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SARS-CoV-2 Genome Structure, Pathogenesis, Issues, and Challenges in Laboratory Diagnosis

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Abstract

Severe acute respiratory syndrome coronavirus 2 causing coronavirus disease 2019 pandemic disease is an enveloped virus, showing genome similarity with bat coronavirus. This virus initially infects the upper respiratory tract, with subsequent spread to the lower respiratory tract. Despite the availability of antigen and antibody detection methods, reverse transcription-polymerase chain reaction (RT-PCR) is the diagnostic test of choice for this novel coronaviral infection. Care must be taken while interpreting the RT-PCR results, as single RT-PCR, especially in early days of infection, maybe false negative. The availability of cartridge-based nucleic acid amplification test has improved the diagnostic facilities in a peripheral setting of developing countries.

Keywords
- SARS-CoV-2
- COVID-19
- RT-PCR
- TrueNAT
- antigen

Introduction

The members of the Coronaviridae family are associated with human and animal infections, mostly affecting the respiratory and intestinal systems.[1] In the past, severe acute respiratory syndrome (SARS) was reported to be caused by coronavirus (CoV-1), now designated as SARS-CoV-1. This virus claimed many lives in China in 2002. Almost a decade later, in 2013, another coronavirus named the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) emerged and caused infection in Middle Eastern countries. The present novel coronavirus (SARS-CoV-2) outbreak, though reported to the World Health Organization (WHO) on December 31, 2019, by Chinese health officials, could be traced back to November 17, 2019. It has been linked to a seafood market in Wuhan, Hubei Province, China. As per the WHO convention held on February 17, 2020, infection/disease caused by SARS-CoV-2 has been named coronavirus disease-19 or COVID-19.[2] Outside of China, on January 13, 2020, the first case of SARS-CoV-2 was reported from Thailand, whereas in the United States, the first case was detected on January 20, 2020. In India, the first case of COVID-19 was reported on January 30, 2020, in Thrissur city of Kerala in a student who returned from Wuhan city of China. Subsequently, India declared a complete lockdown on March 23, 2020, to contain the spread of COVID-19. In the fourth week of January 2022, the total global case reported reached 370 million, with5.65 million deaths.[3] In India, the Indian Council of Medical Research (ICMR) data shows a total of 40,858,241cases with 4,93,218 deaths.[4] Early diagnosis and physical isolation are the mainstays for containing any outbreak/epidemic. This review primarily focuses on the virological aspect of SARS-CoV-2/COVID-19, its pathogenesis, and immunological response concerning knowledge and progress in the diagnostic strategies.

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Genome Structure of SARS-CoV-2

The family of Coronaviridae is comprised of positive-sense, single-stranded RNA (genome of size 26–32 kb) viruses with a diameter of 80 to 120 nm. There are four coronavirus genera: α-CoVs, β-CoVs, γ-CoVsv, and δ-CoVs. Human CoVs belong to α-CoV (HCoV-229E and NL63) and β-CoVs (MERS-CoV, SARS-CoV, HCoV43, and HCoV-HKU1).[4] The whole-genome sequencing of the novel CoV-2 (nCoV-2) revealed that it differs from the previously known human β-CoVs.[5] The nCoV-2 strain detected elsewhere showed 99.6% sequence similarities. However, it has only 50% sequence similarity with MERS-CoV. On the other hand, this new coronavirus shows 88% identity to the bat-derived SARS-CoVs: bat-SL-CoVZC45, and bat-SLCoVXC21. Therefore, the novel β-CoV is named SARS-CoV-2 by the International Virus Classification Commission.

SARS-CoV-2 is a spherical enveloped virus particle. The envelope bears club-shaped glycoprotein spikes. The nucleic acid RNA is associated with a nucleoprotein with a capsid comprised of matrix protein (►**Fig. 1**). Few members of Coronaviridae contain a hemagglutinin-esterase protein (HE).[6] Genome size of SARS-CoV-2, sequenced recently, is approximately 29.9 kb.[7] A typical CoV consists of at least six open reading frames (ORFs) in its genome. The genes for major structural proteins in coronavirus occur in the 5́-3́ order. These genes are spike (S), envelope (E), membrane (M), and nucleocapsid (N).[8] The first ORFs (ORF1a/b) cover almost two-thirds of the whole genome length and encode 16 NSPs (nonstructural proteins). Frame shift mutation between ORF1a and ORF1b produces two polypeptides: pp1a and pp1b. These polypeptides are further processed by virally encoded chymotrypsin-like protease (3CLpro) or main protease (Mpro) and one or two papain-like proteases into 16 NSPs. The single-guide RNA (SgRNA) of CoVs leads to the translation of all structural and accessory proteins (►**Fig. 2**). All the four major structural proteins, S, M, E and N, are encoded by the ORFs near the 3́ terminus.[8] Many accessory proteins, both structural and non-structural such as HE

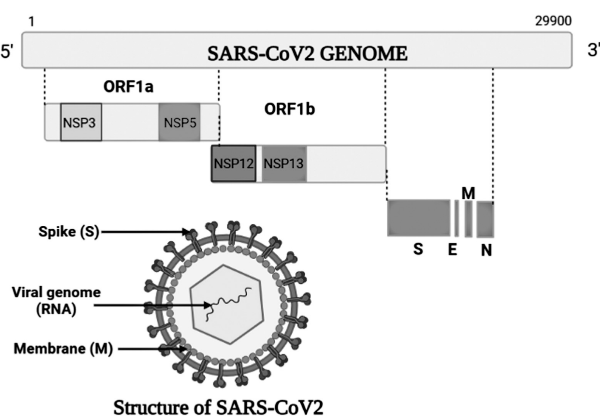

Fig. 1 Structure of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. E, envelope; M, membrane; N, nucleocapsid; NSP12, nonstructural protein 12; ORF1a, open reading frame 1a; S, spike.

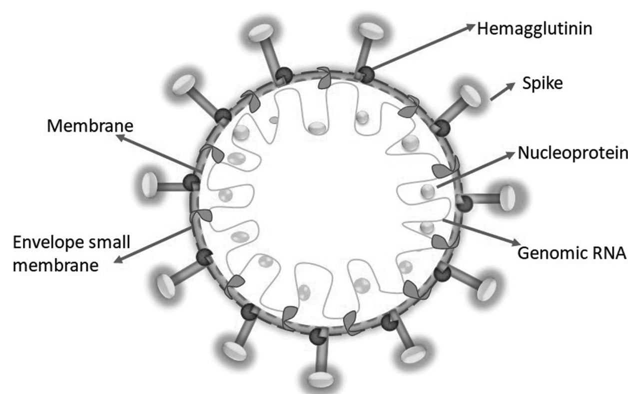

Fig. 2 Genomic structure of severe acute respiratory syndrome coronavirus 2 virus.

protein, 3a/protein and 4b/b protein, carry out essential functions in genome maintenance and virus replication.[9]

The most abundant viral protein is M glycoprotein. Of the two ends of this M glycoprotein, short NH2 domain peeps outside the virus and a COOH terminus remain inside the virion.[6] The other inducer of neutralizing antibodies is the S protein, a type I membrane glycoprotein that constitutes the peplomers. Spike protein is probably responsible for molecular interaction with the host receptors and viral replication. Membrane protein plays a predominant role in the intracellular assembly of virus particles independent of S protein.

The arrangement of N, E and M proteins among β-CoVs differs from other coronaviruses. The untranslated 5́ and 3́ regionś regions (UTRs) are involved in inter and intramolecular interactions. These UTRs are responsible for RNA–RNA interactions and binding of the viral and cellular proteins.[10] The genomic size variation occurs when the first ORF at the 5́ end is analyzed. Pb1ab is the first ORF of the whole genome length encoding nonstructural proteins with the size 7096aa, 7073aa, and 7078aa in SARS-CoV-2, SARS-CoV-1, and MERS-CoV, respectively. Comparative genomic analysis revealed a specific mutation pattern between human coronaviruses: SARS-CoV-2 and bat-SARSr-CoV RaTG13.[11]

Each of the genomes of all the SARS-CoV-2 strains submitted recently contained nearly 29,900 nucleotides (nt), which are assumed to have at least 14 ORFs (5′ to 3′), such as ORF1ab (P; 21,291 nt), spike (S; 3,822 nt), ORF3a (8,28 nt), envelope (E; 228 nt), membrane (M; 669nt), ORF8 (366 nt), and nucleocapsid (N;1,260 nt).[5] The spike gene is highly divergent (93.1% nucleotide identity) when compared with that of bat-SARSr-CoV RaTG13.113.[11] The spike gene encodes a glycoprotein that is crucial for determining host tropism and transmission capacity.

