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Factors affecting SARS-CoV-2 (COVID-19) Pandemic, including Zoonotic, Human Transmission and Chain of Infection. Reducing Public health Risk by Serum Antibody Testing, Avoiding Screening in Unhygienic Places and False PCR Reporting. A Scientific Review

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Abstract

Since December 2019, a rapid increase in the number of SARS-CoV-2 (COVID-19) cases was reported worldwide, despite strict infection control and lock down measures. Current paper investigated the actual facts behind this rapid increase in the number of cases. Study of genomic sequence reveals that domestic and wild animals were likely ancestors and zoonotic source for SARS-CoVs, MERS-CoVs, and SARS-CoV-2. Strong evidence suggest that these viruses already existed and replicated in animals and humans during past several decades, exhibiting diverse mutations, evolutions and self-limiting diseases, except during outbreaks. Serious zoonotic reservoir investigations are required to investigate animal transmission of SARS-CoVs and SARS-CoV-2 to limit current pandemic. This might be the reason of increasing number of cases via animals. SARS-CoV-2 has been retrospectively isolated in different studies in August 2019, several months before Wuhan announced. Hence, there is a possibility that viruses existed, went undetected, infecting subclinically, in past several years, and SARS-CoV-2 antigens and neutralizing antibodies may have been present in humans since long time. This might be another reason of increasing number of cases by screening as mass screening and antigen or antibody testing was not carried out in the past years. Randomized controlled trials are required to investigate human to human transmission by touch, as the current evidence is limited with conflicting results. As all SARS-CoVs are basically respiratory viruses, droplet precautions and infection control measures are essential, especially for hospital staff. Increased number of SARS-CoV-2 asymptomatic, or subclinical cases are detected worldwide. This silent phase of transmission can be beneficial for humans. Lack of symptoms eventually lessen virus transmission and reduce the pathogen's long-term survival and provide humoral herd immunity up to several years. Hence, seropositivity with diverse antibodies develops against mutating SARS-CoVs which will confer strong immunity during epidemics. Strategies such as identification, contact tracing and quarantine are costly and practically difficult. Hence, asymptomatic persons can continue their work with droplet precautions and standard infection control procedures, while symptomatic or sick persons can isolate themselves in their homes without the need for strict quarantine until clinical recovery, with reduced hospital visits and minimizing chances of hospital acquired infections. RT-PCR has low sensitivity and specificity, carries a high risk of handling live virus antigens, and requires difficult protocols. As viral load also sharply declines after few days of onset of infection, this technique might overlook infection. Furthermore, SARS-CoV-2 infection may be present in blood when oropharyngeal swabs are negative by RT-PCR. Additionally, RT-PCR usually gives false negative and false positive results and must be interpreted cautiously. This might be again a reason of increasing number of cases by false positive RT-PCR reporting. Moreover, antibodies against SARS-CoVs develop robustly in serum even by reduced amount of antigens. In contrast to RT-PCR, ELISA for diagnosing antibodies against SARS-CoV-2 demonstrates 100% specificity and 100% sensitivity, even in clinically asymptomatic individuals. These antibodies can be used for serologic surveys, monitoring and screening. However, screening tests for SARS-COV-2 should be avoided in unhygienic public places by nasopharyngeal swabs, which carry a high risk of further transmission, co-infection or superinfection. Such

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highly infectious virus must be isolated and tested in highly sterilized laboratory. Further strict international laws and policies are required to stop the possible spread of experimental viruses, biological warfare and bioterrorism.

Introduction

It was December 2019, when one of the novel coronavirus infections SARS-CoV-2 (COVID-19) outbreak occurred in Wuhan, China. After that, several research papers were published, identifying the characteristics of this virus with nearly one thousand publications worldwide. However, most of these publications are based on the information from one aspect, nor encompassing all the causative factors which are leading to the increasing frequency of the current pandemic. Different aspects, including negative or positive facts have been missed in previous papers. None of these publications have collected all reports with systemic review. To limit SARS-CoV-2 (COVID-19) infections, and to understand all scientific facts, here we have summarized different aspects of this disease with systemic review.

To retrieve the research papers, the software of Reference Manager (Version 12) was used for internet search of literature and electronic databases, including PubMed/Medline, ISI Web of Knowledge, Z.39.50 gateway (international standard client–server communication protocols, and includes gateway to library catalogues). Furthermore, Google Scholar was also included in the search methodology. All the published papers were reviewed thoroughly, in details and the recent information regarding SARS-CoV-2 was extracted.

1. Molecular biology, immunology and genetics

1.1 History and correct nomenclature of the virus for scientific studies

Discovery of coronavirus took place in 1930s after domestic chickens were infected, resulting in acute respiratory infection, and demonstrated to be caused by a virus known as avian infectious bronchitis virus (IBV); later labelled as the coronavirus of the fowl or chicken (*Gallus gallus*). This coronavirus replicates in epithelium of upper and lower respiratory tracts and as well as several other organs of the chicken, and is one of the important causes of economic loss for poultry industry. Virus was detectable in respiratory secretions and feces. This coronavirus was labelled 229-E. Now there is increasing evidence that coronavirus also infects species of birds other than chickens, such as bats, with astonishing molecular diversity [1-6]. Hence, it was then well known that coronaviruses are causing disease in several other animals including pigs, cows, chickens, dogs, and cats; for example Transmissible Gastroenteritis Virus (TGEV) and Porcine Epidemic Diarrhea Virus (PEDV). Recently, a novel coronavirus, called SW1, was identified in a deceased Beluga whale, which cased respiratory and liver failure [7]

The first human coronaviruses (HCoV) were discovered in the 1960s with demonstration that colds could be induced by nasal washing which did not contain rhinoviruses. Later in vitro experiments demonstrated the presence of coronaviruses with similar morphology as IBV [8-11]. Furthermore, it has been shown by scientist that human coronavirus can cause diseases beyond respiratory system, affecting multiple organ systems. This has been demonstrated in studies conducted on patients with multiple sclerosis, where coronavirus RNAs, and antigens were detected from the isolate of brain tissue. Additionally, scientists have proved human coronavirus gene expression in the brains of multiple sclerosis patients and labelled this phenomenon as "Infectious causes of multiple sclerosis" [12-18]. Current research literature demonstrates that all human coronaviruses (CoVs) are thought to originate from animal reservoirs with changes which have occurred in the genome during the course of propagation at different geographical locales. SARS-CoV (from bats via masked palm civet cats in China) and MERS-CoV (camels in the Middle East, seasonal especially during camel birthing) being prominent recent examples [19,20].

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Furthermore, and in fact, it has been demonstrated that novel bat CoVs are likely ancestors for SARS-CoV and MERS-CoV, with discovery of hundreds of coronaviruses over the past decades [21]. It has been demonstrated in research that SARS-CoV originated in Chinese horseshoe bats which contain same genetic sequences of SARS-related CoVs with serologic evidence of a prior infection with a related CoV [22,23].
Hence, it is very clear that coronavirus already existed and replicated in humans during past several decades, in multiple organs exhibiting its antigens during testing. This was also evident from the fact that diagnosis of coronaviruses was unnecessary as the disease was self-limiting and naturally completing its course with the exceptions during the outbreaks.

Initially, W.H.O (World Health Organization, an agency of the United Nations) named this virus as 2019-nCoV. On Feb 11, 2020, WHO renamed the disease as coronavirus disease 2019 (COVID-19), a rather unspecific name, which was not a scientific name, but a traditional approach. However, on the basis of phylogenetic analysis (genome sequence), and using a computational framework of comparative genomics, the Coronavirus Study Group (CSG) of the International Committee on Virus Taxonomy designated the scientific name to this naturally occurring virus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a universal nomenclature approach [24-28]. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses has clearly stated that "The Study Groups quantify and partition the variation in the most conserved replicative proteins encoded in open reading frames 1a and 1b (ORF1a/1b) of the coronavirus genome" and that "Although these viruses were isolated at different times and locations from different human and animal hosts (with and without causing clinical disease), they all belong to the species *Severe acute respiratory syndrome-related coronavirus*, and their relationship parallels that between human individuals and the species *Homo sapiens*". Hence, SARS-CoV-2 should be the recommended name.

