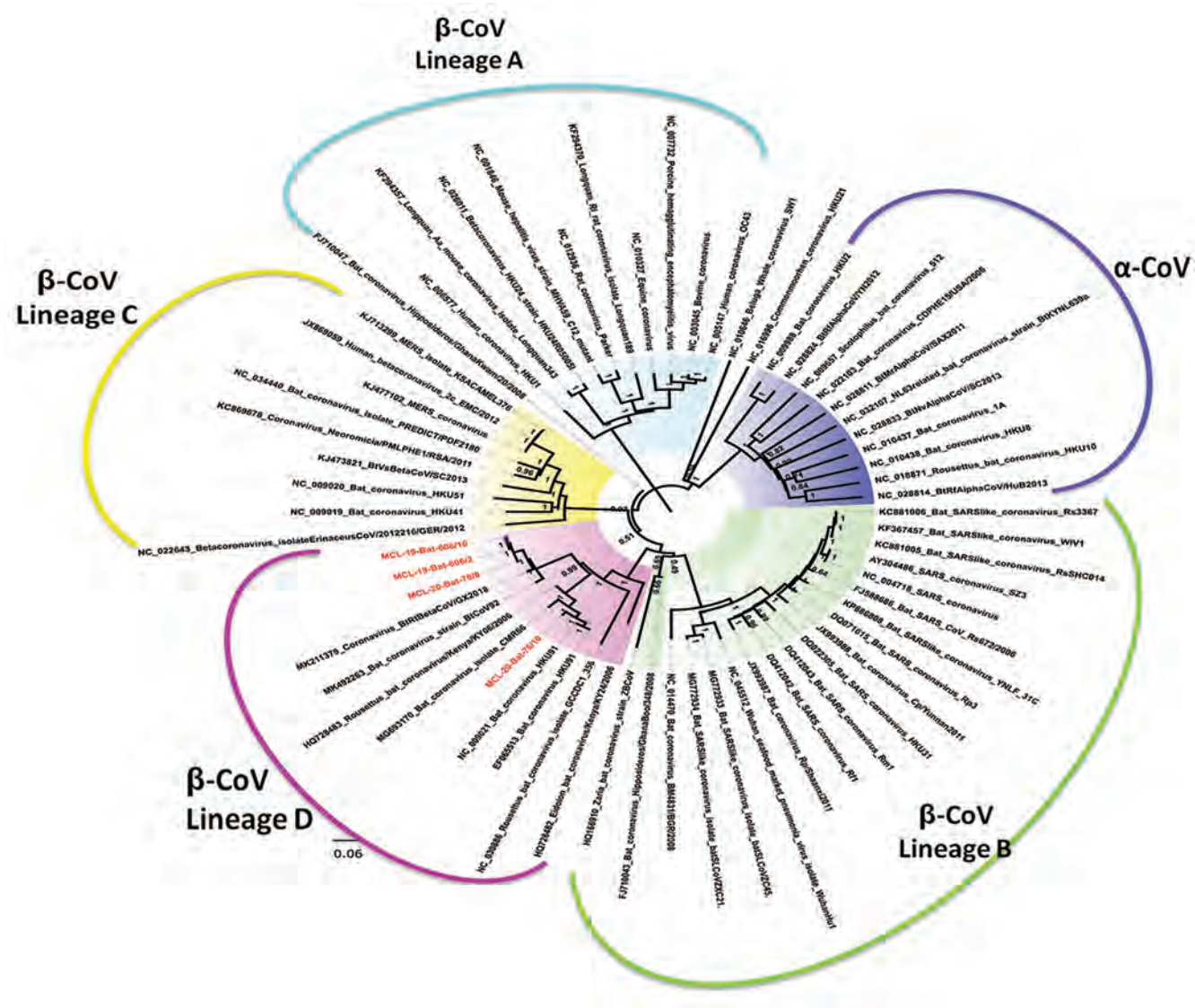

Fig. 2. Phylogenetic tree for the complete genome tree: A neighbour-joining tree was generated using the representative complete genome sequence available in the GenBank sequences. Kimura 2-parameter model was used as the substitution model to generate the tree. A bootstrap replication of 1000 cycles was performed to generate the tree to assess the statistical robustness.

is thought to occur through an intermediate host, in which the virus replicates through yet completely unidentified routes. In India, regions of the Western Ghats, particularly in Kerala, are reported to have habitat for diverse bat populations. The reports of pathogenic human viruses from bat specimens demand enhanced methods to monitor human exposure to various bat species. Investigations in unexplored regions/States should be focused on gaining further insights into CoV diversity within Indian bat populations.

Earlier, we had reported the presence of pathogenic viruses such as the Nipah virus in *Pteropus* bats in India[16]. In the present scenario of changing demography and ecological manipulations, it is challenging to have checks on the encounters of bats with other animals and humans. Therefore, active and continuous surveillance remains crucial for outbreak alerts for bat-associated viral agents with epidemic potential, which would be helpful in timely interventions.

Although CoVs in the subfamily *Coronavirinae* do not usually produce clinical symptoms in their natural hosts (bats), accidental transmission of these viruses to humans and other animals may result in respiratory, enteric, hepatic or neurologic diseases of variable

Table III. Evolutionary divergence for *ORF* 1b, *S*, *N* and *M* genes for the retrieved sequences with other reference sequences. The lower right-check hand matrix of the table depicts the divergence and the upper left-check matrix of the matrix (blue colour) depicts the variation observed in the bootstrap replication

N gene	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D	*M* gene	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D
Alpha		0.15	0.09	0.10	0.08	0.08	0.09	Alpha		0.11	0.12	0.05	0.06	0.05	0.06
Delta	2.08		0.11	0.16	0.09	0.11	0.10	Delta	1.50		0.26	0.08	0.16	0.10	0.11
Gamma	1.57	1.49		0.08	0.08	0.09	0.08	Gamma	1.53	1.84		0.10	0.12	0.11	0.09
L_A	1.84	1.73	1.37		0.05	0.05	0.06	L_A	0.92	1.24	1.30		0.06	0.05	0.05
L_B	1.48	1.37	1.32	1.09		0.03	0.04	L_B	1.05	1.51	1.37	0.92		0.05	0.05
L_C	1.57	1.52	1.42	1.07	0.75		0.04	L_C	0.99	1.35	1.27	0.80	0.82		0.05
L_D	1.64	1.46	1.36	1.27	0.90	0.97		L_D	0.99	1.42	1.23	0.84	0.79	0.82	
ORF 1b	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D	*ORF* 1a	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D
Alpha		0.01	0.01	0.01	0.01	0.01	0.01	Alpha		0.02	0.02	0.01	0.02	0.02	0.03
Delta	0.70		0.01	0.02	0.01	0.01	0.01	Delta	1.32		0.03	0.02	0.03	0.03	0.04
Gamma	0.62	0.67		0.01	0.01	0.01	0.01	Gamma	1.14	1.33		0.02	0.03	0.02	0.04
L_A	0.61	0.69	0.60		0.01	0.01	0.01	L_A	1.22	1.01	1.30		0.02	0.02	0.04
L_B	0.60	0.70	0.65	0.54		0.01	0.01	L_B	1.26	1.42	1.41	1.19		0.01	0.02
L_C	0.58	0.69	0.62	0.53	0.50		0.01	L_C	1.35	1.41	1.44	1.19	0.97		0.03
L_D	0.60	0.67	0.61	0.53	0.50	0.52		L_D	1.26	1.27	1.39	1.09	0.90	1.03	
S gene	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D	*E* gene	Alpha	Delta	Gamma	L_A	L_B	L_C	L_D
Alpha		0.02	0.02	0.03	0.03	0.03	0.02	Alpha		0.12	0.18	0.09	0.15	0.15	0.12
Delta	0.86		0.03	0.04	0.04	0.06	0.03	Delta	1.14		0.47	0.22	0.41	0.28	0.17
Gamma	1.14	0.96		0.04	0.05	0.06	0.04	Gamma	1.59	1.64		0.22	0.24	0.32	0.19
L_A	1.36	1.28	1.43		0.03	0.03	0.02	L_A	1.03	1.58	1.57		0.23	0.21	0.25
L_B	1.33	1.23	1.34	1.19		0.04	0.02	L_B	1.24	1.75	1.40	1.83		0.11	0.14
L_C	1.42	1.32	1.46	1.17	1.03		0.03	L_C	1.37	1.64	1.83	2.18	0.94		0.17
L_D	1.34	1.24	1.41	1.16	1.00	1.11		L_D	1.25	1.42	1.52	1.95	1.16	1.37	

ORF 1a, open reading frame 1a polyprotein; *ORF* 1b, ORF 1b polyprotein; *S*, spike glycoprotein; *N*, nuclocapsid phospoptotein; *M*, membrane glycoprotein; *E*, envelope protein

severity. It is still not understood as to why only certain CoVs can infect people.

There is a need of proactive surveillance of zoonotic infections in bats. Detection and identifications of such aetiological agents will provide leads for the development of diagnostic along with preparedness and readiness to deal with such emergent viruses thereby quickly containing them. The detection and identification of such viruses from bats also recommends cross-sectional antibody surveys (human and domestic animals) in localities where the viruses have been detected. Similarly, if epidemiological situation demands, evidence-based surveillance should also be conducted. There is a need of developing strong mechanisms for working jointly with various stakeholders such as wildlife, poultry, animal husbandry and human health departments.

In conclusion, our study showed detection of pathogenic CoVs in two species of Indian bats. Continuous active surveillance is required to identify the emerging novel viruses with epidemic potential.

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Quick Response Code:

Severe acute respiratory illness surveillance for coronavirus disease 2019, India, 2020

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***Background & objectives*: Sentinel surveillance among severe acute respiratory illness (SARI) patients can help identify the spread and extent of transmission of coronavirus disease 2019 (COVID-19). SARI surveillance was initiated in the early phase of the COVID-19 outbreak in India. We describe here the positivity for COVID-19 among SARI patients and their characteristics.**

***Methods*: SARI patients admitted at 41 sentinel sites from February 15, 2020 onwards were tested for COVID-19 by real-time reverse transcription-polymerase chain reaction, targeting *E* and *RdRp* genes of SARS-CoV-2. Data were extracted from Virus Research and Diagnostic Laboratory Network for analysis.**

***Results*: A total of 104 (1.8%) of the 5,911 SARI patients tested were positive for COVID-19. These cases were reported from 52 districts in 20 States/Union Territories. The COVID-19 positivity was higher among males and patients aged above 50 years. In all, 40 (39.2%) COVID-19 cases did not report any history of contact with a known case or international travel.**

***Interpretation & conclusions*: COVID-19 containment activities need to be targeted in districts reporting COVID-19 cases among SARI patients. Intensifying sentinel surveillance for COVID-19 among SARI patients may be an efficient tool to effectively use resources towards containment and mitigation efforts.**

Key words Containment - COVID-19 - SARI - sentinel - surveillance

In December 2019, an outbreak of a novel coronavirus emerged in the city of Wuhan in Hubei province in Central China[1]. The virus has formally been named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the disease as coronavirus disease 2019 (COVID-19)[2]. On January

30, 2020, the WHO declared the outbreak as a public health emergency of international concern[3]. As on March 31, 2020, 750,890 laboratory-confirmed cases, including 36,405 deaths, have been reported from more than 200 countries/territories/areas[4]. In India, the first laboratory-confirmed case of COVID-19 was reported from Kerala on January 30, 2020. As of March 31, 2020, a total of 2,245 cases and 56 deaths were reported in India[5].