Pathogenesis

SARS-CoV-2 virus enters the respiratory tract through the aerosol and respiratory droplets, primarily affecting the multiciliated cells in the nasopharynx, trachea, or sustentacular cells in the nasal olfactory mucosa. The viral infection usually causes asymptomatic-to-mild symptoms in most

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patients. However, only a small fraction of people suffers serious consequences like pneumonia and gastrointestinal (GI) disturbances. Thus, the vicious cycle of SARS-CoV-2 pathogenesis occurs in viral entry and its multiplication, immune response, cellular damage, and recovery.[12,13]

SARS-CoV-2 Viral Entry and Its Multiplication

The virus enters the human body through inhalation, primarily infecting the upper respiratory tract via the acetyl choline esterase 2 (ACE2) receptor on the cell membrane. Moreover, the virus has an S(Spike) protein on its surface that mediates the target cell surface attachment, its engagement to complimentary receptors, and the membrane fusion. ACE2 receptors are present in the respiratory epithelial cells, GI, renal, cardiac tissues, and blood vessels smooth muscle cells. In addition, the binding affinity of the S protein to ACE2 receptors is much higher for SARS-CoV-2.[12,13]

During the initial phases, the S protein mediates the binding of the SARS-CoV-2 virus with its cellular entry on the surface. The receptor binding domain of the S1 subunit of the S protein binds with the peptidase domain of the human ACE2proteins, whereas the S2 subunit mediates membrane fusion. Subsequent binding of the S1 subunit to ACE2 and transmembrane serine protease TMPRSS2 enzyme cleaves the spike protein at the S2 site so that the S2 subunit fuses the viral and human lipid layers. This fusion results in the release of the viral nucleoprotein in the cellular cytoplasm. Subsequently, the positive sense single-stranded RNA-mediated protein synthesis occurs in the cytoplasm. In addition, the newly synthesized viral particles, assembled in the Golgi apparatus, acquire the lipid component from host cells, and form the envelope. Subsequently, the whole virion is released from the infected cells.[12,13]

Immune Response

Innate immune response against the viral pathogen is a major defense mechanism. During the viral replication, the pattern recognition receptor present in the cell initiates the innate immune response. STAT1, myeloid differentiation primary response protein MyD88, Toll/interleukin-1 receptor (TIR) domain-containing adapter molecule 2 in the toll-like receptor signaling pathway initiate the innate response, that result in release of interleukin-1and other proinflammatory cytokines. In addition, retinoic acid-inducible gene-I-like receptor induces the secretion of interferon. Apart from these, macrophages are crucial defense cells in viral infection. However, SARS-CoV-2 multiplies in the macrophages thus proinflammatory cytokines are released in the circulation.

In addition to the innate immune response, the adaptive immune response against SARS-CoV-2 occurs. The antigen-presenting cell targets the viral antigens to the T and B cells, resulting in the cellular and humoral immune response. Presence of raised proinflammatory T helper 17 (Th17) cells and cytotoxic CD8+ T cells in SARS-CoV-2 infected patients signifies the importance of cellular immune response. In addition, CD4 + T cells mediate the specific immunoglobulin A (IgA), IgM, and IgG humoral immune response. Briefly, the S protein of SARS-CoV-2 binds with antigen-presenting cells and binds with CD4 + T cells through Major Histocompatibility complex II (MHCII); thus, neutralizing antibodies are produced. However, these neutralizing antibodies are short-lived. The memory T and B cells sensitized with SARS-CoV-2 antigen remain for a life-time.

Activation of both innate and adaptive immune response ultimately results in the release of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α), and elevated concentrations of inflammatory markers, including D-dimer, ferritin, and C-reactive protein (CRP) at the site of infection.

Cell Damage

In a small fraction of SARS-CoV-2 infected patients, lung, kidney, heart and muscles are affected severely, where pneumonia, acute respiratory distress syndrome (ARDS), vasculitis and hemorrhage, GI manifestation, cardiac arrhythmia, and clinical manifestations of acute kidney injury occur. These manifestations are common in patients having predisposing risk factors as old age, patients having chronic obstructive pulmonary disease, cardiac, and renal issues, pregnancy, and immunocompromised patients.[14]

Tissue injury in SARS-CoV-2 infections occurs due to direct damage by the virus or improper immune response. The generated response against the SARS-CoV-2 virus is protective on the one side, whereas, on the opposite side, elevated immune response triggers cell damage. In addition to clearing the infected virus, the inflammatory cytokines and chemokines directly damage the cells.

During SARS-CoV-2 infection, an intense innate and adaptive immune response occurs. During this, a massive release of cytokines (storm), especially IL-6, occurs in the circulation.[15] The IL-6 induces the release of acute phase reactant as CRP, hepcidin, serum amyloid A, and fibrinogen.[16] In addition, IL-6 induces the adaptive immune response, where it stimulates the bone marrow to convert the plasma cells into the effective B cells, which subsequently produces antibodies against the specific antigen. These synthesized antibodies bind with antigen and initiate the classical pathway of complement system. During this pathway, chemokines and cytokines as c3a, c5a are released that recruit the inflammatory cells as neutrophil, macrophages at the site of action. In addition, the released c3b initiates the phagocytosis. Apart from IL-6, level of IL-1, IL-8, and TNF-α cytokines is increased, which indirectly results in tissue damage. Macrophages secret the IL-8 that recruits the neutrophil at the site of infection, that again releases the inflammatory cytokines; thus, a cascade pathway results in a massive release of cytokines, resulting in tissue damage.[17]

Recovery

Spontaneous recovery in SARS-CoV-2 infections usually occurs in majority of the patients. This recovery occurs due to the combined effect of clearing of the virus and inflammatory cytokines from the circulation. In addition, at the time of recovery, neutralizing antibodies are present in the circulation. However, the time for complete recovery varies, depending on the pre-existing health and diet. A small fraction of the infected patients has long-term consequences

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such as fibrosis, persistent inflammation, and catabolic syndrome.

Reinfection and relapse are common consequences of SARS-CoV-2 infection. In relapse, the infection occurs due to the sample strain and remains persistent in the immunologically privileged site. However, reinfection usually occurs with a mutant strain of SARS-CoV-2 such as omicron and BF7.

Mode of Transmission

SARS-CoV-2 is transmitted through direct, indirect, or close contact with infected people through infected secretions such as saliva and respiratory secretions or droplets. The virus is transmitted through particles of more than 5 µm as droplets and less than 5 µm as aerosols. These droplets remain on inanimate surfaces for long; thus, transmission can occur indirectly through touching surfaces with ultimate transmission to the mouth, nose, or eyes by contaminated hands.[18]

The Variant of Concern

During the viral replication, a frequent mutation occurs in the genetic code; thus, the newer generated virus has some different phenotypic and genotypic morphology from the previous one. These mutations alter the virus's properties, especially its transmission rate, the severity of the diseases, and diagnostic tools. The newer strains of the viruses are known as variant of concern (VOC) or variant of interest (VOI). VOI is the altered virus having a different phenotype and genotype, where reduced neutralization of the mutated virus is observed with the antibodies targeted against the previous infective virus as there is an alteration in the binding receptors. In contrast, VOC strains have evidence of a higher rate of viral transmission, increased disease severity, reduced effectiveness of the treatment, and diagnostic failure.[19] Variant high consequence are the mutated viruses with clear evidence of reduced effectiveness of preventive/therapeutic strategies and diagnostic failure. Presently, most of the mutated strains are SARS-CoV-2 that are considered VOC.[19]

In India, Indian SARS-CoV-2 Genome Sequencing Consortia reported the presence of global VOCs, B.1.1.7, B.1.351, and P.1 (the alpha, beta and gamma VOCs, respectively). Between December 2020 and February 2021, the delta and kappa variants (B.1.617.2 and B.1.617.1, respectively) comprised 60% of sequences from Maharashtra. During the third wave of SARS-CoV-2 infection in India, the Omicron variant was reported to be a causative strain, which was classified as VOC by the US government SARS-CoV-2 Interagency Group on November 30, 2021.[20]

Laboratory Diagnosis of SARS-CoV-2: Issues and Challenges

Let us consider specific key points before we discuss in detail the laboratory diagnosis of SARS-CoV-2 infection:

It is established that COVID-19 cases are occurring because of community transmission through air droplets. The laboratory diagnosis detects the virus from the infected person in the early days of symptoms. Early diagnosis is the hallmark in containing the spread of infection further through isolation and contact tracing.[21–23] The direct examination under the electron microscope is nonspecific and cumbersome. Isolation of the virus is risky and needs a biosafety level-3 (BSL-3) facility. In addition, antigen detection is not sensitive as the copy number cannot be increased; thus, we can only detect antigens during the symptomatic phase of COVID-19.