1.2 Taxonomy and molecular biology of SARS-CoV-2

Coronaviruses (CoVs; order *Nidovirales*, family *Coronaviridae*, subfamily *Orthocoronavirinae*), are the largest group of enveloped positive sense single stranded RNA viruses, belonging to the *Nidovirales* order, characterized by club-shaped spikes projecting from the cell surface of the virion, with unusually large complex RNA genome [29-31]. Regarding genomic organization, Coronaviruses contain a non-segmented, positive-sense RNA genome of ~30 kilobase (kb). The genome contains a 5’ cap structure along with a 3’ poly (A) tail, allowing it to act as an mRNA for translation of the replicase polyproteins . The organization of the coronavirus genome is 5’-leader-UTR- replicase-S (Spike)-E (Envelope)-M (Membrane)-N (Nucleocapsid)-3’ UTR-poly (A) (UTR=tail untranslated region). Considering Virion Structure, Coronavirus virions are spherical with diameters of approximately 125 nm. Virus particles consist of four main structural proteins, that is, the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, all of which are encoded within the 3’ end of the viral genome [32]. The *Coronaviridae* are further subdivided into four genera, the alpha, beta, gamma, and delta coronaviruses. Seven types of coronaviruses are known to infect humans, casing respiratory symptoms of various severities. HCoV-229E (α-CoV), HCoV-OC43 (β-CoV), and HCoV-NL63 (α-CoV) cause common cold.

Since 2000, there has been three major world-wide health emergencies and severe crises, namely the 2003 SARS (severe acute respiratory syndrome; SARS-CoV, a β-CoV) outbreak, the 2012 MERS (Middle East respiratory syndrome; MERS-CoV; a β-CoV) outbreak, and the SARS-CoV-2 (2019-nCoV; a β-CoV) outbreak. Genomic structures of SARS-CoV and MERS-CoV are presented in figure-1. Surprisingly since 2000, no vaccine has been developed, which is alarming to the global community. Virus initially attaches to the host cell receptor by S protein (the receptor-binding domain, RBD), and this interaction is responsible for further actions and disease process. Aminopeptidase N (APN) receptors are utilized by several α-coronaviruses. However, SARS-CoV and HCoV-NL63 target angiotensin converting enzyme 2 (ACE2) as their host cell receptor; while MERS-CoV enters into human cells via attaching to dipeptidyl-peptidase 4 (DPP4) receptor, then ultimately leading to translation of the replicase gene from the virion genomic RNA [33,34].

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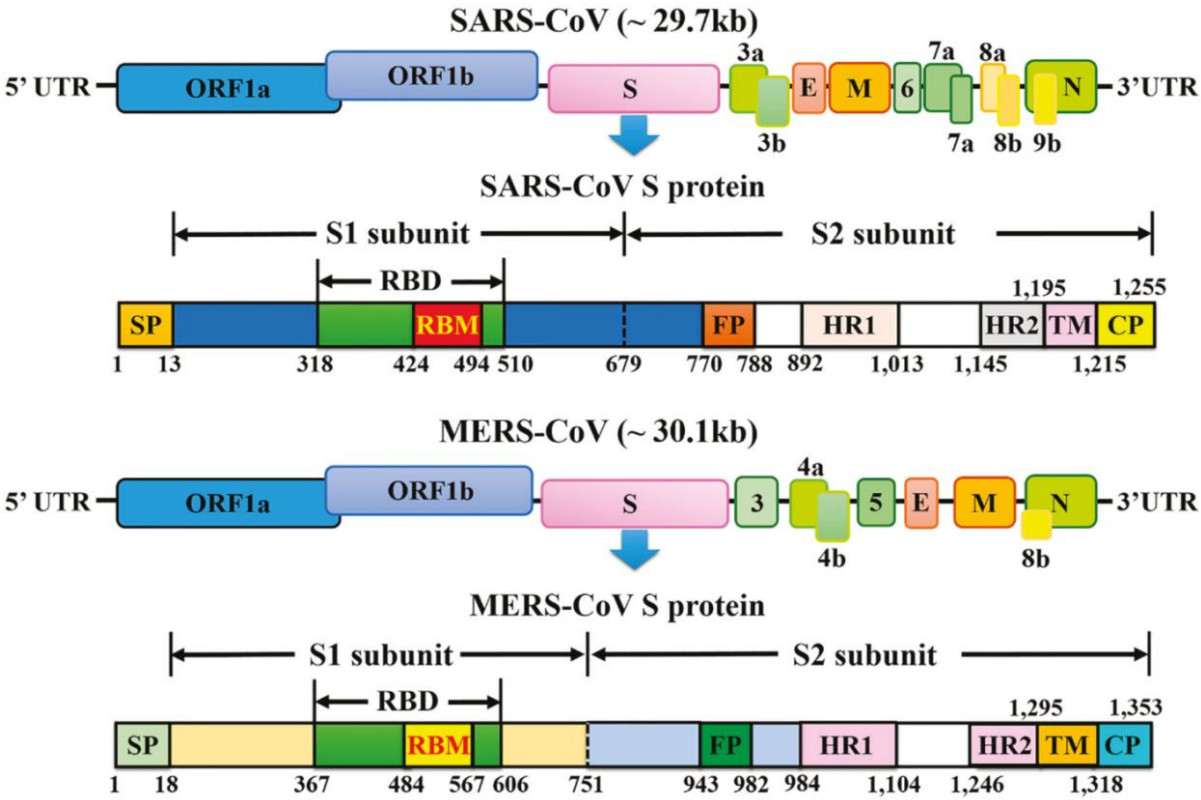

Fig-1. Schematic representation of the genome organization and functional domains of S protein for SARS-CoV and MERS-CoV (Ref: Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. In Coronaviruses 2015 (pp. 1-23). Humana Press, New York, NY.

1.3 Existence of coronaviruses including SAR-CoV-2 in humans for the past several decades by comparative analysis of genomic data

Since the origin of SAR-CoV-2 or HCoV-19 (COVID-19), debates have arrived for its real origin and its historical existence with human's in the past. The fact that this virus is known to humans come from the evidence that SARS-CoV-2 is optimized and showed high affinity to human ACE-2 receptors. In the history, SARS-CoVs also share same ancestor [35]. Coronavirus spike (S) glycoproteins facilitates virus entry into cells and that they are the main target of potentially neutralizing polyclonal antibodies produced by humans. Strikingly, structural similarities were found between SARS-CoV-2 S and SARS-CoV S glycoproteins confirming close relation between them, and both of them recognize ACE-2 to enter target cells. This phenomenon is the most likely due to natural selection and decades of their associations [36-38]. Current evidence also demonstrate that the RNA genomic sequence of SARS-CoV-2 (COVID-19) shows 89% nucleotide homology with bat SARS-CoV and 82% with human SARS-CoV, indicating that bat is the probable zoonotic source and this virus might have been existed since long time in animals and humans [39]. Past evidence also give proof that there may be a zoonotic reservoir or source (a *Rhinolophus affinis* bat and Malayan pangolins or *Manis javanica*), and identification of a potential intermediate host of SARS-CoV-2 will be of clinical significance [40,41]. Furthermore, during its zoonotic course, SARS-CoV-2 has acquired mutations (recombination), making it more complex and difficult to identify. Studying zoonotic infectious pathology of pangolin genome aids in understanding that how SARS-CoV-2 has jumped into humans. [42,43].

Furthermore, researchers have also compared the affinity of different SARS-CoVs. They have come to a conclusion that "2019-nCoV likely uses human ACE2 less efficiently than human SARS-CoV (year 2002) but more efficiently than human SARS-CoV (year 2003). BecauseACE2-binding affinity has been shown to be one of the most important determinants of SARS-CoV infectivity, 2019-nCoV has evolved the capability to infect humans and some capability to transmit among humans. Alarmingly, our data predict that a single N501T

mutation (corresponding to the S487T mutation in SARS-CoV) may significantly enhance the binding affinity between 2019-nCoV RBD and human ACE2. Thus, 2019- nCoV evolution in patients should be closely monitored for the emergence of novel mutations at the 501 position (to a lesser extent, also the 494 position)" [44]. These findings, including with additional research literature [27,28] suggest that the SARS-CoV-2 gradually evolved via mutations and was associated with humans since decades . Hence SARS-CoV-2 antigens and neutralizing antibodies may have been present in humans since long time and this may be the reason that RT-PCR (reverse transcriptase-polymerase chain reaction) and other diagnostic techniques are detecting previous antigens (cross reactivity) which is resulting in an increasing number of positive patients daily in spite of strict lock down policy globally.