In India, the initial COVID-19 testing strategy included people who had international travel history with symptoms, symptomatic contacts of laboratory-confirmed COVID-19 patients and symptomatic healthcare workers managing respiratory distress/severe acute respiratory illness (SARI)[6]. In addition, to track the progression of the epidemic in the early phase, stored samples of SARI patients hospitalized since February 15, 2020 were also tested for COVID-19 under the Virus Research and Diagnostic Laboratory Network (VRDLN). The WHO recommends countries to leverage the existing hospital-based SARI sites to complement the COVID-19 surveillance activities. This will further assist to monitor the intensity of COVID-19 transmission over time and geographical spread and to assess the severity of the disease in the country[7]. Following the evolution of the COVID-19 epidemic, hospitalized SARI patients were included as part of the routine testing strategy[8]. We analysed the SARI surveillance data (February 15 - April 2, 2020) to calculate the weekly COVID-19 positivity, and described the distribution of COVID-19 positive SARI cases by place and individuals' characteristics.

Material & Methods

Forty one sentinel sites were selected to test throat/nasopharyngeal swabs from a sample of SARI patients admitted between February 15 and March 19, 2020. Aggregate data on the number of SARI patients tested and COVID-19 positivity were collected from each laboratory. Since March 20, 2020, testing strategy was revised to include all SARI patients. Line list reported in the VRDLN platform was used to segregate data on SARI patients. The SARS-CoV-2 laboratory test was based on the detection of unique sequences of virus RNA by nucleic acid amplification test such as real-time reverse transcription-polymerase chain reaction (RT-PCR) and targeted the SARS-CoV-2 *E* (envelope protein) and *RdRp* (RNA-dependent RNA polymerase) genes[9].

Results & Discussion

A total of 5,911 SARI patients were tested for COVID-19. Of these, 104 (1.8%) were tested positive for COVID-19. Among the 965 SARI patient samples that were tested retrospectively between February 15-29, 2020 and March 19, 2020, two (0.2%) were positive for COVID-19. When the COVID testing strategy was expanded to include all SARI patients, a total of 4946 samples yielded 102 (2.1%) cases. The positivity increased from zero during the initial weeks to 2.6 per cent in the 14th wk (Table I).

The median age of COVID-19 positive SARI patients was 54 yr (interquartile range: 44-63), and 85 (83.3%) were males; 83 (81.4%) of the affected patients were more than 40 yr of age. Positivity was higher in males (2.3%) and in 50-70 yr of age group (4.4%) (Table II).

COVID-19 cases among SARI patients were detected from 52 districts in 20 States. Majority of the SARI patients were tested from Gujarat (792), Tamil Nadu (577), Maharashtra (553) and Kerala (502)

Table I. Distribution of coronavirus disease 2019 (COVID-19) cases among severe acute respiratory illness (SARI) patients by week, India, 2020

Week	Number of laboratories testing SARI for COVID-19	Number of SARI patients tested	Number of COVID-19 positive (%)
8-9 (February 15 - 29)	16	217	0 (0.0)
10-11 (March 1 - 14)	41	642	0 (0.0)
12 (March 15 - 21)	27	106	2 (1.9)
13 (March 22 - 28)	119	2877	48 (1.7)
14 (March 29 - April 2)	104	2069	54 (2.6)
Total		5911	104 (1.8)

Table II. Distribution of coronavirus disease 2019 (COVID-19) cases among severe acute respiratory illness (SARI) patients by age, gender and per cent positivity, India, 2020

Characteristics	Number of COVID-19 cases (per cent of total)	Number of SARI patients (per cent of total)	Per cent positivity
Gender	n=102	n=5723	
Male	85 (83.3)	3676 (64.2)	2.3
Female	17 (16.7)	2047 (35.8)	0.8
Age groups (yr)	n=102	n=5682	
0-9	2 (2.0)	386 (6.8)	0.5
10-19	0	371 (6.5)	0
20-29	9 (8.8)	1419 (25.0)	0.6
30-39	8 (7.8)	971 (17.1)	0.8
40-49	16 (15.7)	634 (11.2)	2.5
50-59	31 (30.4)	637 (11.2)	4.9
60-69	26 (25.5)	672 (11.8)	3.9
70-79	8 (7.8)	405 (7.1)	2.0
≥80	2 (2.0)	187 (3.3)	1.1

with COVID-19 positivity of 1.6, 0.9, 3.8 and 0.2 per cent, respectively (Table III). COVID-19 positive SARI patients were detected from eight districts in Maharashtra, six in West Bengal and five each in Tamil Nadu and Delhi (Table III).

Of the 102 COVID-19 positive SARI patients, 40 (39.2%) did not report any history of contact or international travel, two (2.0%) reported contact with a confirmed case and one (1.0%) reported recent history of international travel. Data on exposure history were not available for 59 (57.8%) cases (Table IV).

COVID-19 positivity among SARI patients increased from 0 per cent before March 14, to 2.6 per cent by April 2, 2020. In 15 Indian States, more than one per cent of SARI patients were COVID-19 positive. About a third of COVID-19 positive SARI cases did not have any history of contact with laboratory-confirmed case or international travel, and such cases were reported from 36 Indian districts in 15 States. These districts need to be prioritized to target COVID-19 containment activities.

The results of SARI surveillance need to be interpreted against the following limitations. First, the weekly number of SARI patients tested at each laboratory varied between 4 and 24 (13 on an average). Moreover, the proportion of all hospitalized SARI patients tested for COVID-19 by each laboratory was not known. This proportion is expected to be lower during initial weeks of surveillance. However, with the expansion of the testing criteria to include all SARI patients, it is assumed that majority of SARI patients hospitalized in these facilities would have been tested for COVID-19. Second, the data presented pertained to patients seeking care from selected sentinel hospitals that were predominantly in public sector in urban areas and hence might not be representative of the entire district, State or country. However, the trend of COVID-19 positivity among SARI patients could provide reliable information about its spread in the area. Third, diagnosis of COVID-19 positive SARI patients could have been missed due to false negative results of laboratory test based on RT-PCR[10]. Antibody-based testing among RT-PCR negative SARI patients could have increased the yield of COVID-19 cases in this group.

Tracking the spread of COVID-19 is critical to inform response activities including testing, containment and mitigation measures. The current SARI testing strategy will complement and strengthen the routine COVID-19 surveillance activities. Information from hospital-based SARI surveillance would help in setting triggers for escalation/de-escalation of mitigation measures, identify risk

Table III. Distribution of coronavirus disease 2019 (COVID-19) cases among severe acute respiratory illness (SARI) patients by State/Union Territory, India, 2020

State/UT	Number of laboratories testing SARI patients	Number of SARI patients	Number of COVID-19 positive (%)	Number of districts with COVID-19 cases
Gujarat	7	792	13 (1.6)	4
Tamil Nadu	14	577	5 (0.9)	5
Maharashtra	14	553	21 (3.8)	8
Kerala	5	502	1 (0.2)	1
Karnataka	8	320	2 (0.6)	2
Uttar Pradesh	7	295	4 (1.4)	2
Delhi	11	277	14 (5.1)	5
Assam	5	276	1 (0.4)	1
Bihar	2	263	3 (1.1)	2
West Bengal	5	256	9 (3.5)	6
Madhya Pradesh	4	249	5 (2.0)	2
Telangana	4	190	8 (4.2)	2
Rajasthan	4	179	0 (0.0)	0
Haryana	3	161	4 (2.5)	3
Punjab	2	158	1 (0.6)	1
Andhra Pradesh	4	129	4 (3.1)	2
Himachal Pradesh	2	110	0 (0.0)	0
Jharkhand	1	110	1 (0.9)	1
Odisha	3	107	2 (1.9)	1
Jammu and Kashmir	4	79	1 (1.3)	1
Chhattisgarh	1	74	0 (0.0)	0
Puducherry	1	41	0 (0.0)	0
Arunachal Pradesh	0	28	0 (0.0)	0
Chandigarh	2	24	1 (4.2)	1
Meghalaya	1	21	0 (0.0)	0
Manipur	2	20	0 (0.0)	0
Tripura	1	18	2 (11.1)	1
Nagaland	0	18	0 (0.0)	0
Andaman and Nicobar Islands	1	17	0 (0.0)	0
Mizoram	0	11	0 (0.0)	0
Uttarakhand	1	6	0 (0.0)	0
Sikkim	0	3	0 (0.0)	0
Goa	1	2	0 (0.0)	0
Dadra and Nagar Haveli	0	1	0 (0.0)	0

groups for severe disease and measure impact of the response activities. Continued sentinel surveillance for COVID-19 among SARI patients would guide the health departments to prioritize, plan and mobilize their resources in terms of where, when and how to respond.

Table IV. Coronavirus disease 2019 (COVID-19) cases among severe acute respiratory illness (SARI) patients by source of exposure, India, 2020 (n=102)

Source of exposure	Number of cases (per cent of total)
No foreign travel/contact with known laboratory confirmed COVID-19 case	40 (39.2)
Contact with a known laboratory confirmed COVID-19 case	2 (2.0)
History of foreign travel	1 (1.0)
Data not available	59 (57.8)

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Transmission electron microscopy imaging of SARS-CoV-2

Sir,

The description of a novel human coronavirus initially referred to as the Wuhan coronavirus (CoV), currently designated as severe acute respiratory syndrome (SARS)-CoV-2 as per the latest International Committee on Taxonomy of Viruses (ICTV) classification[1] is probably the most recent human pneumonia virus with high outbreak potential. This novel virus was initially identified through next-generation sequencing (NGS) and suggested to have a possible zoonotic origin[2]. Till date, detailed morphology and ultrastructure of this virus remains incompletely understood.

In India, the first laboratory-confirmed infection by SARS-CoV-2 was reported on January 30, 2020 (unpublished data). The throat swab from this case was kept in commercially available transport medium (HiViral™ Transport Medium, HiMedia, Mumbai). A 500 µl aliquot from this specimen that had tested positive for SARS-CoV-2 nucleic acid by real-time polymerase chain reaction (PCR) was centrifuged to remove the debris. The supernatant was removed, fixed at a final concentration of one per cent glutaraldehyde and adsorbed onto a carbon-coated 200 mesh copper grid. Negative staining was done with sodium phosphotungstic acid as described earlier[3]. The grid was examined under 100 kV accelerating voltage in a transmission electron microscope (TEM) Tecnai 12 BioTwin™ (FEI Company, The Netherlands). Imaging was done using a low-dose mode and images were captured using a side-mounted 2k × 2k CCD camera (Megaview III, Olympus, Japan).