Moreover, antibody detection, although immune response usually amplifies the humoral and cellular responses, is not feasible as it appears late in infection. Thus, amplification of nucleic acid remains the most suitable and reliable detection method. Currently, the diagnosis is made using real-time reverse transcription-polymerase chain reaction (RT-PCR), similar to those developed for SARS/MERS-CoV.[24,25] This review primarily focuses on the intricacies of diagnosis must be understood by clinicians, clinical microbiologists, and public health authorities.

In the following section, we describe the steps needed for the laboratory diagnosis of SARS-CoV-2: preanalytical, analytical, and interpretation of results.

Preanalytical Issues

Upper Respiratory Tract Infection and Collection of Samples for Diagnosis of COVID-19 Caused by SARS-CoV-2

The collection of nasopharyngeal secretions appropriately is an essential step. For virus detection, if the sample is not collected properly, it may give false-negative results. It has been reported that after the 5 to 6 days of infection, a significant viral load occurs in the upper and lower respiratory tract.[26–29] The preferred method is the collection of nasopharyngeal secretions with the same swab from both nasopharynges (NP). However, another site to collect the secretions by the second swab is the oropharynx (OP). Subsequently, the collected specimen is placed immediately in the same aliquot of the sterile viral transport medium (VTM).[24,30] However, NP swab from both NP has become a preferred method of collecting the secretions since it is better tolerated and safer for the healthcare provider as they collect the secretions by standing beside the infected person. To collect the OP secretions, the healthcare provider has to be in front of the infected person to visualize the OP. Furthermore, it has been reported that specimens collected from the OP site showed a higher mean cycle threshold (CT) value than specimens obtained from the NP site suggesting a lower viral load in the OP.[31] It has been stated that the OP site collection method is less sensitive than the NP site collection; however; more data is needed to confirm this observation.[31]

The healthcare provider collecting the specimens must be equipped with proper personal protective equipment (PPE) kit to prevent the risk of getting an infection. The NP swab must be inserted into the nasal cavity parallel to the hard

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palate between inferior turbinate and floor of the nasal cavity 1 inch past anterior nares till the patient flinches or resistance is encountered, indicating contact of the swab with the NP. The swab is kept in situ for 10 seconds to allow the secretions to adhere to the flocked tip. Subsequently, the swab is rubbed and rolled before being put into the VTM vial. Ideally, the swab should have flocked nontoxic synthetic fibers such as polyester and synthetic nylon handles.[32] However, in an emergency, other nonflocked swabs and transport media may be used. Cotton swabs are usually avoided as they contain substances that may inactivate the virus and inhibit PCR reaction, thus affecting the test results. After collecting the sample, swabs should be placed immediately in labelled VTM vials; subsequently, these VTM vials should be packed triple-layered and transported directly to the laboratory in icepacks to maintain the temperature. After sample collection, proper doffing is done, whereas PPEs are disposed of properly into the yellow bag and sent for incineration as per guidelines.

The prompt transportation of the collected specimen in the VTM is desirable. Ideally, the specimen may be stored at 4°C for 24 to 72 hours or at −70 to −80°C for long-term storage, thus maintaining the cold chain.[32] It is relevant to mention here that in some cases, specimens collected from saliva or the NP and OP sites may miss the early signs of infection, and also the late stage of infection. In lower respiratory tract infections and patients having acute respiratory distress syndrome, either repeated testing is done or sample is obttained from lower respiratory tract.

Identifying the patient's infection status depends on several factors, such as the site of the specimen, method of collection, the pathogen's burden at the collection site, and the severity of the disease. In addition, repeated sampling is done when the patient has clinical–radiological features of viral pneumonia with a history of potential exposure. Thus, it becomes difficult to interpret the result of a single negative test, especially among healthcare personnels.

Lower Respiratory Tract Infection of SARS-CoV-2

For lower respiratory tract infection, sputum or bronchoalveolar lavage should be collected as they yield the highest viral loads.[33,34] Care must be taken while collecting these samples as the procedures are aerosol-generating, which may infect the healthcare personnel. Some patients with SARS-CoV-2 pneumonia have a high viral load in their fecal samples.[35,36]

Analytical Issues

Safety Issues for Specimen Processing for RT-PCR

As the virus is airborne, the processing should be done in class-2 Biosafety cabinets.[24,25,37] However, it is always better to use a BSL-3 facility equipped with a negative pressure environment. The VTM containing respiratory viral specimens should be transferred to the lysis buffer in the BSL-2 cabinet. The lysis buffer contains a guanidinium-based inactivating agent and a nondenaturing detergent.[38,39] Alternatively, when viral isolation is not required, the VTM may be added with guanidium salt. This process inactivates the viral particles and stabilizes the RNA. The specimen should not be exposed to 56°C as this temperature inactivates the SARS-CoV-2 and disrupts the RNA genome of the virus.

Many self-enclosed systems are integrating nucleic acid extraction, amplification and detection, such as ID NOW (Abbot, San Diego, California, United States), Cobas Liat (Roche Molecular System, Pleasanton, California, United States), and GeneXpert Dx (Cepheid, Sunnyvale, California, United States). Recently, on April 10, 2020, ICMR approved using TrueNAT for E gene screening for this novel SARS-CoV-2 infection. This E gene screening rules out all negative cases, whereas E gene-positive cases are further confirmed by the TrueNAT RdRp confirmatory test, which ICMR approves. This automatic system can easily verify the cases within 2 hours. This testing as a point of care is helpful in peripheral areas of developing countries where secretion collected from NP and middle inferior nasal turbinate can directly be placed in TrueNAT lysis buffer.

This equipment may be used as a point of care test after a clinical specimen gets transferred into a cartridge in a class-2 biosafety cabinet. The cartridge is sealed, and if any spill occurs, it should be decontaminated with an appropriate solution (usually 10% hypochlorite). However, in certain situations, especially when putting the testing system at the point of care, the biosafety cabinet of class-2 may not be available. In this case, the collection and transfer of specimen from a patient should be done by taking full precautions such as using the full protective gear (splash guard, N-95 mask, head cover, gloves, and disposable impervious laboratory coat).

Molecular Detection of SARS-CoV-2

i. The random amplification deep sequencing method is a particular way in the initial identification of SARS-CoV-2. Next-generation sequencing and metagenomic will be necessary to determine this virus's mutations.
ii. Loop-mediated isothermal amplification, multiplexed isothermal amplification followed by microarray-based detection, and CRISPR (Clustered Interspersed Short Palindromic Repeats) assays may also be used in identification.[40]
iii. Targeted real-time polymerase chain reaction

A real-time RT-PCR is recommended for molecular testings at a large scale as amplification and detection are done simultaneously in a closed system. RT-PCR avoids the possibility of PCR product contamination and allows real-time detection. Several coronaviruses are causing respiratory and intestinal infections in humans.[2,41] Out of these coronaviruses, SARS-like bat corona, which includes both SARS-CoV-1 and SARS-CoV-2, is kept under one clade of the subgenus Sarbecoronaviridae.[2,7] Of the several genetic markers, the structural genes are mostly targeted, for example, E, S, M helicase (Hel) and N, for detecting the SARS-CoV-2. In addition, some species-specific accessory genes, namely RNA-dependent RNA polymerase (RdRp) gene, ORF1a and ORF1b, HE, are targeted for confirmation. The WHO recommends

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using E gene for screening, whereas Centers for Disease Control and Prevention (CDC), United States, recommends the N1 and N2 genes. Due to the presence of other endemic coronaviruses and the potential genetic drift of SARS-CoV-2, two targets must be included for the testing. Therefore, mostly N, E, RdRp, and ORF1 genes are chosen for primer designing, and the results should be interpreted carefully.

Recently, the emergence of the Omicron VOC poses a great challenge for its diagnosis as there is an absence of the E gene. Therefore, we can miss the cases, caused by Omicron VOC. Other genes such as N, RdRp, and Orf1 can be targeted in such cases. Recently, CSIR-Central Drug Research Institute has developed an indigenous RT-PCR diagnostic kit, “Om,” to test the Omicron variant of coronavirus.[42]

Postanalytical Issues

Interpretation of Molecular Results

Usually, the current assays with three targets are employed to diagnose a case of COVID-19. To consider any sample positive for SARS-CoV-2, the positive peak for the E gene and with any one of the RdRp and ORF1 is deemed to be positive.[43] However, CDC, United States, initially recommended if both the nucleocapsid targets N1 and N2 are positive, then the sample may be reported as positive for SARS-CoV-2.[44] It is worth mentioning that the viral load does not reflect the severity of disease and also does not help in monitoring any therapeutic responses.[26–28,45,46] Low CT values may be indicative of high viral loads and potentially more infections.[33,47]

Test for Cure and Infectivity

There is a suggestion that if two consecutive paired NP/OP samples collected 24 hours apart become negative by the RT-PCR detection method, the person may be considered free of the infection. Recently, one negative test result is also considered sufficient to discharge the admitted patients. Exceptions have been observed where many patients remain positive and shed viable/live coronavirus. Therefore, self-quarantine for 1 month may be recommended to help in containing the spread of SARS-CoV-2.[19]

During active multiplication in nasopharynx, virus can be isolated fromoropharynx and nasopharyns. However, despite having high viral load, virus may not isolated from their stool samples.[29] Therefore, the present recommendation of ICMR is that the test for COVID-19 should be carried out on day 13 from the time of the isolation of the infected individual. The patient should be discharged from the isolation ward if the test is negative. The RT-PCR method speculates that SARS-CoV-1 could be detected for up to 1 month. A similar period may exist for SARS-CoV-2.[2] Thus, it is highly recommended to have self-quarantined for up to 1 month after the discharge from the isolation ward.