This astonishing fact was revealed in one of the study where positive antibody reactivity with Western blot analysis was observed [45]. It was demonstrated that a strong cross-reactivity of antibodies was observed between SARS-CoV–positive human plasma and SARS-CoV-2 rNP with 45 kD specific band observation. The study was successful in confirming this cross-reactivity via ELISAs (enzyme linked immunosorbent assay). It was concluded that these patients were infected previously with SARS-CoVs in 2002 epidemic. Hence, patients previously infected and positive for SARS-CoVs might show SARS-CoV-2 positivity with RT-PCR which can be labelled as false positive, in the absence of marked clinical infection symptoms and seropositivity for specific antibodies (see below).

The fact of evolutionary relationships is further strengthen by the other studies which reveal that this virus was genetically closely related (89.1% nucleotide similarity) to a group of SARS-like coronaviruses (genus Betacoronavirus, subgenus Sarbecovirus) that had previously been found in bats in China [46,47]. The RBD of its spike protein were also found to be almost identical (73.8–74.9% amino acid identity). Furthermore, it was found that SARS-CoV-2 exhibited 100% amino acid similarity to bat SL-CoVZC45 in the nsp7 and E proteins, suggesting that bats act as natural reservoir for these coronaviruses and using same receptor for cell entry, the ACE-2. Furthermore, the amino acid sequences of the seven conserved replicase domains in "ORF1ab" were also 94.4% identical, with an overall genomic sequence identity of 96.2%, suggesting that the two viruses belong to the same species, *the SARS-related coronaviruses* [8,48].

Interestingly, retrospective investigative studies done in France have shown that SARS-CoV-2 was present in northern France since before Wuhan (China) reported (in December 2019), in a patient with no history of travel to China. The study has reported independent SARS-CoV-2 introductions without local transmission [49]. According to the authors "we used the newly generated genomes to investigate the origins of SARS-CoV-2 lineages circulating in Northern France". They have reported that clinical characteristics of SARS-CoV-2 patients were different; a unique V367F (G22661T) mutation in the receptor binding domain of the Spike protein was observed, which was not observed in other genomes. The study has concluded that SARS-CoV-2 was present earlier in France. Similarly, in another study there is a report of a 42 year old male patient with a history of type-2 diabetes and asthma, traveling record to Algeria in August 2019, absence of travel history to China, was admitted in intensive care unit (ICU) in France with hemoptysis, cough, chest pain, headache and fever, evolving for four days with no etiological diagnosis [50]. With initial examination unremarkable, sputum negative for pathogens, lymphopenia, and the chest computed tomography (CT) imaging demonstrated bilateral pulmonary ground-glass opacities in the inferior lobes. He was discharged after antibiotic treatment. Of interest, one of his children presented with influenza like symptoms. According to hospital policy, all respiratory samples were stored and frozen at –80 °C to be used in future investigations. Medical records and respiratory sample of this patient was again investigated retrospectively for RT-PCR and which was proved to be positive for SARS-CoV-2. Hence, it can be concluded that the disease was already present previously in France and was circulating in asymptomatic individuals.

These observations clearly show that virus might be freely moving in different parts of the world, went undetected, with sub-clinical infections. If studies and screening could have been conducted in the past few years, or perhaps 4 to 5 years back, it may be possible to identify this virus earlier. Surprisingly, no increased death rates have been reported in the past and it may be possible that humans have already developed

immunity and antibodies against this virus (seropositivity). Again, as discussed above, this is the reason for more emerging cases of SARS-CoV-2 (COVID-19) as these subjects were not screened previously. Further studies are required to investigate respiratory samples retrospectively for the past few years (or perhaps several years) to rule out the emergence, propagation and clinical characteristics of SARS-CoV-2 at multicenter level.

1.4 Genomic sequence, Genetic diversity and evolution of Human MERS and SARS CoVs

On January 5th , 2020, the first whole-genome sequence of the SARS-CoV-2 virus (Wuhan-Hu-1) was submitted in the U.S. National Center for Biotechnology Information (NCBI) GenBank. After that, hundreds of researchers explored the details of the genetic sequence of this RNA virus with several hundreds of publications. Most of recent published papers are still investigating the evolutionary history of SARS-CoV-2. Thousands of global SARS-CoV-2 whole-genome sequences are now available on the data sharing platform hosted by the Global Initiative on Sharing All Influenza Data (GISAID). Virus evolution can be now studied or explored by data visualization tools like Nextstrain and CoV-GLUE. Further genomic epidemiology can be explored at GISAID (Global Initiative on Sharing All Influenza Data) database [51].

Nucleotide sequence substitution is one of the most important mechanisms of all viral evolution in nature, responsible for high mutation rate, rapid evolution of RNS viruses and resistance to most vaccines and antiviral drugs. Viruses encode enzymes responsible for replicating their DNA or RNA genomes. Viral RNA polymerases exhibits low fidelity (the intrinsic error rate) approximately 10^{-4} mutations per nucleotide copied, a rate higher than DNA viruses, a phenomenon of virulence which is also explained by quasispecies [52-58]. Study done by Shen Z et al. have reported that "metatranscriptome sequencing on bronchoalveolar lavage fluid (BALF) samples from eight patients with SARS–CoV-2 found that the number of intrahost variants ranged from 0 to 51 with a median number of suggesting a high evolution rate of the virus" [59].

Other studies performed on genomic analysis for diversity and mutations have found eighty-six complete or near-complete genomes of SARS-CoV-2 and has revealed several mutations and deletions on coding and non-coding regions with missense mutations (a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid) including ORF1ab polyprotein, 3’ end of the genome, spike surface glycoprotein, matrix protein, nucleocapsid protein, and three mutations (D354, Y364, and F367) located in the spike surface glycoprotein receptor-binding domain. Moreover, it was reported that because spike surface glycoprotein has an important role in binding to host cell receptors, this leads to variable "antigenicity" and rapid mutations with genetic diversity of coronavirus SARS-CoV-2 [60,61].

In fact, it was found that the mutation rate of SARS-CoV-2 was also comparable to Ebola virus. The mutation rate in the SARS-CoV genome was observed to be $0.80 – 2.38 \times 10^{-3}$ nucleotide substitution per site per year, similar and comparable to other RNA corona viruses, not unusual, and not higher than HIV (human immunodeficiency virus). It should be noted that moderate to high mutation results in a high-level of intra-host RNA genome variants [62-67]. As mentioned above by a review of retrospective investigative studies on respiratory samples for SARS-CoV-2 [49,50], the genomic diversity have been underestimated, and virus was present in different regions, went undetected in asymptomatic individuals (infecting sub-clinically). In other words, SARS-CoV-2 virus was changing genomic sequences, perhaps for several years, with development of immunity in humans without causing increased number of deaths. It may be possible that deaths caused by usual pneumonia or other respiratory illnesses were missed, went undetected by RT-PCR for SARS-CoV-2 for the past years. Cross reactivity between antibodies and antigens proves this scenario that patients currently positive with SARS-CoV-2 might be positive previously with other SARS-CoVs in the past decades [45]

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2. Human to human transmission, the real facts about animal reservoirs and wildlife zoonotic transmissions. A need for serious intervention to stop recurrent pandemics

While considering the infectious diseases, it is important to know the exact mechanism of the disease transfer, animal reservoirs, zoonosis and vectors. If these factors are not studied very carefully, then factors considered for limiting the disease transmission cannot be guaranteed. After pandemic of SARS-CoV-2, several hundreds of news was spreading in social media regarding human to human transmissions by touching. While carefully reading the published research literature, some other conclusions also arise. So far, there are no conclusive case controls or randomized trials which demonstrate definitive human to human transmission in pandemics by usual touching each other. Shen Z et al., have reported that "By investigating a possible person-to-person spread event, we found no evidence for the transmission of intra-host variants" [59]. Moreover, most of the previous information on human-to-human transmission of influenza comes from studies of human inoculation with influenza virus and observational studies. In contrast to influenza and SARS-CoVs, several remarkable human studies of rhinovirus and respiratory syncytial virus transmission are found in the literature and we can come up to a definitive conclusion on their transmission pattern [60].

Some other studies have raised the suspicion of human to human transmission of SARS-CoV-2. One of the paper published in New England Journal of Medicine have raised such suspicion when they investigated one family (patient, his wife and a son) with a travel history from Wuhan and returned to Vietnam. Father and son were positive for SARS-CoV-2 while wife was negative, although they were traveling together. Son was positive for SARS-COv-2 because he shared the bed with his father. This raises the suspicion of droplet infection which he might acquire from his father in a small closed room, while wife remained negative. The authors have reported traveling history to four cities across Vietnam using various forms of transportation systems (including planes, trains, and taxis) with a total of 28 close contacts. However, all of them remain free of infection, which raises the concern regarding human-to-human transmission by usual touching [68].