A total of seven negative-stained virus particles having morphodiagnostic features of a coronavirus-like particle could be imaged in the fields scanned. These included the round shape of the virus with an average size of 70-80 nm and a cobbled surface structure having envelope projections that averaged 15±2 nm in size. One particular virus particle was very well preserved, showing very typical morphodiagnostic features of coronaviruses[4]. This particle was 75 nm in size and showed patchy stain pooling on the surface and a distinct envelope projection ending in round 'peplomeric' structures (Figure A). We used a mild defocussing of the projector lens away from the conventional Fresnel focus in an attempt to image the finer details of the envelope projections. All focusing operations were carried out using a Fourier fast focus transform under the TIA imaging software (FEI Company, The Netherlands). The defocussed image under low-beam current conditions prominently brought out the finer morphology of the SARS-CoV-2 virus surface projection as typical of a coronavirus (Figure B). We further increased the magnification under low-dose image capture and generated a pixel-corrected image of the projection to image single glycoprotein organization. The image revealed the presence of stalk-like projections ending in round peplomeric structures typical of a coronavirus particle (Figure C).

Interestingly, the envelope fringe of the SARS-CoV-2 virus particle imaged by us showed an interesting feature when compared to the classic description of human coronaviruses[5]. This included a relatively shorter size and a possible multi-aggregate of the peplomers (Figure B). This morphological variation could be due to a fixation artefact in clinical material. Imaging the cell culture-derived virus will resolve this point effectively. The limited imaging of a few virus particles has an intrinsic imaging limitation.

Further, imaging thin sections from infected cells by conventional and cryo-ultramicrotomy methods, like that of Tokyasu[6], will further provide more detailed information on the macromolecular assembly and organization of SARS-CoV-2. The use of single-particle reconstruction of the purified virus using CryoEM will also give high-resolution organizational details of

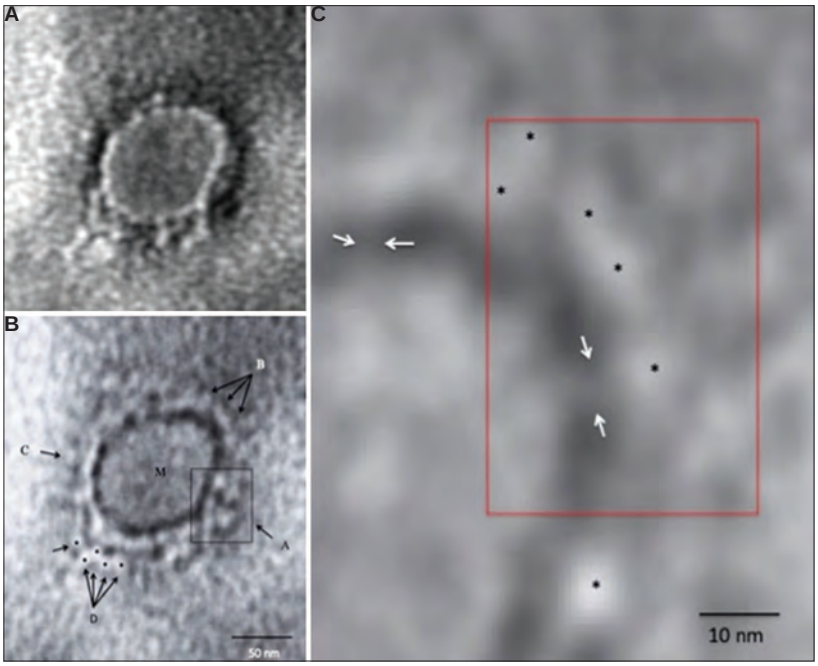

Figure. Transmission electron microscopy imaging of COVID-19. (**A**) A representative negative-stained COVID-19 particle showing morphodiagnostic features of family *Coronaviridae*. (**B**) Defocussed image of the same particle resolving the virus envelope glycoprotein morphology in finer details. The boxed area A shows a tetramer-like aggregate of four distinct peplomers, arrows shown by B show a more orthodox morphology of coronavirus surface projections. M indicates the matrix of the virus particle. C shows a distinct 'peplomer head' with negative stain silhouette. The area D is interesting as possible linear projections could be imaged. Five distinct peplomers could be imaged as shown by the arrows. (**C**) A highly magnified processed image for pixel corrections shows a distinct evidence of direct 'stalk' connecting the peplomer to the virion surface. The peplomers are shown with asterisk and the stalk with an arrow. Magnification bars are built into the micrographs.

the mature virion and complete surface glycoprotein organization. A recent cryoEM study imaging the purified envelope spike glycoprotein of SARS-CoV-2 has reconstructed three-dimensional images at 3.2 Å resolution[7]. While a series of informal reports are available on electron microscopy of the SARS-CoV-2 from Hong Kong University researchers[8], no detailed studies on ultrastructural cytopathology are available till date.

In summary, to the best of our knowledge, this is the first report from India detecting the SARS-CoV-2 virus using TEM directly in a throat swab specimen confirmed by PCR. Although TEM imaging was limited by particle load in the specimen, we could still detect morphologically identifiable intact particles in stored clinical sample without initial fixation. Imaging other specimens such as stool and use of immunoelectron microscopy techniques can improve the detection frequency of virus in direct clinical material. This finding emphasizes the merit of the use of conventional negative-stained TEM imaging in clinical samples along with other diagnostic tests in parallel, especially in situations identifying aetiologic agents[9].

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First isolation of SARS-CoV-2 from clinical samples in India

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has, as on March 31, 2020, spread to over 207 countries around the world[1,2], with a total of 896,475 confirmed cases and 45,525 deaths[2]. The number of reported SARS-CoV-2 cases in India is also on an increase with 1,636 cases and 38 deaths[2]. In the current pandemic situation, the isolation of SARS-CoV-2 is important for developing and evaluating diagnostic reagents, for antiviral studies and for screening of vaccine candidates. Earlier studies showed that SARS-CoV-2 could not replicate in several cell lines, which are routinely used for isolation of respiratory viruses[3]. Human and animal cell lines that were found to support SARS-CoV-1 replication during the first outbreak of SARS in China, 2002[4], are currently being studied. The virus was first isolated in the human airway epithelial cells from clinical specimens as part of early attempts to identify the aetiologic agent of infection[5]. We describe here the successful isolation and characterization of SARS-CoV-2 from clinical samples in India using Vero CCL-81 cells by observing cytopathic effects (CPEs) and cycle threshold (Ct) values in real-time reverse transcription-polymerase chain reaction (RT-PCR), electron microscopy and next-generation sequencing (NGS).

The first three SARS-CoV-2 cases were reported from Kerala during January 27-31, 2020. Later during March 2020, cases were also reported from a group of Italian tourists (n=15) and their contacts in New Delhi, India. Simultaneously, cases were reported in Agra, Uttar Pradesh, which was the outcome of close contact of an infected Delhi-based individual who returned from Italy. The designated COVID-19 testing laboratories of Virus Research Diagnostic Laboratory network (All India Institute of Medical Sciences, New Delhi; Sawai Man Singh Medical College, Jaipur; and King George's Medical University, Lucknow) referred the specimens (throat swab/nasal swab, oropharyngeal swab/sputum) to the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune, after screening for envelope (*E*) gene by real-time RT-PCR was done[6]. A total of 12 SARS-CoV-2 positive specimens having a Ct <30 for the *E* gene were included in the study. Of these, eight samples were from positive cases of Italian tourists and their contacts in New Delhi. The rest of the specimens were from four positive cases at Agra, Uttar Pradesh, and the close contact cases of an infected Delhi-based individual who returned from Italy.

The clinical specimens of the 12 cases were used for infecting Vero CCL-81 which was maintained in Eagle's minimum essential medium (MEM; Gibco, UK) supplemented with 10 per cent foetal bovine serum (FBS) (HiMedia, Mumbai), penicillin (100 U/ml) and streptomycin (100 mg/ml). Likewise, 100 µl was inoculated onto 24-well cell culture monolayers of Vero CCL-81, before growth medium was decanted. The cells were incubated for one hour at 37°C to allow virus adsorption, with rocking every 10 min for uniform virus distribution. After the incubation, the inoculum specimen was removed and the cells were washed with 1X phosphate-buffered saline (PBS). The MEM supplemented with two per cent FBS was added to each well. The cultures were incubated further in five per cent CO_2 incubator at 37°C and observed daily for CPEs under an inverted microscope (Nikon, Eclipse Ti, Japan). Cellular morphological changes were recorded using a camera (Nikon, Japan). From each well of cell culture plate, on the third post-infection day (PID-3) of passage-1 (P-1), 50 µl of supernatant was taken and tested for SARS-CoV-2 using real-time RT-PCR for *E* and RNA-dependent RNA polymerase (*RdRp*) (2) genes as described earlier[7,8]. Similar testing was repeated on the cell supernatant of passage-2 (P-2) at PID-4 for

Supplementary material available from *http://www.ijmr.org.in/preprintarticle.asp?id=282559*

observing viral copy number. Cultures that showed CPE on PID-4 were centrifuged at 4815 × *g* for 10 min at 4°C; the supernatants were processed immediately or stored at −86°C. Further, those that showed CPE were grown in T-25 cm^2 flasks at P-2 and titration was done after serial dilution. Tissue culture infective dose 50 per cent ($TCID_{50}$) values were calculated by the Reed and Muench method[9]. CPEs were observed in 9 of 12 cultures in the P-1. The $TCID_{50}$ values ranged from $10^{5.5}$ to $10^{6.4}$/ml for the different clinical specimens passaged in Vero CCL-81 at P-2. The cells were examined microscopically for cellular morphological changes following inoculation.

Vero CCL-81 cells infected with SARS-CoV-2 strain NIV-2020-770 and uninfected cells (CC) were transferred onto microcavity slides and fixed with acetone. Serum samples (1:25 dilution) from the confirmed COVID-19 cases (POD nCOV-S11, nCOV-S13 and nCOV-S7) and negative serum samples were added followed by incubation at 37°C for 1.5 h[10]. Antibody reactivity was visualized using anti-human immunoglobulin fluorescein-isothiocynate. In immunofluorescence assay of COVID-19 positive patients, three serum samples exhibited specific reactivity against SARS-CoV-2 virus isolate (Fig. 1).

Vero CCL-81 cells that were inoculated with the samples showed evidence of cell rounding and detachment from 9 of 12 clinical samples in P-1 at PID-4. Syncytial cells formed large cell masses that increased in size and number as the infection progressed. Enhanced CPE was noted in P-2 at PID-2. The cells were detached from the tissue culture plate surfaces by PID-3. Similar cellular morphological changes were observed after passaging of the above nine samples up to P-2. No cellular changes were observed in the cell control during both passages. Figure 2 depicts the day-wise changes during the passage of a representative clinical isolate (NIV-2020-770). Virus replication was confirmed using real-time RT-PCR with RNA extracted from the cell culture medium on PID-3. The Ct values ranged from 9.79 to 15.41 (in Vero CCL-81 cells) for the isolates at P-2, which were lower than the Ct values of 16-25.1 in the clinical samples (Table I). The number of virus copies in the isolates at P-1 in Vero CCL-81 cells ranged from 5.18×10^7 to 8.12×10^8 copy/ml and increased 1-26 fold to a range of 1.69×10^8 to 6.77×10^9 in the cell culture supernatants at P-2 (Table I).

On PID-4, enhanced CPE was observed. The P-1 material was reinoculated in a new batch of cells, and it showed progressive enhancement of CPE as observed day-wise. Further, an aliquot of cell culture supernatant was harvested from infected Vero CCL-81 showing CPE and the supernatant used for negative staining as described elsewhere[11,12]. Distinct CoV particles with an average size of 95±10 nm having a distinct envelope fringe could be detected in the fields scanned (Fig. 3), as observed earlier[13].