Additional Methods of COVID-19 Testing

Serological Testing of COVID-19

The S and N proteins on the virus’s outer surface come in direct contact with the immune system. Therefore, the serological method detects serum antibodies against S and N proteins.[48] The spike protein is responsible for receptor binding and fusion. The fusion protein determines the tropism and transmission capabilities.[2,7] The S gene coding this protein has two subunits, S1 and S2. The binding occurs through S1, while the fusion is executed through the S2 domain. SARS-CoV proteins bind to the angiotensin convertase enzyme-2 (ACE-2) receptor, but SARS-CoV-2 binds with greater affinity. This enzyme is abundant in human respiratory, renal, and gastrointestinal cells.[2] The other protein is nucleocapsid, which plays an important role in pathogenesis, replication, and RNA packaging. N protein seems to be an immunodominant antigen as antibodies appear against N antigen.[49]

In about half of the symptomatic infected people, seroconversion usually occurs after 7 days, whereas approximately 97.8% of seroconversion happens after 21 days. However, SARS-CoV-2 viral load is observed in the stool samples, despite the appearance of serum antibodies.[50] It has been noted that antibody detection is not associated with a decline in viral load. A rapid lateral flow assay determines serum (IgM and IgG) antibodies. The serological tests may be able to determine the immune status in the community against SARS-Cov-2. This host response-based detection is unlikely to be useful for early diagnosis.[29,49] However, serology may be helpful as a surveillance tool for assessing herd immunity and confirming the community transmission of SARS-CoV-2 for surveillance purposes.[36]

Different antibody detection kits (lateral flow assay) have various sensitivity and specificity. Recently, ICMR-NIV Pune has developed an enzyme-linked immunosorbent assay (ELISA)-based IgG detection kit against SARS-CoV-2. In India, Zydus Cadila, Gujarat, is producing COVID KAVACH ELISA. However, ICMR has also approved this antibody-based detection kit only for surveillance among the high-risk population such as healthcare workers, frontline workers, or individuals belonging to the containment areas to identify the recovered infected persons.[51]

Antigen Detection

The rapid point of care immunoassay is generally lateral flow-based. These assays may detect antigens of the SARS-CoV-2 virus. Antigen detection would theoretically provide for the rapid and low-cost detection of SARS-CoV-2 but are likely to suffer from reduced sensitivity in early infection. The main concern with antigen detection is missing those cases with low viral load and variability in sampling. Recently, ICMR has approved using STANDARD Q COVID-19 Ag (antigen detection kit) to diagnose SARS-CoV-2 infection. The clinical samples having the SARS-CoV-2 antigen are to be considered infected, whereas RT-PCR should retest the negative results before declaring negative. For this, samples should be collected from the posterior pharynx of the suspected cases. The major advantage of this test is rapid as results are available within 30 minutes and can be executed at the point of care.

In 2021, ICMR approved the home testing of SARS-CoV-2 for only symptomatic individuals and immediate contacts of

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laboratory confirmed-positive cases. ICMR has reported these kits, CoviSelf (PathoCatch) COVID-19OTC Antigen LF device, PanBioCOVID-19 Antigen Rapid Test Device, Covi-FindCOVID-19 Rapid Ag Self Test, AngcardCOVID-19 Home test kit, CliniTestCOVID-19 Antigen Self Test, ULTRA Covi-Catch SARS-CoV-2 Home Test, AbCheck Rapid Antigen Self Test – Nasal, COVID-19 AtHome Test Kit (Nasal), as satisfactory to be used as home/self test kits.[50]

Viral Isolation

This method is not recommended for diagnostic purposes since it requires a BSL-3 level facility. However, it has a role in researching and developing the diagnostic system, vaccines, and antiviral agents.

Disposal of the Generated Biomedical Waste

Different color-coded bins with foot-operated lids are to be kept where segregation and disposal of generated biomedical waste are done. The color-coded bins are labeled with COVID-19 waste. Used PPEs such as goggles, face-shield, single-use splash-proof apron, plastic coverall, and nitrile gloves are to be discarded into the red bag. In contrast, used masks (including triple layer masks, N95 masks), head cover/cap, shoe cover, and disposable linen gown are to be discarded into the yellow bags. VTM, plastic vials, vacutainers, Eppendorf tubes, plastic cryovials, and pipette tips are discarded into red bags.[52]

Conclusions

COVID-19 pandemic can be easily contained by early identification and isolation of the infected person. It has been observed that SARS-CoV-2 viral nucleic acid is usually absent in the serum and urine regardless of illness severity. However, the intact virus is maintained by respiratory specimens, sputum, saliva, NP swab, and OP swab collected in VTM. This can be detected by using the RT-PCR method. To date, this detection method is considered the most sensitive, reliable, and specific in detecting SARS-CoV-2 infection. However, the relevance of the serologic test is limited since it takes a more extended period to have detectable levels of antibodies in the blood samples. The other issue is cross-reactivity with related coronaviruses. However, antibody detection may be useful to determine the immunity of an individual and the community.

Similarly, antigen detection also traditionally suffers from suboptimal sensitivity. ICMR recommends testing three groups: hospitalized patients suspected of COVID-19, patients at high risk of poor outcomes, and persons who had close contact with someone with suspected or confirmed COVID-19. Testing in asymptomatic persons is not practical. Coinfection and secondary bacterial infection in COVID-19 have been reported. The other surrogate diagnostic parameters may be utilized, such as chest imaging by X-ray, computed tomography scan or magnetic resonance imaging, although they are not specific.

Conflict of Interest

None declared.

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Effect of Sleep Restriction during Pregnancy on Fetal Brain Programming and Neurocognitive Development of Offspring: A Review

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Abstract

We spend one-third of our lives in sleep, yet the core function of it still remains an enigma due to underlying complex neural processing in this altered state of consciousness. Sleep requirement varies with phase of development. Neonates spent about 85% of their time in sleep, which is polyphasic in nature. Gradually, this pattern takes the shape of a monophasic sleep in adolescents and adults, with changing micro- and macroarchitecture in every phase. Deprivation of sleep in adults impairs learning and memory, and reduces theta coherence among hippocampus and amygdale during sleep. However, sleep loss during pregnancy can affect the ontogenetic development of networks for sleep–wakefulness and the cognitive development of offspring. Even in normal pregnancy, poor sleep quality, reduced rapid eye movement (REM) sleep, and sleep fragmentation are common observation during the last trimester of pregnancy. Delta power, a marker for the homeostatic drive for sleep, in the NREM sleep during the last trimester of pregnancy and postpartum is increased. However, further sleep loss during late pregnancy is a growing concern. Neonates that are born to the total sleep-restricted dams present significant alterations in their emotional development (symptoms of hyperactivity, increased risk-taking behavior during periadolescence) and immature sleep–wakefulness patterns. The REM sleep restriction during late pregnancy elicits depressionlike traits in neonates, which persist until middle age. For a healthy development of brain and body, thorough understanding of the dynamic nature of sleep in relation to age and state (pregnancy) is instrumental in preventing the above-mentioned conditions of prenatal origin. Although sleep is essential for an active brain (for work during day), it remains an underestimated phenomenon. This review highlights the importance of sleep during pregnancy for a healthy brain network programming in offspring.

Keywords

- REM sleep restriction
- total sleep restriction
- hyperactivity
- depression
- sleep loss during pregnancy
- cognition
- human
- rat
- behavior

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Introduction

Sleep is an integral component of our life, yet it remains an enigma due to underlying complex neural processing in this altered state of consciousness. After the discovery of the rapid eye movement (REM) sleep in 1953, it was categorized into two distinct states, nonrapid eye movement (NREM) and REM sleep.[1] Subsequently, there was tremendous progress in understanding neurophysiological correlates of the NREM and REM sleep.[2,3] It was soon realized that sleep loss is a common problem in the current 24×7 societies, due to changing lifestyles. Further evidences from human and animal studies indicate that sleep loss is a potential risk of hypertension, metabolic disorders, insulin resistance, cardiovascular diseases, neurocognitive decline, and even cancer. Almost every function of the body gets affected by lack of sleep. It is emphasized that sleep is a dynamic process that undergoes quantitative and qualitative changes from birth until the fag end of life.