Although it is still unclear whether the transmission through person-to-person contact occurs via large respiratory droplets, due to coughing and sneezing, as in SARS, or via fomites [69], however, precautionary measures are recommended. During the outbreak of MERS-CoV, The Fifth Meeting of the International Health Regulations Emergency Committee concerning MERS-CoV concluded that although the subject of transmission was serious, there was no evidence of sustained human-to-human transmission; and increased number of cases were due systemic weaknesses in infection prevention and control principals [70]. It was mentioned above regarding interesting retrospective investigation done on respiratory samples by Gambaro et al., and others [49,50] that, even before Wuhan reporting, SARS-CoV-2 strains were already introduced and present in France in patients without history of travel to china and without local human to human transmission, perhaps in significant number of asymptomatic individuals for several months, and SARS-CoV-2 went undetected.

It is well known that strains of human coronavirus OC43 and 229E cause approximately 33 % of common colds and hospital-acquired upper respiratory tract HCoV infections (nosocomial infections) in premature newborns. Studies have also demonstrated detectable minor infectivity of HCoV-229E for up to three hours on various hospital surfaces and also on sterile latex surgical gloves, which are significant vector sources for hospital-acquired viral infections. However, rapid loss of virus infectivity was observed after disinfecting, drying or cleaning. These and other studies have shown that health care facilities are the source infection transmission, and proper infection control protocols should be followed [71,72] .

Although there is considerable conflicting and limited data, it has been also shown that SARS and MERS are usually transmitted by large respiratory droplets (≥10 µm in diameter) during coughing and sneezing [73]. From the past research experiences of nCoVs (novel coronaviruses such as SARS and MERS outbreaks), there is sufficient evidence of droplet transmission of infection, but no or limited evidence of sustained human-to-human transmission (through routes such as contact) [74] . Moreover, our current knowledge on transmission is based on a small number of cases and further clinical case control or randomized trials are required to

understand transmission physics of nCoVs. Studies which have done intensive follow-up of close contacts with index cases have also failed to demonstrate onward transmission due to the limited evidence [75,76]. Similarly, research reports from SARS-CoV-2 outbreaks also have shown that high reporting of cases was due to health care associated transmission, overcrowding in hospitals with lack of diagnostic tools, which raised the bias and high incidence reporting was notoriously unreliable. Hence, alone contact transmission may not be as important as influenza virus survives on hand for less than five minutes. Although hand washing has been demonstrated to reduce transmission of respiratory illness, but there is no specific scientifically based evidence which shows clear benefits of repeated hand washing for several times a day during epidemics [77,78]. However, as a precautionary tool, especially in health care facilities or hospital, it is advisable for health care staff to wash their hand while dealing with sick patients. Hence, it can be concluded that droplet spread is the sole or primary route of transmission for all influenza viruses, including SARS-CoVs.

Last but not the least, if current SARS-CoV-2 transmission pattern was a new, based on its mutations, then it can be considered to have a unique transmission pattern from human to human. However, this was not the case and currently there is no evidence to validate such theory in reality. However, and conversely, a recent study has demonstrated that SARS-CoV-2 genomic mutations did not increase the virus transmission capability. They conducted research from the data of 75 countries and did virus genomic analysis from 15,000 SARS-CoV-2 patients by a phylogenetic index. Instead, they concluded that these mutations are neutral primarily induced by RNA editing by human immune system, and this high rate of mutations do not appear to be benefiting the virus. Currently there is no evidence that more transmissible lineages of SARS-CoV-2 will emerge [79]. Hence, these mutations appear to be a natural process and pathogen to human interactions and adaptations, occurring since long time, with no effect on current virus transmissibility.

In summary, it can be concluded that SARS-CoV-2 is not a new true contagious disease and which, is not transmitted "*solely*" by direct contact with an infected people, for example hand shake, etc., unlike other contagious diseases (e.g., chickenpox, smallpox, measles, leprosy, ringworm, gonorrhea syphilis, etc.,). However, studies do confirm SARS-CoV-2 transmission via Droplet infection (though coughing, sneezing and spitting of infected people) like other respiratory diseases, e.g., pneumonia diphtheria, influenza, tuberculosis, common cold, whooping cough, etc., which are more common in more crowded living conditions. In conclusion, mode of transmission of such diseases including influenza is via droplet [80]. Similarly, nosocomial infections and opportunistic airborne transmission (which may occur through fine particle aerosols) should also be considered. It was also reported that human-to-human transmission is usually documented in health care facilities during outbreaks or pandemics due to poor infection control measures [81-84].

The nosocomial spread in tertiary care hospitals is because of the fact that receptor for the SARS S-glycoprotein, ACE2, is found in the lower respiratory tract and, while admitted in hospital, procedures such as intubation contribute to nosocomial spread [85]. Under all these conditions, precautionary measures are essential in hospital settings and intensive care units to avoid spread of droplet infections (droplet precaution), such as wearing mask, gloves, gown, keeping distances between beds at least eight meters, and avoiding poor hygienic conditions [86-89,69]. Moreover, and interestingly, it has been shown that most of the patients with SARS-CoVs infection had a milder clinical course of the disease and usually do not transmit the virus because of insufficient adaptation of the civet cat virus RBD to human ACE2 receptors, which is required for propagation of the infection of human cell lines [90,91] .

During pandemics, it has also been also shown that, as commonly thought, quarantine is of unproven reality. There are no research based trials on quarantine regarding influenza like viruses and previous experience with influenza and SARS-CoVs epidemics suggests that large scale quarantine of a given population is logistically and technically very difficult [92]. Interestingly, and conversely, one study has found that neither contact tracing nor isolation of infected people would prevent an epidemic of certain viruses, including the coronaviruses [93]. Hence, quarantine will not be necessarily must in diseases which are not contagious and there are no recommended guidelines published so far for such activities, but only precautions and infection control measures with quality assurance will be appropriate for health care workers working inside the

hospitals [73]. It should also be noted that effectiveness of quarantine or isolation will be of limited value under poor isolation facilities, failure of infection control procedures and protocols for isolation ward and for isolate patients. Moreover, the direct and indirect costs involved in quarantine measures are of considerable importance which has economic, international trade, and environmental effects [94-97].

It has been observed that some quarantine and isolation centers and quarantine camps are so small or narrow that health care workers are frequently affected by droplet infections and if seronegative for SARS-CoV-2 subjects are kept there for temporarily monitoring, these normal individuals also may get infection during the isolation period in these isolation centers or camps because of poor or substandard infection control procedures which is alarming and dangerous. Nowadays, on commercial basis, five star hotels are also being offered which is not cost effective and unaffordable for the majority. It is the prime duty of governmental and international health agencies to look into this subject and to provide standard infectious control protocols and facilities for their citizens of all classes, without bias because SARS-CoV-2 is currently considered a pandemic health issue.

Although quarantining is carried out for the sake of caution or safety, however, current evidence does not support this concept because research studies have demonstrated that subclinical infection in SARS-CoVs is almost nonexistent with almost absence of mildly symptomatic cases, without any serious health effects. Study done by Leung et al. have done serologic survey for immunoglobulin IgG against SARS-CoV in a representative sample of close contacts of all SARS patients by viral lysate enzyme linked immunosorbent assay (ELISA) and Positive results were confirmed with immunofluorescence assay (IFA) and neutralization tests (sensitivity of 100% and specificity of 95%). They reported that " of 1,068 samples analyzed, 2 (0.19%, exact 95% CI 0.02%–0.67%) had a positive titer (1:25 to 1:50 on IFA compared to at least 1:100 in most recovered SARS cases) for SARS-CoV IgG antibody. Neither participant with a positive sample reported a chronic medical condition or being sick with febrile or respiratory illness from February to August. Both seropositive participants arose from two superspreading events in Hong Kong, i.e., Prince of Wales Hospital nosocomial outbreak and Amoy Gardens environmental point source community outbreak" [98-100] . They have discussed further about interspecies transmission from animals to humans that asymptomatic infection was observed in Guangdong animal traders (palm civets) , who demonstrated an animal SARS-CoVs seropositivity rate of 72.7% (95% CI 49.8%–89.3%) in the absence of prior clinical disease [101]. They have concluded that "SARS rarely manifests as a subclinical infection, and at present, wild animal species are the only important natural reservoirs of the virus". In fact, animal reservoirs and zoonotic transmission are well known for SARS-CoVs since decades [102].