Next-generation sequencing was performed on SARS-CoV-2 positive clinical samples (100

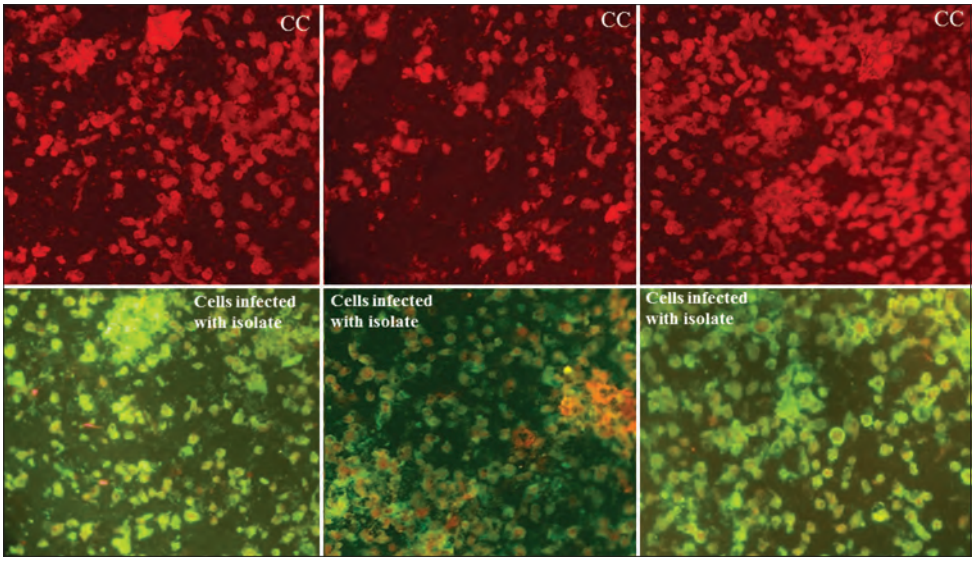

Fig. 1. Immunofluorescence images (red panel) showing uninfected Vero CCL-81 cells probed by positive patient serum samples after post infection day of 13th (left), 11th (middle) and seventh (right) and with SARS-CoV-2 strain NIV-2020-770 infected Vero CCL-81 cells probed by positive patients serum (green panel) showing the reactivity of virus and antibody.

Table I. Cycle threshold (Ct) of SARS-CoV-2 positive clinical specimens and respective viral copy number in isolates in different passages for two different cell culture types using real-time reverse transcription-polymerase chain reaction (RT-PCR). *E* gene was targeted in all

Serial number	Sample ID	Isolate ID	Ct (copy number) of viral RNA in real-time RT-PCR		
			Original (clinical) samples by qRTPCR (Ct)	Vero CCL-81 passage-1 Ct (copy number)	Vero CCL-81 passage-2 Ct (copy number)
1	nCoV-763	NIV-2020-763	18.07	10.56 (4.08×10^9)	11.14 (2.77×10^9)
2	nCoV-770	NIV-2020-770	18	15.15 (1.96×10^8)	11.62 (2.02×10^9)
3	nCoV-772	NIV-2020-772	20.2	14.00 (4.18×10^8)	10.93 (3.19×10^9)
4	nCoV-773	NIV-2020-773	25.1	17.15 (5.18×10^7)	15.41 (1.69×10^8)
5	nCoV-781	NIV-2020-781	22.1	14.91 (2.27×10^8)	10.0 (5.91×10^9)
6	nCoV-C132	NIV-2020-C132	16	13.68 (5.12×10^8)	10.73 (3.64×10^9)
7	nCoV-777	NIV-2020-777	23.3	13.31 (6.57×10^8)	9.99 (5.92×10^9)
8	nCoV-C31	NIV-2020-C31	25	12.99 (8.12×10^8)	9.79 (6.77×10^9)
9	nCoV-C32	NIV-2020-C32	16	13.21 (7.01×10^8)	10.25 (5.05×10^9)

Serial numbers 1-7: Italian tourists who arrived in Delhi, India and an Indian contact of the cohort; Serial numbers 8-9: Close contacts in Agra, Uttar Pradesh, of an infected Delhi-based person who returned from Italy. qRT-PCR, quantitative RT-PCR

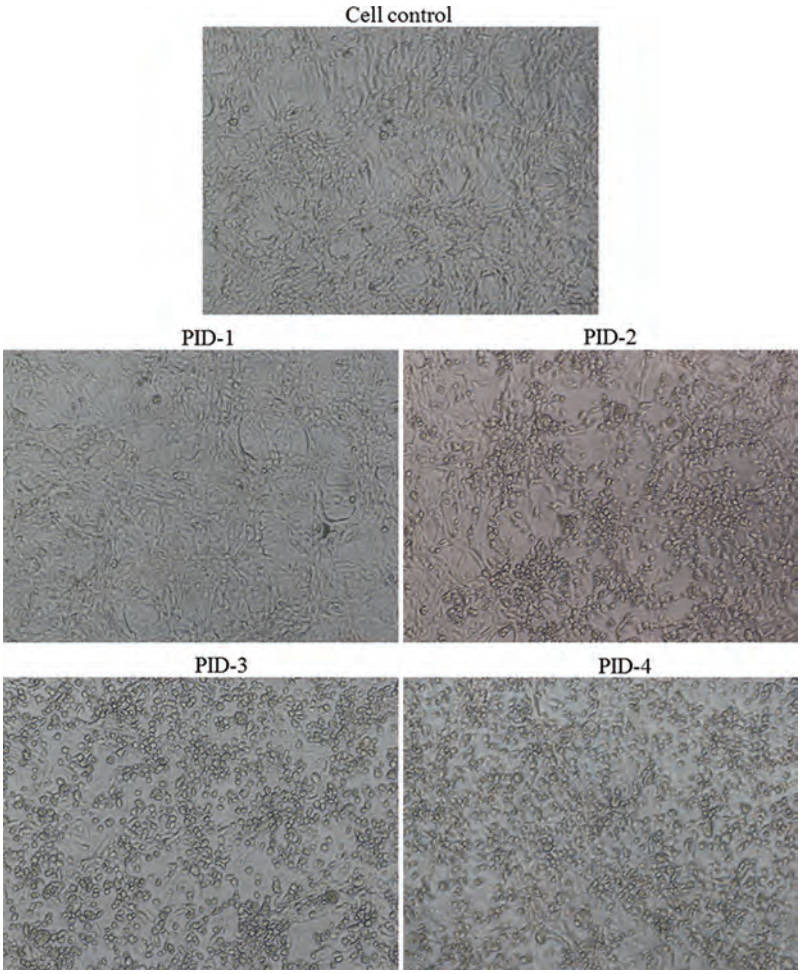

Fig. 2. Cytopathic effect of the SARS-CoV-2 isolate (NIV-2020-770) demonstrated in Vero CCL-81 cells on different post-infection days (PID).

Table II. Per cent of the reads mapped, total reads and the per cent of genome coverage recovered for the clinical samples and the isolates

Sample type	Sample/isolate details	Total reads	Per cent of reads mapped	Per cent of genome recovered	Position of nucleotide in genome[17]	
					8782	28144
Isolate	NIV-2020-763	10,054,258	94.8	100	C	T
	NIV-2020-770	4,384,130	99.0	100	C	T
	NIV-2020-772	3,482,648	98.4	99.9	C	T
	NIV-2020-773	5,952,758	94.2	99.9	C	T
	NIV-2020-777	3,949,748	98.7	100	C	T
	NIV-2020-781	2,226,464	91.6	99.9	C	T
	NIV-2020-C32	4,159,878	99.0	100	C	T
Clinical sample	nCoV-763	8,721,610	84.9	99.9	T	T
	nCoV-770	5,197,614	93.1	99.9	T	T
	nCoV-772	4,222,912	81.7	99.8	C	T
	nCoV-773	9,951,190	19.98	99.8	C	T
	nCoV-777	8,808,756	26.93	99.8	C	T
	nCoV-781	15,688,460	35.5	99.9	C	T
	nCoV-C32	2,772,158	88.5	100	C	T

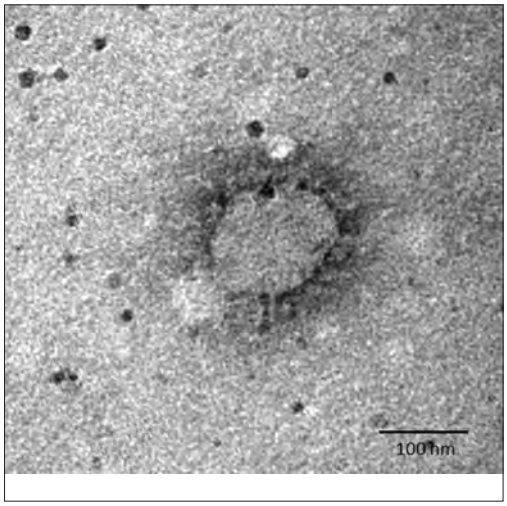

Fig. 3. Transmission electron microscopy imaging of SARS-CoV-2. A negative-stained SARS-CoV-2 viral particle, demonstrating spike morphology of glycoprotein along with peplomeric projections, a feature typical to the family *Coronaviridae*, is seen.

µl) included in the study and the tissue culture fluid (50 µl) of virus isolates at PID-3 as described earlier[14,15]. Reference-based mapping as implemented in the CLC genomics workbench 11.0 (CLC, Qiagen) was used to retrieve the sequence of the SARS-CoV-2. BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) identification of the viral genome sequences retrieved from the clinical samples and their isolates had 99.98 per cent identity with the SARS-CoV-2 isolate Wuhan-Hu-1 (Accession No. NC_045512). Details of the sequences obtained including the per cent of the reads mapped, total reads and the per cent of genome coverage recovered for the clinical samples and the isolates are provided in Table II. Partial sequences were retrieved from the clinical samples (nCoV-C 132 and nCoV-C 31) and were not included in the analysis.

MEGA software version 7.0.11[16] was used for the multiple alignments of the sequences retrieved in this study and the sequences from the Global Initiative on Sharing All Influenza Data (GISAID) database (*https://www.gisaid.org/*) (Supplementary Table). A neighbour-joining tree was generated using the best substitution model (Kimura 2-parameter model) with a bootstrap of 1000 replicates. As per Tang *et al*[17], the circulating SARS-CoV-2 can be grouped into two types (S and L type) based on the two different single-nucleotide polymorphisms (SNPs) at positions 8782 and 28144 in the genome. The S type possesses TC SNPs while the L type possesses CT SNPs at positions 8782 and 28144, respectively. In the present study, it was observed that two sequences from clinical samples (nCoV-763 and nCoV-770) had TT SNPs, while the other sequences had CT as the SNP (L type) (Table II). The TT SNPs have been observed in few of the GISAID sequences, including one of the Kerala genome sequences (nCoV-19/India/31 January 2020) submitted by us earlier. All the isolates of the clinical samples were of L type.