In the 21st century, concerns were raised on poor sleep quality during pregnancy and adverse pregnancy outcomes.[4–6] Even though several factors including malnutrition, substance abuse (cocaine, marijuana, etc.), alcohol consumption, oral hygiene, human immunodeficiency virus (HIV)/acquired immunodeficiency virus (AIDS), infection, stress, and smoking during pregnancy were identified risk factors for the development of fetus, sleep loss during pregnancy as a risk factor was never given importance (►**Fig. 1**). World Health Organization has reported that about 10% of women during pregnancy and 13% of women who have just given birth to babies experience mental disorders including anxiety and depression (www.who.int/mental_health/maternal-children).[7] In developing nations, this percentage escalates by another 5 to 6%. Globally 10 to 20% of children and adolescents also experience mental disorders. These neuropsychiatric conditions are the leading cause of disabilities in youngsters and they severely influence their development, educational attainments, and abilities to live fulfilling and productive lives (www.who.int/mental_health/maternal-children).[7] It is always a difficult task to conduct controlled sleep deprivation experimental studies during pregnancy in human subjects due to the ethical and practical reasons. Amidst this scenario, an extensive study was initiated in the rodent model to study the effects of sleep deprivation during pregnancy on the cognitive development of offspring under the Cognitive Science Research Initiative program of the Department of Science and Technology (India) in 2012.[8–18]

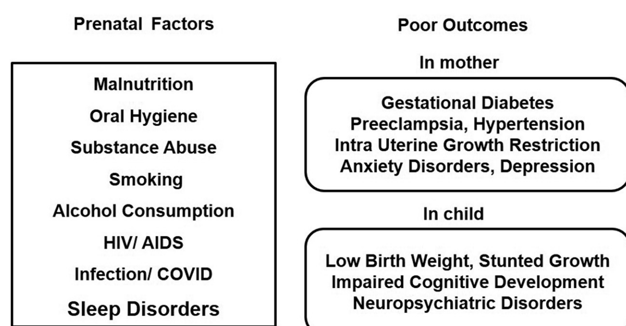

Fig. 1 Factors during pregnancy for poor outcomes on maternal and child health.

Rat Model to Study Effects of Sleep Restriction during Pregnancy

Even though rats are nocturnal animals, they are accepted models for studies involving sleep restriction during pregnancy and ontogenetic neurocognitive development in offsprings.[8–18] Similar to humans, pregnancy in rats consists of three trimesters with a total gestation period of 3 weeks. The developmental profile of sleep–wakefulness (S-W) in normal rat dams was assessed by recording electroencephalogram (EEG) and electromyogram (EMG) for 24 hours, starting from prepregnancy days and during pregnancy to postpartum (lactation) and postweaning (PW) days, along with monitoring of their anxiety.[11] For this, female rats were chronically implanted with EEG and EMG electrodes under anesthesia and were allowed to recover completely.[11] After complete postoperative recovery, 24-hour control recordings of their S-W were taken on 2 consecutive days. These female rats were subjected to mating with age-matched males. This is followed by recording their S-W on various gestational days of pregnancy (GD), postpartum days (PD), and PW were taken, some of which are shown in this article. Sleep fragmentation was observed during the third trimester of pregnancy (GD 19) and postpartum days (PD 4, PD 10) with a concomitant rise in the NREM sleep delta power (►**Fig. 2**). Delta power during the NREM sleep is a measure of the homeostatic drive for sleep. Delta power in the NREM sleep was calculated from EEG traces, using a fast Fourier transform. Increased NREM sleep delta power during late pregnancy–lactation continuum was evident as a result of sleep fragmentation (►**Fig. 2**). During normal pregnancy, the postpartum sleep and anxiety decreased compared to the antepartum levels.[11]

In the rat model, during the last trimester of pregnancy (GD 15–20), REM sleep restriction (REMSR) was carried out using the classical platform method along with a sham control for 22 h/d from 11 a.m. to 9 a.m. subsequent day (8), whereas the total sleep restriction (TSR) was carried out by the gentle handling procedure wherein the rats were manually woken up whenever they got drowsy (9 a.m. to 2 p.m.).[9,10] The effects of REMSR and TSR were studied in the rat model for (1) vocalization pattern to assess the emotional development in pups from postnatal days (PNDs) 1 to 21,[8,9,12] (2) ontogenetic profile of S-W from PNDs 1 to 21 and mathematical modelling for developmental assessment,[13–15] and (3) behavior testing of phenotypic traits of anxiety, depression, etc., for alteration in cognitive development during periadolescence (PNDs 26–46) in three groups.[9,10]

Cries as Initial Markers of Emotional Development in Human Neonates

Cry (vocalization) at the time of birth in human is the first robust acoustic signal that marks successful switching from the fetal life in amniotic fluid to an air-breathing life.[19] The Apgar score in neonates consisting of five features (appearance, pulse, grimace,

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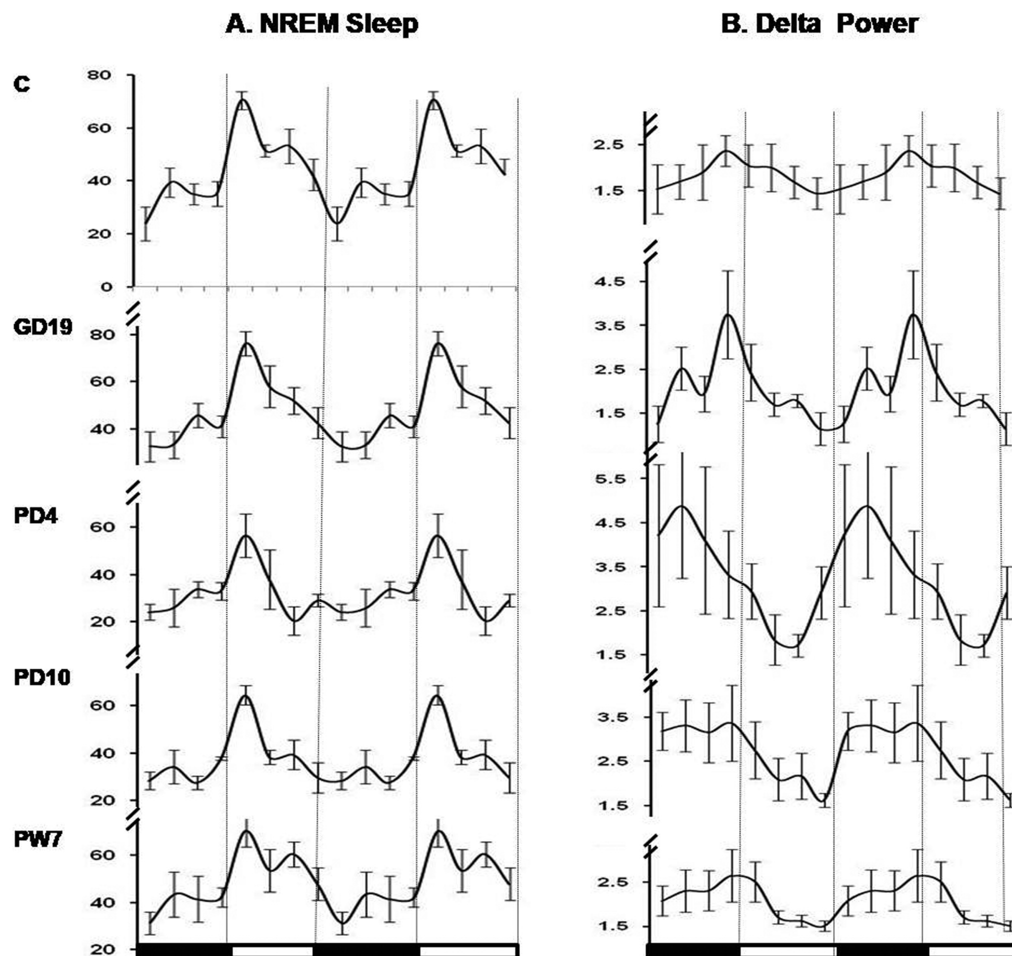

Fig. 2 Pattern of nonrapid eye movement (NREM) sleep and delta power over day and night during pregnancy and after parturition in rats. (**A**) NREM sleep (%) in 3 hourly bins across day and night for control (C), gestational day 19 (GD19), postdelivery day 4 (PD4), 10 (PD10), and postweaning day 7 (PW7). In the horizontal axis, the *dark bar* denotes dark period beginning at 6 p.m. and the *light bar* shows light period beginning at 6 a.m. (**B**) Normalized delta power is shown for respective bins. (Adapted from Sivadas et al.[11])

activity, and respiration) provides a quick assessment of their health profile at the time of birth. Since the language is not developed in newborn, cry remains an important signal for caregivers/mothers and play important biological functions initiating mother–neonate interactions and care giving, which is crucial for optimal physiological and psychological development of infants.