There is an evident and strong risk of pathogens emerging from animal reservoirs (including wild and domestic animals) that have a serious potential to infect humans. This risk is increased when humans are in close and continuous contact with animals. On the other hand, Insect vectors are also another source of infections for several other infectious diseases. It is also well known that coronavirus (CoVs) are long having been associated with animals, infecting them and causing serious respiratory disease. MERS-CoV severely infects Rhesus macaques (*Macaca mulatta*), and symptoms of this animal are similar to human infections [103-105]. Further evidence for the origin of SARS-CoV-2 from the bats comes from the fact that this virus uses the same cell surface receptor, the ACE2, as discussed above [40,59]. Recent studies have confirmed and isolated coronavirus from several other animals as well, including domestic rabbits [106-109]. Moreover in a recent study it has been shown that novel SARS-CoV-2 (COVID-19) has a 96 % genomic resemblance with a bat Rhinolophus affinis, and considered the origin of epidemic due to animal to human zoonotic transmission [110].

Studies published early in 2020 have given strong evidence that now domestic animals, such as ferrets and cats are prone to develop SARS-CoV-2 infection with rapid transmission. Their nose, throat, and lower respiratory secretions demonstrate high viral titers and neutralizing antibodies (against s-protein) are detectable in their sera [111-113] . This alarming fact should be considered by global health authorities. This might be the reason of increased number of SARS-CoV-2 cases in the community.

Hence, to control transmission to humans, animal reservoirs must also be investigated. Further extensive research at multicenter level is required to know more about coronavirus' animal reservoir and to explore the exact mechanism of zoonotic transmission and chain of infection to prevent the spread of SARS-CoVs to humans.

It is one of the great responsibilities of international health organizations to conduct research and to make decisions or guidelines only on published randomized controlled trials for patients' safety, security and minimizing the health risk. For example, statement for direct contact transmission was based on one of a research letter (not the original research), which was done only for 10 days on three patients [114,115] . The authors of this study themselves have stated in their letter that "This study has several limitations. First, viral culture was not done to demonstrate viability. Second, due to operational limitations during an outbreak, methodology was inconsistent and sample size was small." Most of such studies conducted are assumed or presumed, which leads to further spreading of false news in social media which poses public health risks due to malpractice. Hence, international health agencies should conduct original research trials in their research facilities worldwide and not to rely on pilot studies or letters. Also, they should do research to explore more about animal reservoirs and mechanisms of coronavirus zoonotic transmission to humans.

3. Development of antibodies against SARS-CoVs in Humans and their serologic detection for diagnosis with high specificity and sensitivity without the risk of handling live virus

Neutralizing antibodies (IgG, IgM, and IgA) develop rapidly with high level of efficacy and safety, against SARS-CoVs in human population conferring immunity [116-118]. It has been shown that the enzyme-linked immunosorbent assay (ELISA) can be used for serosurveillance of corona virus related SARS-CoVs which can detect antibodies in clinically asymptomatic individuals with 100% specificity. This is highly specific and can detect SARS-CoVs antibodies even when blood is infected by other organisms.

The ELISA is developed by epitope mapping, using synthetic peptides from the spike, membrane, and nucleocapsid (S, M, and N) proteins of the virus protein sequences of SARS-associated coronavirus. As compared to ELISA, RT-PCR (reverse transcriptase-polymerase chain reaction) will be inferior in such cases because viral load sharply declines after nine days of disease onset and RT-PCR carries a high risk of handling infectious live virus. RT-PCR may also be exhausting as it requires strict criteria for confirmation of positive results, the test has to be done on at least two different samples, with controls (positive and negative), have to be repeated at least twice and confirmed with different laboratories if the result is positive. Furthermore, RT-PCR requires sophisticated instruments or equipment, highly trained laboratory personals, with very high laboratory quality-control standards and which may be not possible when mass screening for population is considered.

The techniques for identification of seroconversion to SARS-CoVs by ELISA is one of the definitive and accurate criteria, is preferred and standard for retrospective detection of infection by SARS-CoVs. Immunoassay for SARS-CoVs includes ELISA or Western blot (with virus whole antigen or recombinant antigens), IFA (immunofluorescence assay; using whole virus), and detection of specific neutralizing antibodies by precise methods. All SARS-CoVs patients demonstrate increase in IgG (immunoglobulin G) within few days and anti-SARS-CoVs IgG persistently remain by more than three to six months. [119-130]. In research studies, diagnostic sensitivity of ELISA has been shown to be 100% with specificity of 100% with no cross-reactivity detected with common non-coronavirus respiratory pathogens, such as OC43 and 229E. Immunological studies and serological assays have demonstrated that detection of antibodies in plasma or serum via ELISA allow for screening and diagnosis of SARS-CoV-2 (COVID-19) as early as two days after onset of symptoms, without the risk of handling live infectious virus. Moreover, research trials have demonstrated that serological assays or studies helps study the immune responses to SARS-CoV-2 in a qualitative and quantitative manner and can be used for cohort or population studies or screening to determine seroprevalence or previous exposure in a given population [119, 131]. Researchers have shown that such serosurveys are essentially

diagnostic and determine the precise rate of infection in an affected region and determine that how many people are immune to SARS-CoV-2 in affected area, which may be an important strategy for health care and hospital workers.

It should be noted that immune responses to SARS-CoV-2 are identical to SARS-CoVs and MERS-CoVs. In fact, it has been shown that individuals infected with coronaviruses or SARS-CoVs specifically demonstrate neutralizing antibodies in the serum with protection from reinfection and acquired immunity up to years [128,132-134]. Hence, by detecting antibodies in a given population, we can safely determine that such people in a given community have acquired immunity against SARS-CoV-2 and "can go safely back to their work and normal routine". In fact, antibody response to SARS-CoVs and MERS-CoVs is usually robust, healthy and persistent, significantly correlating with viral or antigen load [134,135]. Similarly, Antibodies (IgG and IgM) to the new SARS-CoV-2 has also been demonstrated to be persistence for long periods [136]. Moreover, a reduced amount of viral antigen is sufficient to trigger a humoral antibody response, and is not blunted further by antibody response or reaction [137]. Additionally, studies have shown that S-proteins (spike proteins) of SARS-CoVs are the main target of Neutralizing antibodies and that sensitivity of S-protein based ELISA was higher in detecting antibodies and is optimally recommended as screening test.
Furthermore, neutralizing activity was detectable in 89% of patients recovered at 36 months, and even at that time S-protein antibody detection rate was 100%, indicating sustainable neutralizing activity. Hence, antibodies confers not only persistent immunity, but also helpful in screening and diagnosing the SARS-CoVs [138] . Additionally, current research studies have demonstrated that SARS-CoV-2 infection may be present in blood when oral swabs are negative for SARS-CoV-2 and the researchers have recommended serological testing for confirmation and future epidemiological testing at large scale.

Serological assays (microneutralization assays) with specificity and sensitivity without cross reactivity are now developed and approved in the USA for screening antibodies to SARS-CoV-2 and distributed to 200 laboratories worldwide [131, 139 -143]. Further research trails are also required to study and confirm the details of above mentioned research trails. Hence, understanding the basic mechanisms of acquired immunity is an urgent issue which will make future strategic planning and relaxation in social distancing protocols [144] .
The passive immunization or the technique of using polyclonal or monoclonal antibodies, which have neutralizing activity against SARS-CoVs, via convalescent serum (convalescent plasma therapy) from SARS patients to control the severe disease and its transmission, have possible benefits. Passive transfer of immune serum has been tested in experimental mouse model of SARS-CoV infection. Furthermore, commercial production of SARS antiserum containing antibodies has also been applied in clinical practice [23,145-151].
However, for mAb (monoclonal antibody) prophylaxis against SAR-CoVs or SARS-CoV-2 has conditions which must be met before initiating this therapy. First, it is a time consuming method and availability of neutralizing antibodies (Nabs) in the market is difficult and challenging. Secondly, within the group of SARS-CoVs, RBD demonstrates highest variability isolated from animals [6,23]. Single mAb may not be enough to protect against all genomic strains of the SARS-CoVs, and genotyping of SARS-CoVs is recommended to select optimal or appropriate neutralizing mAbs when a new outbreak is detected. It is, therefore, advisable to investigate prior whether SARS-CoV quasispecies generation in vitro cell culture will affect the outcome of virus neutralization antibodies and assays. [152-155].