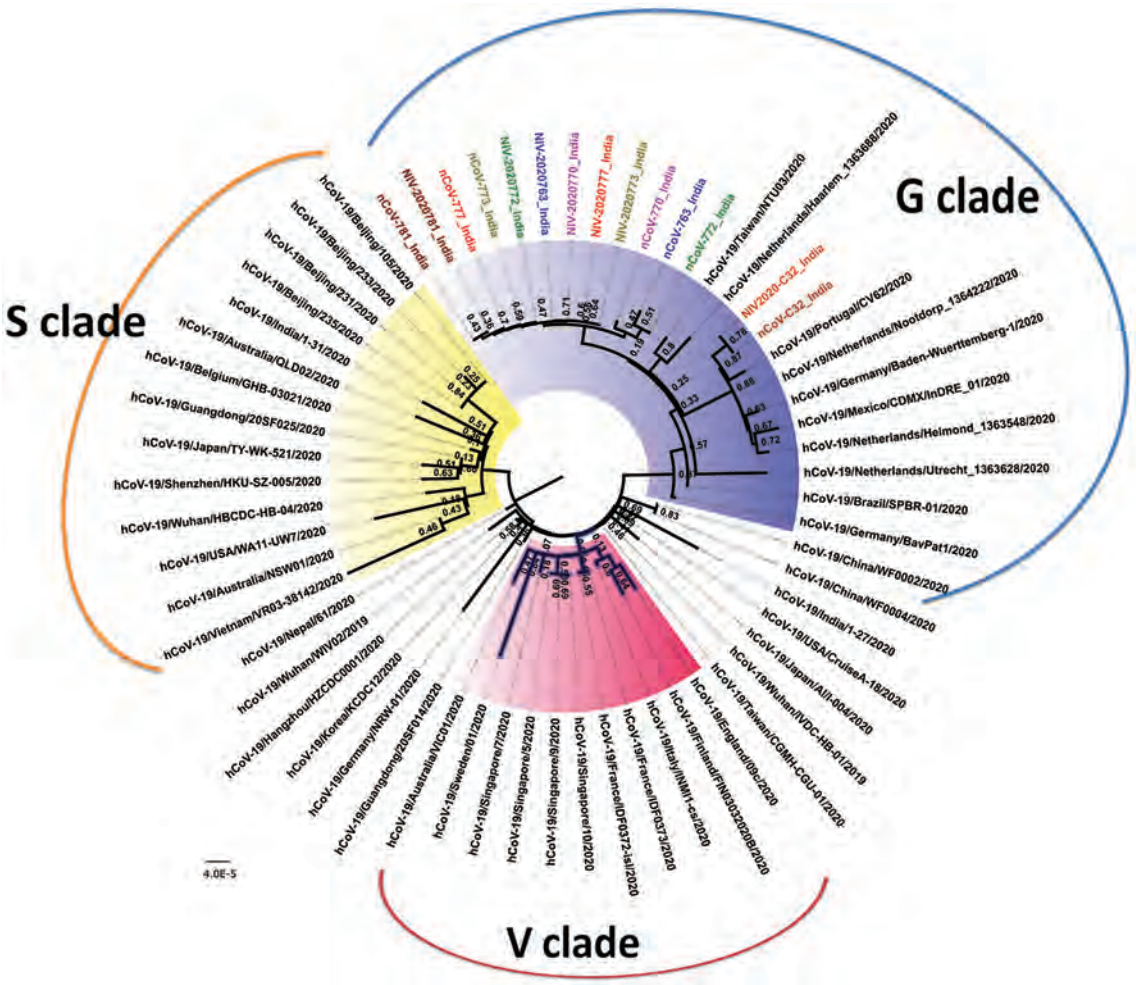

Fig. 4. Neighbour-joining tree of SARS-CoV-2. The phylogenetic tree is generated using the best substitution model. A bootstrap of 1000 replicates was used to assess the statistical robustness of the tree. Same colours are used for sequences derived from a clinical sample and the respective isolate. Clinical samples are labelled with initials as nCoV while the isolates are labelled with initials as NIV. The clades are represented by different colours in the core region (S - yellow, V - pink, G - blue and unclassified - not coloured).

Specific amino acid mutations in the nsp3 region, spike protein and ORF8, in general, lead to the formation of V, G and S genetic variants/clades, respectively, as per the recent classification followed by GISAID. It was observed that the clinical samples, as well as the isolates, had the mutation D614G in the spike protein, classifying the study samples and isolates into the G clade (Table II and Fig. 4). No specific substitutions were observed in any of the isolate sequences with respect to the corresponding clinical sample sequences, as these were sequences from a low passage. The sequences of the clinical samples and the isolate from the contact of the infected Delhi-based individual, who returned from Italy, further showed two mutations, R203K and G204R in the nucleocapsid protein (*N*). Although all strains demonstrated 99.6 per cent identity with the original Wuhan Hu-1 sequence, the role of unique SNPs and mutations in identifying the source of infection needs to be explored.

After the first isolation of the virus in the human airway epithelial cells reported by China[5], countries such as Australia[18], Korea[19], Germany[20] and the USA[21] have also isolated the SARS-CoV-2 strain. In India, initial attempts to isolate the virus from the first three cases did not succeed due to low titres in the clinical specimens. This is the first successful virus isolation of SARS-CoV-2 in the Vero CCL-81 cells in India from nasal and throat swabs of persons with a travel history from Italy and their contacts. Isolation of SARS-CoV-2 from clinical samples will be helpful to address key questions of correlating the differential cell line susceptibility and viral replication efficiency, especially important for clinical samples with low

viral titres. Isolation of the virus in such a pandemic situation would help to develop indigenously designed reagents such as positive controls, virus antigen and antibodies, which could lead to the indigenous development of sero-diagnostic assays. These assays would be critical for conducting population-based serosurveys. Propagation in culture will also facilitate antiviral susceptibility studies and vaccine efforts in India.

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Development of *in vitro* transcribed RNA as positive control for laboratory diagnosis of SARS-CoV-2 in India

The WHO has recommended reverse transcription-polymerase chain reaction (RT-PCR) for the confirmation of coronavirus disease 2019 (COVID-19) diagnosis. Real-time RT-PCR assays with automated extraction systems are required to process large numbers of specimens. Corman *et al*[1] have reported three real-time RT-PCR assays [based on the RNA-dependent RNA polymerase (*RdRp*) gene, envelope (*E*) gene and nucleocapsid (*N*) gene] for detecting beta coronaviruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1], and additionally, Chu *et al*[2] have reported two real-time RT-PCR assays based on *ORF* 1b and *N* gene that are highly conserved among Sarbeco viruses.

At the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), Pune, we adopted a real-time RT-PCR assay for screening (*E* gene assay) and confirmation (*RdRp*, *N* and *ORF* gene)[1] along with housekeeping *Rnase P* gene. Sequences and source of primers and probes used in this study are given in the Table, and the performance of screening assay was assessed using *in vitro* transcribed (IVT) RNA for SARS-CoV-2 targeting *E* gene, whereas the confirmatory *RdRp* assay used purified RNA of SARS-coronavirus Frankfurt 1 strain. Limited supply of positive controls was available from the WHO from Charité Laboratories, Berlin, *via* European Virus Archive Global (EVAg). A need was sensed to provide positive control to all laboratories in the national network of Viral Research and Diagnostic Laboratories (VRDLs) for real-time RT-PCR. Thus, positive controls for the screening and confirmatory assays were generated in-house.

Table. Primer and probe sets used in this study

Assay type	Name	Sequence (5'-3')
E gene screening assay	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT†
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA†
	E_Sarbeco_P1	FAM-ACACTAGCATCCTTACTGCGCTTCG-BHQ‡
RNase P gene (internal control) screening assay	RNaseP -F1	AGATTTGGACCTGCGAGCG†
	RNaseP -R1	GAGCGGCTGTCTCCACAAGT†
	RNaseP -P1	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ‡
RdRp gene confirmatory assay	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG†
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA†
	RdRP_SARSr-P2 (Specific for Wuhan-CoV)	FAM-CAGGTGGAACCTCATCAGGAGATGC-QSY‡
HKU ORF gene confirmatory assay	HKU-ORF1b-nsp14F	TGGGGYTTTACRGGTAACCT†
	HKU-ORF1b-nsp14 R	AACRCGCTTAACAAAGCACTC†
	HKU-ORF1b-nsp14 P	FAM-TAGTTGTGATGCWATCATGACTAG-QSY‡

Source: †Eurofins Genomics India Pvt. Ltd., Bengaluru; ‡Invitrogen, USA

Using whole-genome sequence of the first Indian COVID-19 case, forward primers with T7 promoter tag at the 5′ end, were designed to amplify full-length *E* gene, *N* gene and partial *RdRp* and *ORF* 1b regions. Gene-specific PCR was carried out to amplify the desired PCR product. Amplicons were purified using Qiagen direct PCR purification kit (Qiagen, Hilden, Germany). IVT was synthesized using T7 Riboprobe (Promega, USA) as per the kit protocol. Ten-fold serial dilutions of each transcribed RNA products were tested with respective gene primer probe sets for specific detection and limit of detection.

Gene-specific desired amplification was also observed in conventional RT-PCR (*E*: 550 bp, *N*: 1254 bp, *RdRP*: 344, *ORF*: 388) (Fig.1). Further, the IVT RNA of each gene was serially diluted 10-fold (10^1 to 10^{10}), and the performance was tested with gene-specific primer probe by real-time RT-PCR. All the transcribed RNA showed amplification with specific primer probe. The limit of detection for *E* gene was 10^6 yielding a cycle threshold (Ct) at cycle 29, *RdRP* (p1) was 10^5 with 27 Ct, *RdRp* (p2) was 10^6 with 29 Ct and *ORF* 1b10^6 with 28 Ct, whereas *N* gene showed 10^3 with 25 Ct (Fig. 2A-E).

When the assay was first set up at the National Influenza Centre of ICMR-NIV, Pune, the IVT RNA for E and SARS coronavirus Frankfurt 1 strain were received from EVAg. The real-time PCR screening assay (*E* gene) was also established at the 13 VRDLs as part of ICMR's efforts to expand testing to VRDLs closer to major airports[3]. However, due to screening of low number of samples, the repeated use of positive controls was made. The supply of IVT RNA as positive control for *E* gene from EVAg was limited and had non-consistent performance when diluted further. In addition, the control for RdRp assay was from a SARS coronavirus Frankfurt 1 isolate, which yielded weak signal with *RdRp* Wuhan-specific probe. This necessitated the development of an indigenous IVT RNA for *E* and *RdRp*. In addition, majority of the WHO screening protocols (5 of 6) are based on *N* gene targeting different nucleotide positions and require multiple specific positive controls[4]. Hence, an IVT RNA was designed for entire *N* gene which would be compatible for multiple protocols.