Calls in human babies are marked by phonation, hypophonation, and hyperphonation. An altered vocalizing pattern with abnormal calls is related to brain damage in human babies.[20] Hyperphonation, which is marked by the shifts in pitch, is relevant for translational analysis of neurobehavioral dysregulation assessment in human infants. The two-component step calls of rat pups appear analogous to hyperphonation of human babies. Human studies have linked maternal stress with adverse neural developmental in children.[21] The decreased REM sleep in pregnant women increased the risk of postpartum depression in mothers.[5]

Cries as robust acoustic signals contain hidden information about their state of well-being.[19,20,22,23] For new parents and caregivers, it is a huge challenge to decode the underlying message in cries. It is a well-known fact that language is not developed at birth and babies are born with mechanisms of cry to convey their discomfort and call for attention. The vocalization pattern in cry varies, but it is hard to differentiate the cry types. Pediatricians follow the Wessel rule of 3 for colic-related excessive cries, according to which infantile colic is defined as inconsolable crying for 3 hours per day at least three times a week for at least 3 weeks. But still in the majority of infants, this compliance is not observed. In a few recent studies, attempts were made to label the cry types made in normal physiological state and various conditions like malnutrition, preterm birth, hypoxia, pneumonia, sudden infant death syndrome, laryngomalacia, etc.[24–33] It is also noted that voice recognition is based on individual-specific voice traits like finger prints as the individual differences in human voice pitch are preserved.[34]

Ultrasonic Vocalizations Pattern to Assess the Emotional Development in Pups from Postnatal Days 1 to 21

Rodents also communicate through ultrasonic vocalizations (USVs) in the range of 20 to 100 kHz, which are inaudible to

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human ears in addition to making a few audible calls during any painful handling. After birth, the rat dam (mother) keeps the pups huddled for maintaining their body temperature, nursing, and for frequent grooming (fecal removal training, somatosensory stimulation). USVs are recorded using special microphones in the isolation paradigm wherein pups are taken away from their mother for 2 minutes in a soundproof chamber.[8,9,16] Pups born to either REM sleep-restricted (22 h/d, 11 a.m. to 9 a.m. from gestational day 15 to 20) mothers (rat dams) or total sleep-restricted (5 h/d, 9 a.m. to 2 p.m., from gestational day 15 to 20) mothers, from PND 1 to 21, showed a distinct pattern of USVs in terms of both quantity and quality (►**Fig. 3**). In control pups, the number of USVs is low after birth, but it begins to increase gradually peaking on PND 9 followed by a decrease until PND 21. Compared to controls, pups born to the REM sleep-restricted mother not only made lesser calls on the PNDs 1 to 11 but also showed development delay in vocalization patterns.[8] However, the pups born to total sleep-restricted dams cried more compared to controls on initial PNDs 1 to 9 that appeared reaching to normalcy on later days.[9] Normal pups show a specific pattern of call types, making simpler constant frequency calls during initial PNDs. They gradually gained the ability to produce frequency modulation with progression of age.[16] Moreover, USVs reduced to minimal in normal pups (C) around the PND 21 when separation from the mother is no more a stress to them as they become more independent in their habits, and depending less on mothers for their needs. But pups born to total sleep-restricted dams made a higher pitch (increased bandwidth) in upward call type and also in the two-component step calls, which help in seeking more maternal attention (►**Fig. 3**). This is similar to abnormal calling patterns reported in pups born to mothers undergoing malnutrition during the prenatal period.[35]

Possibly the altered USVs in the absence of optimal nest environment and maternal care during early postnatal period can signal the emotional deficits during later life. The exact biological communication hidden in the individual call types are not yet deciphered, but it would be interesting to decode these acoustic signals to understand the nature of these deficits. Reverse communication also exists and pup vocalizations do influence the mother's behavior as the USVs of the pups shape the mother's responsiveness toward the pups.[36] Alterations in USVs in pups could be due to prenatal factors alone or with a contribution from postnatal factors. However, it seems that the brain development during early postnatal period is important in determining cognitive functions[37] and also impaired neural development during fetal life may increase the susceptibility to emotional disturbances.[21,38]

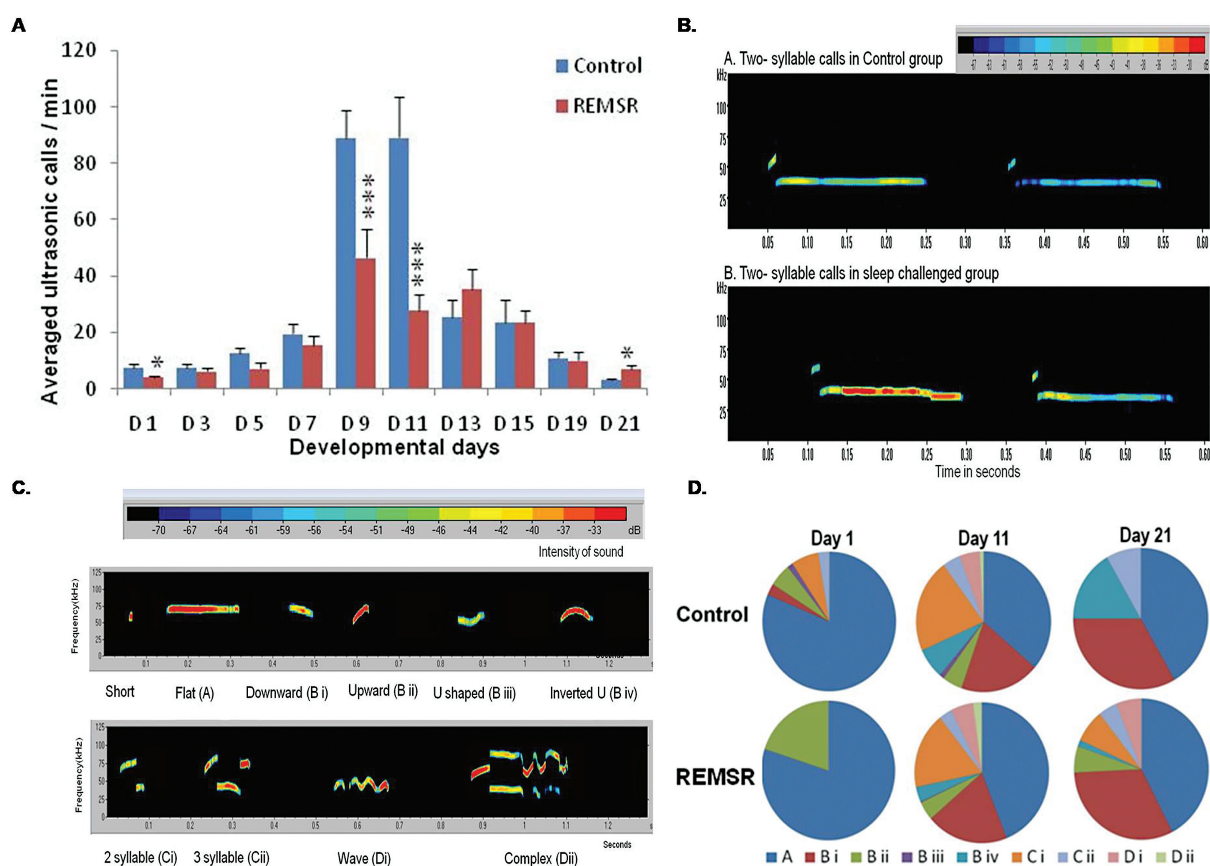

Fig. 3 Qualitative and quantitative pattern in ultrasonic vocalizations (USVs) in pups during various postnatal days born to control and rapid eye movement (REM) restricted rat dams. (A) Developmental patterns in USVs in pups born to control and REM restricted dams from postnatal days 1 to 21. (B) Higher intensity in call type (2 syllable) in pups from the REM sleep restriction (REMSR) dams. (C) Various categories of USVs. (D) Changes in call types in pups on days 1, 11, and 21 in control and the REMSR group. (Adapted from Gulia et al.[8])

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Effects of Sleep Loss during Pregnancy on Sleep–Wakefulness during Early Development in Offspring

In altricial species including humans, babies are born immature. The fetal brain undergoes fast development during the third trimester of pregnancy that continues to develop until the adolescent period to achieve optimal cognitive abilities for survival. Fetus sleep exists in a primitive form consisting of the active sleep (AS; precursor of REM sleep) and the quiet sleep (QS; precursor of NREM sleep). Term-born human neonates spend about 80 to 85% of time in sleep, of which nearly 50% of the component is AS. With development, the percentage of AS gradually keeps on decreasing and reaches about 20 to 25% in young adults. Sleep pattern is also polyphasic wherein babies wake up regularly across 24 hours, as circadian rhythms are not yet developed.[39]