Moreover, effect of passive antibody treatment and its immune response while protecting against SARS-CoVs is debatable and controversial because patients who died of SARS demonstrated acute antibody and immune response. As compared to active immunization and until vaccines are available, great caution is required in case of plasma therapy because neutralizing antibodies are associated with strong antibody agglutination reaction with hypercytokinemia, pulmonary pro inflammatory accumulation and fatal acute lung injury (ALI) [156]. In summary, however, human antibodies to SARS-CoVs are protective for both donors and patients, provided the procedure is done according to standard recommendations. If vaccines are developed to confer permanently immunity, SARS-CoV-2 infection can be eliminated [157]

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4. Asymptomatic transmission from human to human, the asymptomatic fraction, self-limiting course: influenza in context

Here the literature and significant studies from 1800 onward were reviewed. Influenza was and still considered one of the most important causes of morbidity and mortality in elderly patients. In a period of 1957-1960, over 86,000 deaths occurred in the United stated as a result of influenza. Patients above the age of 65 with associated diseases were at higher risk [158,159]. The death rate from influenza in England and Wales were relatively low during the past decades because of general use of antibiotics. After the first outbreak in 1847-1848 with English death rate of 459 per million, the rate declined after 1850s. In the pandemic of influenza 1918 – 1919 during World War 1, the crude death rate of 3129 per million were observed.

Worldwide, this Spanish flu (La Grippe) was one of the deadliest pandemic in human history and about 500 million people (approximately one third of the world population) were infected with H1N1 influenza virus. In one year, more people died of influenza than four year plaque from 1347 to 1351. The observed death rate was about 100 million worldwide. 675,000 deaths occurred only in United States. After 1936, it has been remarkably constant. [160,161]Hence, decline in attack episodes can be observed in any pandemic infectious disease, due to several factors, including humoral immunity, and treatments (antibiotics or vaccines) [162]. The protection may be greater if influenza virus strains and genomic sequence of RNAs match closely the vaccine strain. Globally, it has been reported that there is a higher mortality rate from seasonal influenza associated severe respiratory illness (H3N2 and H1N1 viruses) with a death rate of 290 000-650 000 [163-165]. Currently, during influenza epidemics in the United States, annually more than 36,000 death and 114,000 hospitalizations are observed [166].

As organizations relied more on vaccinations and mass screening was not done, there is possibility that people infected previously might harbor same antigenic genomic sequence as that of SARS-CoV-2 with humoral immunity or antibodies. In fact, and as mentioned above, in different studies it was demonstrated that SARS-CoV-2 existed in France before the reports came from Wuhan, China [49,50] . Recently not much is known about influenza and therapies to prevent it. Currently, still new strategies are required for mitigating the severity of influenza pandemic and are now top health priority. Research and data from influenza pandemics has demonstrated that preventive measure during the peak of pandemic can significantly reduce the attacks, but has little effect on overall pandemic rates. Prophylaxis or vaccines can reduce influenza rates up to 75%.

Reviewing details by medical literature, it can be concluded that clinical characteristics of pandemic of SARS-CoVs or SARS-CoV-2 is similar in nature to previous influenza pandemics or attacks, requiring same precautions. Future pandemic strains of virus and pandemic transmission are uncertain in both cases, until further details are available [167-171]. In the absence of vaccination, silent transmission or asymptomatic transmission has an importance regarding the community health stability. An infection without any signs or symptoms is called asymptomatic fraction, asymptomatic or subclinical disease [172].

A study done by researchers at Princeton has found that this asymptomatic or silent phase of transmission can be a successful evolutionary strategy for viruses and SARS-CoV-2 (COVID-19). A silent infection has definitive short-term advantages. The study has provided some insights of silent transmission on the virus' long term survival and how it could be beneficial to humans. In other words, lack of symptoms eventually lessen virus transmission and reduce the pathogen's long-term survival, which are called "host-parasite interactions"[173].

Hence, silent infection has advantages of providing humoral immunity, in spite of applying other strategies such as identification, contact tracing and quarantine which have economic impact and difficult implementation. An asymptomatic individual can go to his normal work or routine and may come in contact with many susceptible people, giving a chance to develop immunity. Interestingly, a study conducted in South Korea has used a unique model of epidemiology to study the dynamics of the pandemic by using SIR model (S, susceptible; I, infectious ; R, recovered). They concluded that Public information disclosure targeting SARS-

CoV-2 positive cases were more effective in limiting transmission than lockdowns in the region with 50 percent lower economic losses [174]. Similarly, strict lock down and qurantine policy should be revised as there are further studies demonstrating high percentage of asymptomatic individuals. A study conducted on cruise ship passengers has described 81% asymptomatic and the researchers have emphasized to ease lockdown restriction as some other strategy have to be adapted in future [175].

In contrast, a symptomatic person with fever and cough can self-isolate himself by staying at home for few days until recovery. By this way overall transmission can be reduced over time, and in fact, evolution favors such behavior. This methodology is of immense importance in epidemics where the virus is constantly changing its genomic characteristics, virulence and antigenicity with treatment complexities and vaccine development difficulties; in this scenario, only very sick people can isolate themselves in their homes and the remaining healthy subjects (asymptomatic, subclinical or asymptomatic fraction) can continue their work, with precautions, conferring wide variety of immunity to the community with seropositivity of diverse antibodies, and hence, limiting the epidemics. This technique may also substitute convalescent plasma therapy on large scale, which is costly, a time consuming complex laboratory process, and impossible to apply when a large population or nation is considered with the difficulty that single monoclonal antibody will not be enough to protect against all genomic strains of the SARS-CoVs [145,148]. However, passive immunization or convalescent plasma therapy by neutralizing antibodies (NAbs) may be reserved for neutralizing antigens and viremia load in severely ill SARS-CoV-2 patients admitted to ICU and considered or requiring mechanical ventilation [176,177].

In another study, researchers have shown that there are several factors which affect early transmission by a newly infected host. Infection can persist longer in individuals who are less symptomatic, with reduced transmission. Three infectious stages are defined: fully asymptomatic, less symptomatic, or fully symptomatic which are possible results of evolution. Hence, bio-stability can be achieved with time when humoral immunity develops or even by active vaccine immunizations. Furthermore, and as mentioned above, by a review of further intensive literature details, no definitive experimental studies can be found regarding person-to-person spread of flu by touching. In fact, not much is known about influenza in this context. Under such conditions, and as has been seen in past few decades, asymptomatic carriers play an important role in human adaptation for viral diseases and development of acquired immunity. It has been observed that 50% - 77% of infections with seasonal flu are asymptomatic (subclinical, with mild temperature rise), which may be due to pre-existing partial immunity [166,178,179]. The influenza virus shedding can occur in asymptomatic individuals, and disease transmission may also occur, but not at the same rate as observed in symptomatic patients. This phenomenon is practically observed since even with high infectious control measures, epidemic flu progression may slow down, but cannot stop. However, it is advisable to take droplet precaution during working in public places and hospitals.

It should be noted that any viral disease, if become seasonal, will be less virulent as most of the people in the community will developed variety of divergent antibodies against diverse antigens. Studies have reported that SARS-CoV-2 is the 7th coronavirus which may become seasonal [144] . The other six were: four human coronaviruses causing seasonal common cols (alphacoronaviruses NL63 and 229E; betacoronaviruses HKU1 and OC43); the remaining two, SARS-CoV and MERS-CoV, from zoonotic reservoir, yet not become endemic so far. However, as we discussed above, SARS-CoV-2 might be circulating worldwide, unnoticed in previous years. There is a high possibility that SARS-CoV-2 cased some sort of seasonal flu or perhaps outbreak in certain communities or regions, and went undetected. There is a need to study the humoral response to SARS-CoV-2 if it becomes seasonal.

There are reports of influenza outbreak in the past several decades with several reports repeatedly. But their previous events were clinically controlled and stable. As soon as the people of the community develop antibodies or herd immunity, even by vaccines, there is a less chance of influenza outbreak or becoming pandemic. One study has revealed that 65% and 75% of the children between the age group two to four years were seropositive for 229E and NL63, respectively. Moreover, by 6 years almost all children were seropositive.