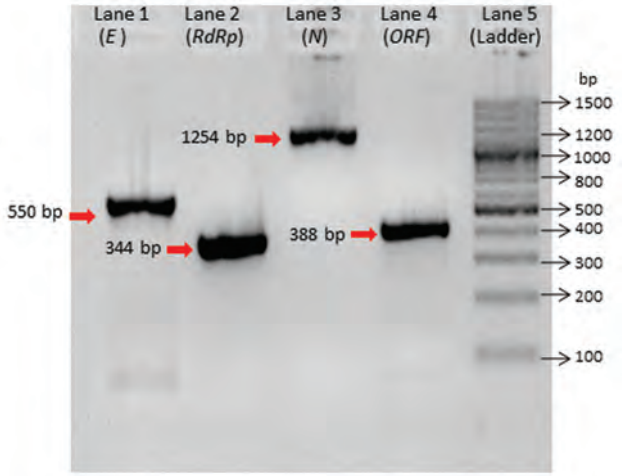

Fig.1. Positive DNA amplification of envelope (*E*), RNA-dependent RNA polymerase (*RdRp*), nucleocapsid (*N*) and open reading frame (*ORF*) 1b-nsp14 for *in vitro* transcribed preparation. Lane 1: *E* gene (550 bp), lane 2: *RdRp* gene (344 bp), lane 3: *N* gene (1254 bp) positive, lane 4: *ORF* (388 bp), lane 5: 100 bp DNA ladder.

We demonstrated the successful use of IVT RNA for *N* gene recommended in various protocols available on WHO site. The partial *RdRp* IVT RNA worked well with both the *RdRp* probes described in Charité, Berlin, Germany[5], especially Wuhan-specific *RdRp* probe 2, which could be used as confirmatory test. All the IVT RNA had good yield and performed well with specific primer probe.

In conclusion, gene-specific IVT RNA was synthesized for all the gene targets used in real time PCR. These IVTs were used effectively by all VRDLs as positive control. Successful establishment of diagnostic system including in-house positive control was beneficial to provide timely diagnosis and accelerate clinical management and isolation of SARS-CoV-2 patients and to control further spread.

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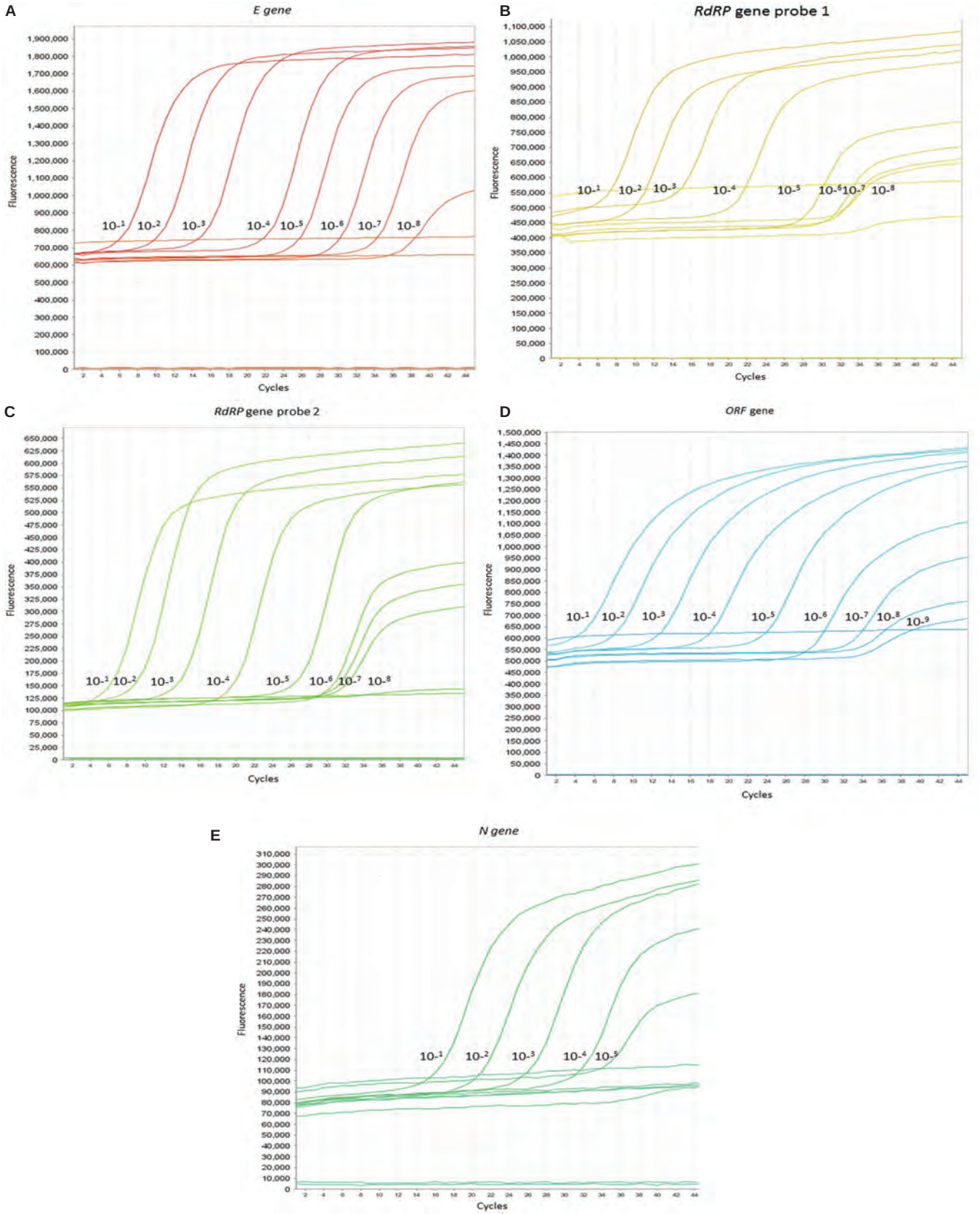

Fig. 2. (**A-E**) Multicomponent amplification graph for 10-fold dilutions of *in vitro* transcribed RNA: (**A**) *E* gene, (**B**) *RdRp* gene probe 1, (**C**) *RdRp* gene probe 2, (**D**) *ORF* gene, (**E**) *N* gene. The X axis represents number of cycles and Y axis represents amount of fluorescence.

***Conflicts of Interest*:** None.

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Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy, Iran & China, & Italian tourists in India

The single-stranded RNA genome of the 2019 novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) about 29.9 kb in length and encoding about 9860 amino acids, was annotated to possess 14 open reading frames (ORFs) and 27 proteins[1,2]. The *orf*1ab and *orf*1a genes at the 5′-terminus of the genome encode the pp1ab and pp1a proteins, respectively, together form 15 non-structural proteins (nsps), nsp1-nsp10 and nsp12-nsp16. The 3′-terminus of the genome encodes four structural proteins, the spike surface glycoprotein (*S*), the small envelope protein (*E*), membrane protein (*M*) and nucleocapsid protein (*N*). There are eight accessory proteins denoted as 3a, 3b, p6, 7a, 7b, 8b, 9b and ORF14[2].

The epidemiology of the SARS-CoV-2 since its emergence in December 2019 has been ever expanding, with increase in the number of cases and its spread globally[3,4]. The number of SARS-CoV-2 cases in India as on March 31, 2020 was 1,071, with mortality crossing 29[4]. In this context, it is vital to understand the genetic nature of circulating SARS-CoV-2. In India, as per the guideline of the Ministry of Health and Family Welfare, suspected samples of SARS-CoV-2 were collected and tested at the designated Viral Research and Diagnostic Laboratories (VRDL)[5]. As a part of this activity, a total of 15 SARS-CoV-2 positive specimens were obtained during the first week of March 2020, from Italian tourists and travellers from Italy and their contact cases in India. Further, in an effort to screen Indian nationals in Iran to enable their evacuation, during March 5 to 17, 2020, throat swabs were collected from 1,920 individuals; of whom 281 were positive. In addition, a team of Indian doctors visited Italy and collected a total of 380 swabs of Indian citizens; of whom four positive specimens were identified. In an earlier study, the authors identified the first three cases of SARS-CoV-2 in Kerala, India, as imported cases from Wuhan, China, and presented the first two full-genome sequences along with the potential B-cell and T-cell epitopes on the spike protein[6]. Further, in another study, the SARS-CoV-2 viruses were isolated in Vero CCL-81 cells[7]. The present study was undertaken to understand and compare the genetic makeup of representative samples of the imported cases of SARS-CoV-2 to India from Wuhan, China, those of Italian tourists in India and the Indians evacuated from Iran and Italy.

Throat swab/nasal swab specimens collected from the 1,920 individuals in Iran were tested at the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV) Pune, using real-time reverse transcription-polymerase chain reaction (RT-PCR) protocols to detect *RdRp* (1), *RdRp* (2), *E* and *N* genes as described elsewhere[8]. Next-generation sequencing (NGS) was performed on a total of 41 SARS-CoV-2 positive clinical samples from Italy and Iran. Table I presents the details of the full genomes obtained (n=19) as a part of this study as well as the two earlier genomes retrieved from the Kerala samples (n=2) from those who had the travel history from China[6,7].

Multiple sequence alignment of 21 full genomes obtained and 1563 full-genome sequences (Supplementary Table) available at the Global Initiative on Sharing All Influenza Data (GISAID) database (as of March 26, 2020) was carried out in MAFFT v.7.450[9]. The phylogenetic tree was constructed using MEGA v.6[10], employing the neighbour-joining method with the composite likelihood method and 1000 bootstrap replications. An initial tree was constructed based on a total of 1586 sequences. This

Table I. Cycle threshold (Ct) values of real-time reverse transcription-polymerase chain reaction (RT-PCR) for the *E* gene of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) along with the per cent of the reads mapped and the genome size recovered for the clinical samples

Sample ID	Travel history/details	Ct value of *E* gene for clinical samples	Per cent of relevant reads	Genome length (bp)	GISAID ID
hCoV-19/India/1-27/2020*	Wuhan, China travel history of Indian citizens (Group A)	34.5	0.36	29,854	EPI_ISL_413522
hCoV-19/India/1-31/2020*		28.98	0.80	29,851	EPI_ISL_413523
hCoV-19/India/1073/2020	Specimens collected at Iran from Indian citizens (Group B)	25	1.53	29,855	EPI_ISL_421662
hCoV-19/India/1093/2020		23	0.10	29,847	EPI_ISL_421663
hCoV-19/India/1100/2020		23	0.79	29,862	EPI_ISL_421664
hCoV-19/India/1104/2020		22	34.88	29,890	EPI_ISL_421665
hCoV-19/India/1111/2020		22	3.36	29,861	EPI_ISL_421666
hCoV-19/India/1115/2020		22	3.04	29,864	EPI_ISL_421667
hCoV-19/India/1125/2020		25	0.18	29,873	EPI_ISL_421668
hCoV-19/India/1616/2020		23	0.60	29,857	EPI_ISL_421669
hCoV-19/India/1621/2020		18	5.28	29,860	EPI_ISL_421671
hCoV-19/India/1644/2020		22	1.23	29,855	EPI_ISL_421672
hCoV-19/India/1652/2020		24	0.17	29,847	EPI_ISL_424363
hCoV-19/India/3118/2020	Indian contacts of an Indian citizen having travel history to Italy (Group C)	24	3.30	29,857	EPI_ISL_424364
hCoV-19/India/3239/2020		20	22.58	29,862	EPI_ISL_424365
hCoV-19/India/770/2020‡	Italian tourists who arrived in Delhi, India and an Indian contact of the cohort (Group D)	18	93.08	29,862	EPI_ISL_420545
hCoV-19/India/773/2020‡		25.1	19.98	29,858	EPI_ISL_420549
hCoV-19/India/777/2020‡		22.1	26.93	29,856	EPI_ISL_420551
hCoV-19/India/781/2020‡		22.1	35.47	29,871	EPI_ISL_420553
hCoV-19/India/31/2020	Close contacts in Agra, of an infected Delhi-based person who returned from Italy (Group E)	25	2.13	29,860	EPI_ISL_426179
hCoV-19/India/32/2020‡		16	88.50	29,903	EPI_ISL_420555

GISAID, Global Initiative on Sharing All Influenza Data
Source: *Ref. 6, ‡Ref. 7

tree was used to reduce the dataset to 121 sequences, on the basis of country and the genetic variant identified based on the GISAID classification. Comparison of the sequences of this study with respect to the Wuhan Hu-1 reference strain was done to identify unique mutations, if any.