It is a challenging task to study newborn rat pups owing to their tiny size and unossified skull, lack of thermoregulatory abilities, and closed eyes/ears at birth. For studying their S-W, acute implantation of EEG and EMG electrodes was carried out, and pups were studied in a special fabricated environmental chamber in which temperature and humidity are regulated.[13–15] Similar to human neonates, rat pups also show relatively shorter bouts of sleep episodes compared to adults. In these newborn rats, AS constitutes a substantial period of time (about 70–75%) and they spent comparatively lesser time in QS (about 10%). This pattern is observed during the initial week and thereafter the AS reduces, while the percentage of QS rises. After 10 days, AS decreases dramatically, while QS increases exponentially. Also, the bouts of AS become shorter, while the length of QS increases. A higher percentage of AS is found in immature newborns albeit there are species differences.[13] Behavioral events in rats during AS in the initial PNDs 1 to 7 include rapidly occurring myoclonic twitches and jerky limb movements. Myoclonic twitches and subsequent hippocampal neocortical communication probably assist in the development of cortical neural networks and somatosensory responses.[40,41]

Sleep restriction during the last trimester of pregnancy provided insights into brain plasticity for sleep networks in growing neonates. REMSR or TSR during the third trimester of pregnancy not only affected the natural ontogenetic development of sleep in utero but also delayed and disrupted the normal development of S-W.[13,14,17] Compared to age-matched control, pups from both groups of sleep-restricted dams displayed a higher percentage of AS and a lesser percentage in QS. Longer duration of the S-W cycles in neonates and infants of sleep-restricted mothers indicated vulnerability of this parameter during fetal development. Reduced S-W cycles are also observed in preterms (younger than 30 weeks of gestational age) as compared to the control group.[42] The circadian rhythms in these preterm neonates are also less robust as they exhibit a multitude of ultradian frequencies, while full-term neonates show a distinct circadian frequency depicting fast initial adaptation in the first week of life to a 24-hour day.[43,44] In altricial species where babies are born immature (e.g., human babies born between 27 and 37 weeks), AS is high at the time of birth.[45,46] On the contrary, full-term pups of sleep-restricted dams also display immature patterns of sleep at the time of birth.[13,14] This clearly indicates that maternal sleep restriction during the last term of pregnancy delays the maturation of S-W in utero. This may not be due to maternal stress during sleep restriction, as infants of mothers who consumed alcohol showed reduction in the AS and increase in wakefulness.[47,48] Mothers exposed to cigarette smoke during pregnancy (passive smoke) had babies with impaired arousal patterns and were more susceptible to sudden infant death syndrome.[49,50]

Altered Sleep Microarchitecture during Development

In the developing brain, the EEG power spectral analysis is an established method to understand the power distribution in different EEG frequency bands. Such analysis performed during postnatal days 15 (D15) and 20 (D20) during QS and AS sleep provided an interesting insight into the development of these bands.[17] In control pups, during QS, delta power decreases from D15 to D20, whereas powers in theta, alpha, and beta bands increase. However, pups born to REMSR dams exhibited lower delta power than controls on D15, while there was high power on other frequencies (beta and theta) in the QS traces. Besides this, the delta power did not decrease during D20 but remained higher, while the alpha, beta, and theta powers decreased significantly. During AS, there was higher delta power, but lesser theta and beta powers on D15. Higher power in the theta band and lower powers in alpha and beta on D20 indicated imbalance and delayed development of networks involved in EEG. Similar trends are observed in preterm fetal growth restricted neonates.[51] Alterations in thalamocortical cortical network organization can reduce the percentage of delta power. Decreased delta powers during slow wave sleep are also evident in depressed patients[52] with increased high-frequency activity,[53] which may be considered as markers of a susceptibility to stress and psychiatric disorders. Compared to full-term newborns, the premature group exhibited a higher frequency and larger amplitude of beta activity and also better inter- and intrahemispheric relationships. In human neonates, high-density EEG (noninvasive) can be used to examine brain maturation in infants who are at risk of neurodevelopmental deficits.

Clues from Modeling of Distribution Patterns of S-W Bouts during Development

Mathematical models are used to predict the precise developmental accounts of the neural foundations of sleep.[54] In human adults, sleep bouts follow an exponential distribution, whereas wake bouts follow a power law distribution and similar patterns are true in adult rats, cats, and mice.[54] Exponential processes imply probabilistic state changes typically governed by a constant rate of change over time. As sleep follows the same exponential dynamics, the distribution of S-W transitions may thus hold important clues to understanding the

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mechanisms underlying sleep architecture in health and disease.[55] Power law distributions arise from multiple components of a system interacting in a complex manner and often follow a similar profile across multiple measurement scales.

We also tested the generalizability of the S-W bout distribution from PNDs 1 to 20 in pups born to total sleep-restricted dams. The duration of sleep bouts exhibited exponential distributions similar to previous reports.[13,54] Decreased slope in the log-survival plots for bout durations of sleep in the TSR group on all the studied PNDs not only featured a delay in the development of sleep networks but also depicted the vulnerability of sleep maturation process. Strikingly, due to underdeveloped wake networks during early PNDs 1 to 10, the survival plots for wakefulness were least altered. However, during PNDs 15 and 20, compromised wakefulness distribution suggested that a sleep bout is a stochastic process having a steady probability of transition into wakefulness, which decreases through development, thereby achieving longer episodes of sleep. Wake bouts in very young rats also follow an exponential distribution during initial development (PNDs 1–10), but the distribution of wake bouts followed the power law from PND 15 onward. The probability distribution during the third week of development indicated that an already lengthy wake bout is likely to persist a little longer. Sleep and wake bout lengths show no memory for the duration of any previous episode, so that transitions between these states resemble an alternating renewal process.[56] These modeling studies were useful in understanding the development of sleep architecture, fragmentation, and associated network formations.

Detection of sleep changes in neonates may be of great clinical significance and can be viewed as early markers of psychobehavioral disorders. Decreased QS during early PNDs (1–10) and comparatively increased AS during all postnatal developmental days in the offspring of REM sleep-restricted mothers reflect downregulation of the maturational process. Pups of REM sleep-restricted mothers had reduced REM sleep latency during PNDs 15 to 20 that are indicative of depression-like traits. Early onset of REM sleep is commonly observed in the subjects with depression and narcolepsy. Moreover, the impaired sleep regulation including altered sleep homeostasis, reduced REM latency, reduced slow-wave sleep, and increased REM duration are commonly observed in depressed patients.

Possible Disruption of Neural Networks for S-W in Newborns after Maternal Sleep Restriction

In general, the AS mechanisms located in the brainstem mature first and the brain mechanisms for QS expression develop comparatively later. But QS is also affected by maternal sleep restriction. The reduced QS percentage in the pups of REM sleep-restricted dams indicated suppression of QS organization. Furthermore, the persistence of higher level of AS/REM sleep until PND 20 probably indicated the existence of the immature brain lacking inhibitory control over the AS/REM sleep.[6] Greater amount of QS observed in infants of depressed mothers is considered a conservation–withdrawal phenomenon since information processing for both auditory and visual stimulation is altered in depressed individuals during deep sleep. In addition, decreased wakefulness also indicate a delay in natural development of wake-promoting regions in the brain, including the locus coeruleus, which contributes to the developmental transition in the S-W pattern.

Circadian rhythms for S-W develop at around 2 months of age in humans. Since melatonin is one important hormone for sleep regulation, maintenance of an optimal maternal circadian rhythmicity via the suprachiasmatic nuclei would be essential to program the developing master oscillator of the fetus melatonin cycle. Chronodisruption and disturbed melatonin cycles have a negative impact on the maturing fetal oscillators, which may contribute to psychological and behavioral problems in the offspring. Preterm infants that may be deprived of some exposure to a normal maternal melatonin cycle show delayed development of the circadian rhythm.

Changes in the Behavioral Traits of the Pups (Emotional Testing in Elevated Plus Maze)

At the time of birth, the testing of UVs is the suitable method to understand their emotional profile. However, postweaning, the emotional behavior during the periadolescent window care carried out through elevated plus maze (EPM) with a video-tracking system. As the name suggests, a maze in shape of plus sign is kept elevated from the surface of the floor. Two arms of the plus maze are open, while the remaining two are closed ones. Rodents, being burrowing animals, like to spend more time in the closed chamber as compared to the open arms. Various parameters of the EPM test conducted individually for each rat (5 minutes) provide their emotional status.[10] The pups born to the TSR dam showed distinct hyperactivity during the periadolescent period.[10] They also enhanced novelty-seeking and risk-taking behavior. These findings suggest that maternal sleep loss during the prenatal period might result in emotional instability and behavioral deficits in pups during postnatal life. Weanlings from the REMSR group did not show such changes. However, these rats (REMSR group) showed increased immobility time and decreased latency to swim in the forced swim test, indicating persistence of depressionlike symptoms.