This may be the result of repeated re-infection. This also supports the evidence that children possess seroprotection against homologous virus genotypes. Furthermore, In future, if adult from such population is infected with more severity, then this indicates lack of cross-protective antibodies or antigen exposure. Hence, herd immunity from variety of antigens with resopositivity with divergent antibodies is the most important factor controlling outbreaks in community. However, older people with comorbid conditions are usually susceptible for any type of influenza infections and special care must be given with vaccines to reduce influenza morbidity and mortality. [180-182]

Current evidence suggests that in most of the patients, SARS-CoVs and other coronaviruses follows a self-limiting course. Severe disease is the most likely among older patients and those with comorbidities. Currently no specific antiviral therapies are available and studies have also shown that antiviral therapies did not accelerate clinical improvement and were proven unsuccessful [183-185]. In the current pandemic, cellular and humoral immunity with specific monoclonal antibodies developed by humans are the only natural source which can prevent infection spread as early as hours to two days and will remain up to several months to years, conferring immunity [156, 186]

With the passage of time when more humans develop immunity against SARS-CoVs including SARS-CoV-2, more resistance will be demonstrated by the herd immunity (either by acquired immunity or active immunization by vaccines). Similarly, if repeated viral infections occur in Humans, they can be combat later by memory B-cells, a response from immune system [187-191] .

5. RT-PCR in the diagnosis of SARS-CoV-2, sample specimens and practical realities and possibilities of false positive or false negative, with high risk of handling live virus antigens

Before going in details of RT-PCR (or RT-qPCR), it is important to discuss the sample specimens which are being considered for RT-PCR. Because of respiratory complication of SARS-CoV-2, that is pneumonia, suggesting that mainly lower respiratory tract is infected and affected. Recently, research papers have been published regarding accuracy of different respiratory specimens, including broncho-alveolar lavage fluid (BALF), sputum and nasal swabs. In the current epidemic, nasopharyngeal swabs are being considered the first choice for taking samples for RT-PCR. This is because it is easy (although sometimes painful to reach to pharynx via nose) and cheap. BALF is practically difficult, require a trained staff, suction device, and is more painful at the same time and cannot be performed at large scale. On the other hand, studies have shown that (apart from BALF) sputum samples demonstrated highest positive (90%) rate at all clinical stages of SARS-CoV-2, followed by nasal swabs (73%) and then the throat swabs (61%). However, BALF showed 100% positivity [192]. Hence, studies have not recommended throat swabs for the virus detection and considered sputum as most accurate for laboratory diagnosis. Currently, RT-PCR method is used also for diagnosing SARS-CoVs from nasopharyngeal or oropharyngeal swabs.

Although RT-PCR (reverse transcriptase-polymerase chain reaction) has improved ability to detect pathogen and efforts are done to develop PCR for pan-species, Genus and family (which is not practical). However still with highly variable RNA viruses it is unsuccessful and not possible for PCR to detect the genomic sequences and is highly "biased" with which only known pathogens or antigens can be detected. This is because the primers chosen will alter nucleic acids which will be amplified. Moreover, RNA viruses exhibit such an extensive genomic diversity that it is very difficult to design primer sets for PCR with high sensitivity and specificity. Because of inadequacy of any single RNA primer pair to detect all human viruses (or range of viruses), several RNA primer sets are chosen to run sample analysis; but this increases the cost of the diagnostic methodology with complexity of the assay with difficulties of comparing results among different laboratories.[193-195]

In contrast, NGS (next generation sequencing) allows greater, unbiased and massive sequencing of genetic material. Furthermore, serologic assays are more sensitive and important in scenarios where genome (RNA) is difficult to isolate or is no longer present, and for cohort epidemiological studies [196-200] . As discussed above, RT-PCR has some other drawbacks (as viral load sharply declines after nine days of disease onset) and difficulties, requires re-confirmation, strict quality control, and carries a risk of handling infectious live virus. Mutation rates are also high among RNA viruses and make them difficult to identify.

One of the most important issues with RT-PCR is the reporting of false negative and false positive results; as viral RNA genome in sufficient quantity is required to be detected by RT-PCR. Other important factor regarding false results is that the RT-PCR utilizes primers, which can be affected by the variances or genomic diversity of viral RNA of SARS-CoV-2 (COVID-19). False negative results occur due to mutations in the primer and probe target regions in SARS-CoV-2 genome. Furthermore, currently several types of SARS-CoV-2 RT-PCR kits have been developed rapidly and available in the market with different quality. They were brought into the market without strict quality control measures. In fact, the sensitivity and specificity of real-time RT-PCR test is not very accurate or 100% like that of serology test [201,202]

On February 12th 2020, a sudden rise in new cases of SARS-CoV-2 was observed. Researchers have shown that such increase in numbers was due to change in the diagnostic methodology, and indeed at that time, more than seven types of SARS-CoV-2 test kits were developed and approved rapidly and there were several factors attributed to their false negative rate (FNR). As an example, using primers in the ORF1ab gene and N genes are affected by the variation in the viral RNA genomic sequences. Additionally, regarding the natural history and collection of specimens, sampling techniques also contribute to high FNR. It was also demonstrated that in one testing scenario, FNR from one testing was as high as 30% to 50% in SARS-CoV-2 patients. It has been also reported that one of the patient was not confirmed SARS-CoV-2 infection by RT-PCR three times within three weeks until BALF was done and next generation sequencing (NGS) with RT-PCR were positive for SARS-CoV-2. This will not only increase the cost of testing, but also underestimate the true positive cases; and during the outbreak or pandemic, it is a serious issue.[203,204]. Moreover, there are events of false positive reports when tested on animals such as goat [205]. Such reports and testing kits should be further investigated to eliminate the public health risk. Hence, it is a serious issue and urgent call to rapidly improve the quality and standard of testing kits with their operative techniques for the accurate diagnosis of SARS-CoV-2.

High false positive rates are also a public health risk because such people will self isolates themselves and their office work will be affected. It is now well known that sensitivity and specificity of RT-PCR are very poor and false results are common. Recently, a study was conducted on RT-PCR false predictively among high risk patients who were exposed in inpatient departments and also health care workers to rule out SARS-CoV-2 infections [206]. They have reported false negative probability of 67% at initial days of infection and that precautions must be taken while interpreting the results. Hence, if suspicion is high, the RT-PCR should not be used alone for ruling out the diagnosis, but rather serological testing (especially IgG, which is produced rapidly after infection), should also be carried out and considered with clinical course of the disease [207].

If RT-PCR is considered for testing, then lower respiratory tract will be recommended (sputum, BALF), if possible, instead of nasopharyngeal swab because of genomic sequence analysis of SARS-CoV-2 and ACE2 viral receptors. Correct specimen selection is an important during pandemics. Blood specimens with serology testing are preferable over nasopharyngeal swabs. There are further reports globally that nasopharyngeal swabs (upper respiratory tract) were negative for pandemic (H1N1; novel swine-origin influenza A) 2009 influenza virus, but proved to be positive on BALF and some patients requiring admission to intensive care units (ICU) and mechanical ventilation were positive by bronchoscopic specimens [192, 208, 209]. These studies have important practical clinical implications that patient with influenza like illness with pneumonia and unexplained diagnosis should undergo bronchoscopic specimens.

An interesting recent study done on 1014 patients' cohort with SARS-CoV-2 (COVID-19) in radiology and image processing department has demonstrated that positive rates of RT-PCR throat swab assay were 59% while 88% for chest CT imaging with 97% sensitivity for the diagnosis of suspected SARS-CoV-2 cases. This study has concluded that CT imaging is more reliable and practical than RT-PCR in pandemic areas; RT-PCR showed low positive rates, lack of sensitivity, long processing time with unpredictable stability [210-214]. Similarly, a study discussed above on cruise ship passengers also reported a significant false-negative rate with RT-PCR testing [175].

Hence, serum based testing methods for SARSCoV- 2-specific immunoglobulin-M will be more sensitive and specific. It was demonstrated that serologic test are gold standard for coronavirus testing during epidemics and showed a sensitivity of 0.96 (95% CI, 0.91 to 0.98) and a specificity of 0.96 (CI, 0.92 to 0.97) which was based on SARS-CoVs disease clinical course, with the time from symptom onset to attending the clinic was 3.3±2.6 days[204] .

Studies have reported that patients showing symptoms of SARS-CoVs clinically and radiologically were further confirmed by RT-PCR with positivity of 68.2 %, while serology confirmation was 100% [215] . It is also worth noting that viral titers significantly diminish within few days after development of clinically significant disease both in humans and animals, indicating that serum antibody detection will be appropriate. Hence, according to these observations, serological testing further improves clinical case detection and must be used in combination with other detection methods [216-220].