Phylogenetic trees based on full-genome sequences deposited and available at GISAID revealed the diversification and the clustering of sequences into groups, based on the genetic variants. Specific amino acid substitutions in the nsp3 region, spike protein and ORF8, in general, lead to the formation of V, G and S genetic variants/clades, respectively. The S clade corresponds to the C28144T nucleotide polymorphism that results in a non-synonymous substitution Leu84Ser in ORF8. Clades V, G and a group of unclassified strains possess mainly C28144 and are referred to as the L type[11]. The phylogenetic analyses of the study strains and the other global sequences revealed that the SARS-CoV-2 sequences derived from Italy (n=8) in this study, clustered in clade G, while the SARS-CoV-2 sequences (n=11) of Indians evacuated from Iran belonged to the unclassified group which also included one of the SARS-CoV-2 sequences imported from Wuhan (hCoV-19/India/1-27/2020) (Figure). The other sequences imported from Wuhan (hCoV-19/India/1-31/2020) possessed Leu84Ser in ORF8b, classifying it in clade S.

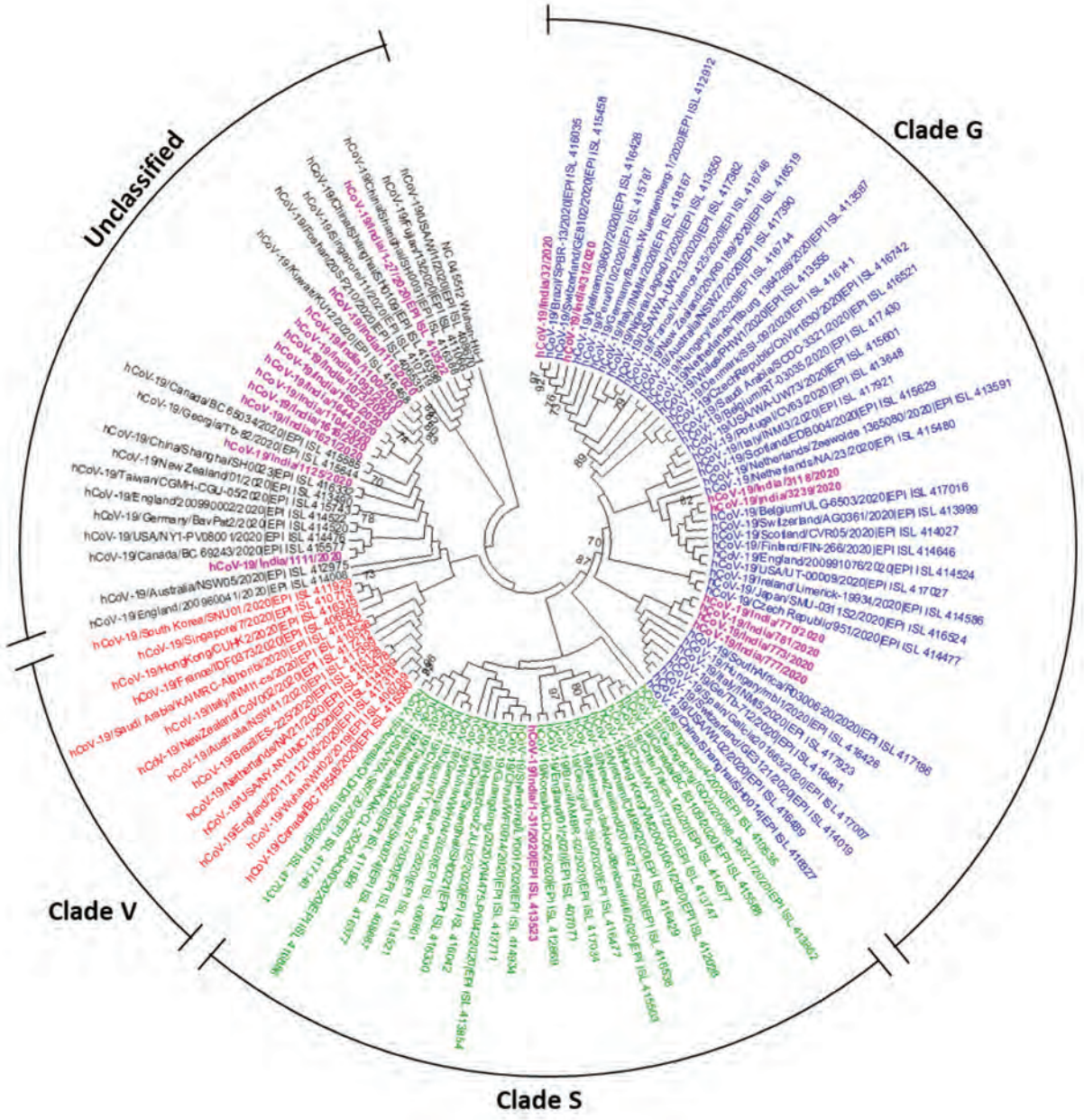

Figure. Phylogenetic tree of selected representative full-genome sequences of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-derived from coronavirus disease 2019 positive patients' clinical samples who had travel history of Wuhan, China, Italy and Iran by neighbour-joining method. Strains sequenced at ICMR-NIV are shown in magenta colour. The clades as per Global Initiative on Sharing All Influenza Data (GISAID) nomenclature are indicated in blue (clade G), red (clade V), green (clade S) and black (unclassified).

The sequences of Italy origin were noted to segregate into at least two subgroups. The percentage nucleotide divergence (PND) within these sequences was found to be 0.01 per cent. The SARS-CoV-2 sequences from the Italian tourists (n=6) showed relatedness to other European SARS-CoV-2 sequences from Scotland, Finland, England, Spain, Ireland and the Czech Republic along with a Shanghai, China, strain as the outgroup (Figure). Two other sequences (hCoV-19/India/3118/2020 and

hCoV-19/India/3239/2020) clustered more closely with sequences from Belgium and Switzerland. The two sequences (hCoV-19/India/31/2020 and hCoV-19/India/32/2020) from the Agra contacts of the Italy-returned Delhi based individuals were more distinct and showed clustering in a strongly supported subgroup consisting of strains from Brazil and the European countries including Switzerland, Germany, France, Hungary and The Netherlands.

The variable amino acid sites based on the alignment of the 21 sequences of this study with respect to Wuhan Hu-1 strain are shown in Table II. All the Italy-origin sequences possessed the substitution D7711G/D614G in the S protein, characteristic of the G clade, along with another mutation P4715L (nsp12-323) that is also shared with many other countries. Mutation S1515F (nsp3-697) was specific to the Italian cohort strain; D8726G (M-3) was specific to hCoV-19/India/3118/2020 and hCoV-19/India/3239/2020 (Indian contacts of an Indian citizen having travel history to Italy), similar to sequences from Scotland, Belgium, Finland, Switzerland and England. The mutations, R9455K and G9456R (N-203 and 204), were found to be specific to the two strains, hCoV-19/India/31/2020 and hCoV-19/India/32/2020 but shared with a few more countries. A recent study has identified the earliest Italian importation of SARS-CoV-2 to a case from Shanghai, China, and has also identified at least two circulating variants in Italy[12]. Thus, it is likely that the former strain (Italian cohort) has its origin from China, whereas the latter strain (contacts in Agra, n=2) appears to have been from a European cluster involving an entry into Germany that preceded the first cases in Italy by almost a month[12,13].

Analysis of the strains from the SARS-CoV-2 positives in Iran (Figure) showed that these sequences (n=11) clustered with other strains having a global spread inclusive of Canada, USA, several European countries, New Zealand, Australia and Southeast Asian countries noted in this group (moderate support of 64%). The PND among these study sequences was found to be 0.24 per cent. Common mutations shared among SARS-CoV-2 sequences in the group included R207C (nsp2-27), V378I (nsp2-198), M2796I (nsp4-33) and L3606F (nsp6-37). A mutation V9082F (ORF7a-74) was unique to four of the study sequences (hCoV-19/India/1073/2020, hCoV-19/India/1093/2020, hCoV-19/India/1115/2020 and hCoV-19/India/1100/2020) that clustered with a strain from Kuwait, KU12. The KU12 strain was also noted to possess this mutation. To date, there are no other sequences from Iran in the GISAID database. However, a phylogenetic study[14] of full-genome sequences has identified distinct SARS-CoV-2 link to travellers returning from Iran to Australia and New Zealand. Some of these representative sequences were included in this study as well.

In terms of the overall divergence of SARS-CoV-2, the strains in this study were 99.97 per cent identical to the earliest strain Wuhan Hu-1. However, it is vital to track the evolutionary dynamics of the strains *vis-à-vis* the strains circulating globally and monitor any specific changes in the functional sites of the major viral proteins.

Delineation of circulating strains into three major evolving clades has been reflected in GISAID, with clade G apparently being one of the dominant ones. From the start of the pandemic, severity or transmission patterns have not been associated with any clade in particular. A limitation of this study was the non-availability of full genomes from other parts of India. This would enable a pan-India comparison of the circulating strains in the country. Overall, the present study revealed genetic variants in India that were similar to strains circulating in the specific regions of their origin. Continued surveillance of SARS-CoV-2 strains in India is warranted to get the complete picture of all circulating strains and identify changes that could be associated with increased virulence.