Moreover, it has been demonstrated that in some exposed individuals RT-PCR may be negative and at the same time can have serologic evidence of antibodies against viruses. The study has shown that there was a 4-fold rise in antibody titer without signs and symptoms (subclinical infection) with RT-PCR negative (false negative) [172].As discussed above, serological diagnosis of coronavirus Infection is a good and recommended option. An indirect immunofluorescence assay (IFA), and enzyme linked immunosorbent assay (ELISA) are used to measure developing antibodies during infection. The most appropriate test is immunochromatographic assay (ICA) [221-223]. With technological advancements in molecular biology and recombinant engineering, it is now possible to calculate and quantify virus particles with high accuracy in a given sample. These techniques include flow cytometry, dynamic light scattering, quantitative capillary electrophoresis [224-227].

Furthermore, diagnosing coronavirus infection on RT-PCR is also not as simple as is thought because these human coronaviruses (such as SARS-CoV, MERS-CoV and SARS-CoV-2) are huge (around 30 kb); and production or generation of CoV infectious clones are hampered due to their huge size genome and the toxicity of some CoV replicase gene sequences during its propagation in bacteria. These problems can be overcome by bacterial artificial chromosomes (BACs) in vitro ligation of cDNA fragments , and using vaccinia virus as a vector for the propagation of CoV full-length cDNAs [228-231]. Moreover, usually 3-4 days are required for the final results, and then again test is repeated to confirm. The accessories and supplies (swabs, testing kits, and testing media) become short while doing mass screening. Guide RNA can also recognize other interspersed RNS genomic sequences, false positive results appear with loss of specificity. Inadequate sampling with low virus quantities, timing and site of collection (upper or lower respiratory tract), poor collection or handling, performance of kits (or substandard kits) are other issues which affect PCR results.

In conclusion, RT-PCR results must be cautiously interpreted. RT-PCR is also a complex, expensive and time consuming technique if followed properly, specifically, with quality control measures, which is not possible at mass screening level.

6. Testing SARS-CoV-2 in unhygienic, unsterilized public places under, a dangerous issue of Human to Human transmission

For the past approximately five months, it has been observed that SARS-CoV-2 (COVID-19) testing is being done in public places, which are unhygienic and unsterilized. If it is believed that CARS-CoV-2 is highly infectious and transmittable, then precautions are necessary. A highly infectious virus should be isolated and tested in a highly sterilized laboratory. If not tested in sterilized laboratories, then the infection control principles are violated [232-234]. However, for the past few months the tendency of testing was observed to be increased in crowded public places, on the roads such as in motorcycles and cars, drive thru, field camps, markets which are unhygienic and source of reinfection. Taking nasopharyngeal samples in unsterilized conditions or public places there is also a serious risk of co-infection or superinfection by other viruses and bacteria [235-239].

Recently, it was observed that use of gloves by public was not according to recommendations. Usually, health care professionals and public assume that after wearing gloves they are fully protected. They usually touch objects freely, ignoring that they themselves will be infected by touching objects, their face and others as well. If a viral disease is of a serious concern and spreads by touching, then the use of gloves in such a manner will be dangerous. In this way, the gloves will be inoculated by several viruses and bacteria, and will further spread infections by ignorant people in the community. In such as case, hand sanitization will be more appropriate. There are reports globally that if gloves are not used in an appropriate way, then they will be the source of infection and its further transmission [240-245].

It was also observed that the health care personals take precautions for themselves only and not for the public. Health staff is touching cars and other materials during testing, while at the same time not following sterilization and infection control principles, changing their disposable gloves and gowns which is a dangerous and serious issue. While testing, if a person is assumed to be positive, then the health care staff will transmit the infection to the negative one if strict infection control principles are not followed. This is difficult to achieve in crowded public places, on the roads and small camps. Similarly, collecting and transferring the sample to test tube in open air may inoculate airborne viruses, causing cross reactivity with other viruses and false positive results. All these may be the other reasons of increasing frequency of infections all over the world, despite severe lockdown. While searching on the internet, several famous organizations, institutions and newspaper websites were discovered with more than 500 pictures showing testing being done in public places, outdoor camps which were unsterilized. Few are cited in section of references [246]. It is the urgent responsibility of international health agencies, health care authorities, officials and policy makers to understand and control such unhealthy and unethical behaviors. Furthermore, it was also observed that after severe lockdown of several hours, in some countries few hours are given to the citizens to purchase food, during which more crowding is observed. This is again a cause of increasing numbers of SARS-CoV-2 despite of precautions [247]. These situations and problems should be balanced.

7. Controlling Bioterrorism, biological warfare, laboratory manipulation of Virus and preventing laboratory acquired viruses: a need for strict global consensus

Although currently there is no authentic source evidence of biologically construct virus regarding SARS-CoV-2, however, in the past few years there are repeated reports of experimentally laboratory acquired SARS-CoVs and other viruses as well. The studies have also concluded that the transmission of virus was due to lab technician error. Similarly, there are published reports that laboratories are conducting experiments on reverse genetics and construction of a virus with similar RNS genomic sequence of 1918 Spanish Influenza Pandemic Virus [248-254]. Although these experiments are done to study the characteristics of virus, however, there is a high risk of transmission from the laboratories. Of importance, microbial bioterrorism may lead to thousands of deaths with high morbidity and mortality. Bioterrorism not only produce fear, anxiety and terror, but also has great psychological impact on society, with long lasting effects [255-261].

International health agencies should take notice of such experiments. Moreover, to limit the transmission and pandemic of laboratory construct virus, following precautions are recommended:
1. Experiments on virus construction should not be done and must be prohibited in usual clinical research laboratories, but there should be "specialized virus experimental construction labs" and a specific name should be given so that international health agencies are aware of their experimental works.
2. Such specialized virus experimental construction laboratories should report to international health agencies prior to initiate experiment, and must take approval to do such work.
3. Such experimental labs must be in closed or sealed vicinity, with all available facilities such as housing, markets, etc., for the personals working in such localized research area.
4. Laboratory personals should not be allowed to leave this sealed vicinity or area without the governmental permission, with high surveillance and must report their route or movement areas. Furthermore, name of all laboratory personals should be in exit control list (ECL) on the airports to avoid their travel (without permission), to other countries to limit the virus transmission, bioterrorism and pandemics [262-269] .

Conclusion and Recommendations

By the review of the genomic sequences of SARS-CoVs, it is concluded that these viruses existed in humans and animals since decades. Studies have demonstrated that SARS-CoV-2 infection existed in other regions several months before Wuhan announced, in August 2019. Hence, there is possibility that SARS-CoV-2 circulated in different parts of the world, infecting subclinically, and went undetected. This might be a reason of increasing number of cases all over the world, despite severe lockdown. Regarding SARS-CoV-2, animal sources cannot be excluded and animals might be a potential reservoir and can play active role in virus transmission. This is a second reason of increasing cases in certain communities. Hence, active research is required to investigate animal transmission of SARS-CoVs and SARS-CoV-2. Randomized controlled trials are required to investigate human to human transmission by touch, as there are very few studies with limited evidence and conflicting results. As all SARS-CoVs are basically respiratory viruses, droplet precautions and infection control measures are essential, especially for hospitals and health care staff. Asymptomatic transmission from human to human usually occurs in communities. This asymptomatic or silent phase of transmission can be a successful evolutionary strategy for viruses including SARS-CoV-2. Lack of symptoms eventually lessen virus transmission and reduce the pathogen's long-term survival and silent infection has advantages of providing humoral immunity. Strategies such as identification, contact tracing and quarantine have economic impact and difficult to implement. Under such circumstances, asymptomatic persons can continue their work with droplet precautions, and infection control measures while symptomatic or sick persons can isolate themselves in their homes until clinical recovery. RT-PCR has low sensitivity and specificity, carries a high risk of handling live virus antigens, usually gives false negative and false positive results and must be interpreted cautiously. This might be a third reason of increasing number of cases by false positive RT-PCR reporting. Conversely, highly specific antibodies are developed against SARS-CoVs and SARS-CoV-2, confer immunity, and can be used for serologic surveys, monitoring and screening with high specificity and sensitivity. However, testing and screening of SARS-COV-2 should be avoided in unhygienic public places by nasopharyngeal swabs, which carries a high risk of further transmission, and such highly infectious virus must be isolated and tested in highly sterilized laboratory. Further strict international laws and policies are required to stop the possible spread of experimental viruses, biological warfare and bioterrorism.

Conflict of Interest

The authors have declared no competing conflict of interest

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