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Table II. Variable amino acid positions in the Indian full-genome sequences

Amino acid position in genome	207	378	476	671	1515	2079	2144	2796	3606	4715	4798	5538	7505	7535	7711	8027	8726	9082	9214	9455	9456
NC 045512 Wuhan-Hu-1	R	V	I	I	S	P	P	M	L	P	A	T	R	S	D	A	D	V	L	R	G
hCoV-19/India/1-27/2020|EPI ISL 413522	.	.	.	T	.	.	S	.	.	.	V	.	I	.	.	.	.	.	.	.	.
hCoV-19/India/1-31/2020|EPI ISL 413523	.	.	V	.	.	L	.	.	.	.	.	I	.	.	.	V	.	.	S	.	.
hCoV-19/India/1073/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	F	.	.	.
hCoV-19/India/1093/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	F	.	.	.
hCoV-19/India/1100/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	F	.	.	.
hCoV-19/India/1104/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1111/2020	.	I	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1115/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	F	.	.	.
hCoV-19/India/1125/2020	.	I	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1616/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1621/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1644/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/1652/2020	C	I	.	.	.	.	.	I	F	.	.	.	.	.	.	.	.	.	.	.	.
hCoV-19/India/3118/2020	.	.	.	.	.	.	.	.	.	L	.	.	.	.	G	.	G	.	.	.	.
hCoV-19/India/3239/2020	.	.	.	.	.	.	.	.	.	L	.	.	.	.	G	.	G	.	.	.	.
hCoV-19/India/770/2020	.	.	.	.	F	.	.	.	.	L	.	.	.	.	G	.	.	.	.	.	.
hCoV-19/India/773/2020	.	.	.	.	F	.	.	.	.	L	.	.	.	.	G	.	.	.	.	.	.
hCoV-19/India/777/2020	.	.	.	.	F	.	.	.	.	L	.	.	.	.	G	.	.	.	.	.	.
hCoV-19/India/781/2020	.	.	.	.	F	.	.	.	.	L	.	.	.	.	G	.	.	.	.	.	.
hCoV-19/India/31/2020	.	.	.	.	.	.	.	.	.	L	.	.	.	.	G	.	.	.	.	K	R
hCoV-19/India/32/2020	.	.	.	.	.	.	.	.	.	L	.	.	.	.	G	.	.	.	.	K	R

Wuhan Hu-1 strain of severe acute respiratory syndrome coronaviruses 2 (SARS-CoV-2) is used as the reference strain. Strains and mutations specific to China, Iran and Italy are shown in orange, violet and brown colour, respectively. In case of Iran and Italy, only those amino acid sites are shown where at least two of the sequences share the same mutation. R, arginine; V, valine; I, isoleucine; S, serine; P, proline; M, methionine; L, leucine; A, alanine; T, threonine; D, aspartic acid; G, glycine; C, cysteine; F, phenylalanine; K, lysine

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Indian J Med Res 151, February & March 2020, pp 261

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Contents

Pages

First isolation of SARS-CoV-2 from clinical samples in India
Prasad Sarkale, Savita Patil, Pragya D. Yadav, Dimpal A. Nyayanit, Gajanan Sapkal, Shrikant Baradkar, Rajen Lakra, Anita Shete-Aich, Sharda Prasad, Atanu Basu, Lalit Dar, Veena Vipat, Sidhartha Giri, Varsha Potdar, Manohar Lal Choudhary, Ira Praharaj, Amita Jain, Bharati Malhotra, Pranita Gawande, Kaumudi Kalele, Nivedita Gupta, Sarah S. Cherian & Priya Abraham ... 244
DOI: 10.4103/ijmr.IJMR_1029_20

Development of *in vitro* transcribed RNA as positive control for laboratory diagnosis of SARS-CoV-2 in India
Manohar Lal Choudhary, Veena Vipat, Sheetal Jadhav, Atanu Basu, Sarah Cherian, Priya Abraham & Varsha A. Potdar ... 251
DOI: 10.4103/ijmr.IJMR_671_20

Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy, Iran & China, & Italian tourists in India
Varsha Potdar, Sarah S. Cherian, Gururaj Rao Deshpande, Padinjaremattathil Thankappan Ullas, Pragya D. Yadav, Manohar Lal Choudhary, Rohan Gughe, Veena Vipat, Sheetal Jadhav, Savita Patil, Dimpal Nyayanit, Triparna Majumdar, Atul Walimbe, Shivshankar Gaikwad, Hitesh Dighe, Anita Shete-Aich, Sreelekshmy Mohandas, Deepika Chowdhury, Gajanan Sapkal, Atanu Basu, Nivedita Gupta, Raman R. Gangakhedkar, Sidhartha Giri, Lalit Dar, Amita Jain, Bharati Malhotra, Priya Abraham & NIC Team ... 255
DOI: 10.4103/ijmr.IJMR_1058_20

Contents *Pages*

Full-genome sequences of the first two SARS-CoV-2 viruses from India
Pragya D. Yadav, Varsha A. Potdar, Manohar Lal Choudhary, Dimpal A. Nyayanit, Megha Agrawal, Santosh M. Jadhav, Triparna D. Majumdar, Anita Shete-Aich, Atanu Basu, Priya Abraham & Sarah S. Cherian ... 200
DOI: 10.4103/ijmr.IJMR_663_20

Strategic planning to augment the testing capacity for COVID-19 in India
Nivedita Gupta, Tarun Bhatnagar, Kiran Rade, Manoj Murhekar, Raman R. Gangakhedkar, Anu Nagar & ICMR COVID Team ... 210
DOI: 10.4103/ijmr.IJMR_1166_20

Laboratory preparedness for SARS-CoV-2 testing in India: Harnessing a network of Virus Research & Diagnostic Laboratories
Nivedita Gupta, Varsha Potdar, Ira Praharaj, Sidhartha Giri, Gajanan Sapkal, Pragya Yadav, Manohar Lal Choudhary, Lalit Dar, A.P. Sugunan, Harmanmeet Kaur, Ashok Munivenkatappa, Jayanthi Shastri, Krishnasamy Kaveri, Shanta Dutta, Bharti Malhotra, Amita Jain, Kammilli Nagamani, G.B. Shantala, Sharmila Raut, M.M. Vegad, Ajanta Sharma, Aashish Choudhary, Megha Brijwal, Anukumar Balakrishnan, Jayaswamy Manjunatha, Manish Pathak, Sivasubramanian Srinivasan, Hasina Banu, Himanshu Sharma, Parul Jain, Pakalpati Sunita, R. Ambica, Babita Fageria, Disha Patel, Gitika Rajbongshi, Neetu Vijay, Jitendra Narayan, Neeraj Aggarwal, Anu Nagar, Raman R. Gangakhedkar & Priya Abraham ... 216
DOI: 10.4103/ijmr.IJMR_594_20

Detection of coronaviruses in *Pteropus & Rousettus* species of bats from different States of India
Pragya D. Yadav, Anita Shete-Aich, Dimpal A. Nyayanit, Prachi Pardeshi, Triparna Majumdar, R. Balasubramanian, Padinjaremattathil Thankappan Ullas, Sreelekshmy Mohandas, Hitesh Dighe, Pradeep Sawant, Savita Patil, Dilip Patil, M.D. Gokhale, Basavaraj Mathapati, A.B. Sudeep, Sreekant Baradkar, Abhimanyu Kumar, Rutuja Kharde, Malvika Salve, Yash Joshi, Nivedita Gupta & Devendra T. Mourya ... 226
DOI: 10.4103/ijmr.IJMR_795_20

Severe acute respiratory illness surveillance for coronavirus disease 2019, India, 2020
Nivedita Gupta, Ira Praharaj, Tarun Bhatnagar, Jeromie Wesley Vivian Thangaraj, Sidhartha Giri, Himanshu Chauhan, Sanket Kulkarni, Manoj Murhekar, Sujeet Singh, Raman R. Gangakhedkar, Balram Bhargava & ICMR COVID Team ... 236
DOI: 10.4103/ijmr.IJMR_1035_20

Correspondences

Transmission electron microscopy imaging of SARS-CoV-2
Sharda Prasad, Varsha Potdar, Sarah Cherian, Priya Abraham, Atanu Basu & ICMR-NIV NIC Team ... 241
DOI: 10.4103/ijmr.IJMR_577_20

Contents

Pages

COVID-19 in India: Moving from containment to mitigation
George M. Varghese & Rebecca John ... 136
DOI: 10.4103/ijmr.IJMR_860_20

Responding to COVID-19 pandemic: Why a strong health system is required
Tanzin Dikid, Sushma Chaudhary, Kapil Goel, Preeti Padda, Rajesh Sahu, Tripurari Kumar, S.K. Jain, S.K. Singh & Jai Prakash Narain ... 140
DOI: 10.4103/ijmr.IJMR_761_20

Review Articles

The 2019 novel coronavirus disease (COVID-19) pandemic: A review of the current evidence
Pranab Chatterjee, Nazia Nagi, Anup Agarwal, Bhabatosh Das, Sayantan Banerjee, Swarup Sarkar, Nivedita Gupta, Raman R. Gangakhedkar ... 147
DOI: 10.4103/ijmr.IJMR_519_20

Perspectives for repurposing drugs for the coronavirus disease 2019
Sarah S. Cherian, Megha Agrawal, Atanu Basu, Priya Abraham, Raman R. Gangakhedkar & Balram Bhargava ... 160
DOI: 10.4103/ijmr.IJMR_585_20

Biorisk assessment for infrastructure & biosafety requirements for the laboratories providing coronavirus SARS-CoV-2/(COVID-19) diagnosis
Devendra T. Mourya, Gajanan Sapkal, Pragya D. Yadav, Sujeet Kumar M. Belani, Anita Shete & Nivedita Gupta ... 172
DOI: 10.4103/ijmr.IJMR_763_20

Policy Guidelines

Guidance for building a dedicated health facility to contain the spread of the 2019 novel coronavirus outbreak
Anup Agarwal, Nazia Nagi, Pranab Chatterjee, Swarup Sarkar, Devendra Mourya, Rima Rakeshkumar Sahay & Rajesh Bhatia ... 177
DOI: 10.4103/ijmr.IJMR_518_20

Protocol

Lopinavir/ritonavir combination therapy amongst symptomatic coronavirus disease 2019 patients in India: Protocol for restricted public health emergency use
Tarun Bhatnagar, Manoj V. Murhekar, Manish Soneja, Nivedita Gupta, Sidhartha Giri, Naveet Wig & Raman Gangakhedkar ... 184
DOI: 10.4103/ijmr.IJMR_502_20

Original Articles

Prudent public health intervention strategies to control the coronavirus disease 2019 transmission in India: A mathematical model-based approach
Sandip Mandal, Tarun Bhatnagar, Nimalan Arinaminpathy, Anup Agarwal, Amartya Chowdhury, Manoj Murhekar, Raman R. Gangakhedkar & Swarup Sarkar ... 190
DOI: 10.4103/ijmr.IJMR_504_20

R.N.I. Regd. No. 2223/57

Indian J Med Res 151, February & March 2020, pp 111-262

Special Issue - India & COVID-19

Guest Editors: Drs Rajesh Bhatia & Priya Abraham

Contents *Pages*

Editroial

Time to revisit national response to pandemics
Rajesh Bhatia & Priya Abraham ... 111
DOI: 10.4103/ijmr.IJMR_846_20

Viewpoint

Tracking the impact of interventions against COVID-19 in absence of extensive testing *Active surveillance for SARI is urgently needed*
Ganesan Karthikeyan ... 114
DOI: 10.4103/ijmr.IJMR_864_20

Perspectives

The research community must meet the coronavirus disease 2019 challenge
Poonam Khetrapal Singh ... 116
DOI: 10.4103/ijmr.IJMR_832_20

Public engagement is key for containing COVID-19 pandemic
Rajesh Bhatia ... 118
DOI: 10.4103/ijmr.IJMR_780_20

COVID-19: Impact on health of people & wealth of nations
Prakash N. Tandon ... 121
DOI: 10.4103/ijmr.IJMR_664_20

Ethics preparedness for infectious disease outbreaks research in India: A case for novel coronavirus disease 2019
Roli Mathur ... 124
DOI: 10.4103/ijmr.IJMR_463_20

Need for integrated surveillance at human-animal interface for rapid detection & response to emerging coronavirus infections using One Health approach
Rajesh Bhatia ... 132
DOI: 10.4103/ijmr.IJMR_623_20