Drastic reduction in the USVs until PND 11 and higher calling rate during PND 21 are the earliest signal of behavioral alterations in REMSR rats. When these rats grew up to the adult stage, they showed deficits in emission of mating vocalizations (data from our unpublished findings) that mark persistence of long-term effects in the offspring from the REMSR mothers.

Sleep Deprivation–Induced Learning and Memory Impairment in Adults (Rats)

Sleep deprivation–induced impairment in learning and memory in adult rats are manifested through altered functional connectivity among the hippocampus, amygdala, and prefrontal cortex during REM sleep.[17] In these set of

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experiments, in addition to EEG and EMG electrodes for recording S-W, microelectrodes were stereotactically implanted deep into the brain (pons, amygdala, and prefrontal cortex).[17] Their REM sleep is characterized by the presence of theta waves and P waves (in rats) similar to the ponto-geniculo-occipital (PGO) waves in humans, in addition to desynchronized EEG and muscle atonia, in addition to several other physiological changes.[57,58] The PGO waves originate from the pontine area and spread to the geniculate and occipital areas and are associated with learning and memory. Total sleep deprivation of 24 hours (both components of sleep) elicited changes in the P waves, and theta coherence among the hippocampus, amygdala, and the prefrontal cortex, affecting the reference and working memory in rats.[12] This study emphasized the crucial role of sleep in maintaining an appropriate synchrony between various neuronal networks during REM sleep. It is comparatively easier to conduct such studies in adults, but it is always very challenging in neonates and infants.

It is getting well established now that prenatal disruptions in sleep patterns can give rise to various neuropsychiatric disorders in the offspring. Sleep can be seen as an epiphenomenon for neurocognitive network activities as any acute and chronic sleep disruptions can influence brain activity, resulting in impaired behavior.[17,59] Sleep deprivation during pregnancy suppressed the hippocampal long-term potentiation in rat offsprings.[60] All these studies strongly indicate the role of prenatal sleep in fetal programming.[61] Moreover, recent reviews strengthen the prenatal origin of neuropsychiatric diseases especially depression and anxiety.[15,17,18,62–65] More genome-wide association studies are required to tease out the effects of prenatal and postnatal contribution in neuropsychiatric diseases in humans.[66]

Sleep loss during pregnancy is a potential risk factor for various psychological disorders in the offspring, which is also emphasized through a few recent meta-analyses.[10,16,67–69] These findings from the rat model illustrate the importance of adequate sleep during late pregnancy for proper neuropsychological development in the offspring (►**Fig. 4**). Lack of NREM sleep during late pregnancy can lead to the development of hyperactivity and increased risk-taking behavior in the offspring. REM sleep deprivation during pregnancy can lead to depressionlike symptoms in babies and deficits in sexual behavior in later life due to impaired USV communication during mating. The neuropeptide orexin contributes in shaping an appropriate arousal system.[70] It is to be noted that spontaneous penile erections (in males) and clitoris engorgements (in females) are observed during REM sleep

3rd Trimester of Pregnancy in Rats

	REM sleep restriction	Total sleep restriction
PND1:	Low body weight Decreased rate of USVs Reduced QS %, increased AS % Long SW cycles	Low body weight Increased rate of USVs Reduced QS %, increased AS % Long SW cycles
PND10:	Low body weight Reduced no. of USVs Reduced QS %, increased AS % Long SW cycles	Normal body weight Increased no. of USVs Reduced QS %, increased AS % Long SW cycles
PND20:	Low body weight USVs still persist Increased AS % Long SW cycles Reduced latency to AS	Normal body weight USVs negligible Normal AS & QS % Long SW cycles Low arousal index
PND25-45:	Low body weight Depression like traits Impaired social communication	Reduction in body weight Persistent Hyperactivity Increased risk taking behavior

Fig. 4 Summary of effects of sleep restriction on various parameters across different postnatal days (PND). AS, active sleep; QS, quiet sleep; SW, sleep–wakefulness; USVs, ultrasonic vocalizations.

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irrespective of age. Our group have provided evidences for involvement of the septal neurons in these sleep-related erections in adults, indicating a wider role of sleep in organizing the vital functions of the body.[71–74] Further, knocking the peripheral warm receptors postnatally, by injecting capsaicin in neonatal rats, the central warm receptors produced adaptive changes to defend the high ambient temperature by increasing NREM sleep in adults.[75] It will be interesting to explore in future studies if prenatal sleep disruption also influences erectile and thermoregulatory networks. All these elegant studies unearth the importance of sleep during late pregnancy for a healthy brain programming and functioning. Moreover, further electrophysiological, molecular, and genetic studies in neonatal life would help in understanding complex interactions of several neural networks in brain as sleep, thermoregulation, sexual functions, and behavioral regulation are overlapping.[76–80]

Conclusion

Evidences from both the controlled studies carried out in rodent models and the epidemiological studies in human subjects emphasized on the fact that decreased sleep quality during pregnancy is a potential novel risk factor for neurocognitive development of the offspring.[4–6,18,67–69,76] It is a challenging task to take optimal care of newborns with a relatively immature brain. There is pestering need to conduct research in humans as well as animal models to explore the effects of unavoidable photic and other stimulations in the neonatal ICUs (the preterm human babies) on the development of an optimal arousal system as these critical conditions of postnatal hyperstimulation may affect autonomic nervous and alter behavior in growing babies. On the other hand, underdiagnosed term born babies with an immature brain (difficult to diagnose without EEG parameters) pose another challenge. Extensive meticulous research is needed to study the effects of disruptions of postnatal sleep in babies on the developmental timelines due to vulnerability of networks for S-W, autonomic nervous system, and motor and somatosensory networks in relation to onset of neuropsychiatric disease. It is already pointed out that various prenatal factors including environmental and psychosocial stress are detrimental to the fetal developmental programming, which is reflected during later developmental trajectories of life.[72–75] Since the newborn requires maternal care for a reasonably long time in humans, it is essential to tease out the role of sleep and mechanisms for developmental disruption of the brain in utero and during postpartum growth. Further research is needed to understand the role of sleep in the pre- and postnatal genesis of neuropsychiatric diseases. Also, since the process of the development of the brain is so complex, preventive measures must be implemented through formulation and implementation of the strong public health policies. A cry detector innovative app based on artificial intelligence may be developed to detect the early development abnormality in human neonates as a noninvasive tool. It is high time that we give priority to good-quality sleep during pregnancy and also during the remaining phases of life in the current era for a healthy life and well-being.

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Conflict of Interest
None declared.

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The Underutilization of Forensic Microbiology: A Narrative Review

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Abstract

One of the main reasons of death in India is infection. At many centers, determining the infectious cause of death during autopsy is not regularly done. Although it is still a neglected field, postmortem microbiology has the potential to be a crucial tool for identifying the reason and circumstances of unexpected death. In addition to its use in forensic autopsies and medicolegal investigations, this tool can help with the detection of novel pathogen presentations, estimation of drug resistance, identification of bioterrorism agents, and a better understanding of infectious diseases like toxic shock syndrome, Human Immunodeficiency Virus (HIV), and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). In India, there has not been much use of microbiology in postmortem, and there is a dearth of specific guidelines or recommendations by regulatory agencies.

In an effort to highlight the value of microbiology in postmortem, this narrative review focusses on suggestions made by a group of academicians from Europe in February 2016 and how they may be used in an Indian context. Based on the Indian Council of Medical Research and the Centers for Disease Control and Prevention standards, we have briefly discussed about postmortem in coronavirus disease 2019 related deaths.

Keywords

- forensic microbiology
- postmortem microbiology
- postmortem transmigration
- agonal spread
- COVID-19

Introduction

One of the main reasons of death in India is infection.[1] At many centers, determining the infectious cause of death during autopsy is not regularly done. Although it is still a neglected field, postmortem microbiology has the potential to be a crucial tool for identifying the reason and circumstances of unexpected death. In addition to its use in forensic autopsies and medicolegal investigations, this tool is useful for identifying emerging pathogens, novel presentations of well-known pathogens, estimating drug resistance, identifying bioterrorism agents, and gaining a better understanding of infectious diseases like toxic shock syndrome and Human Immunodeficiency Virus (HIV).[2] In context of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which caused an unprecedented pandemic, the importance of postmortem microbiology was unmatched. It assisted in not only figuring out the cause of sudden death (SD) but also in comprehending the etiopathogenesis of this epidemic.[3] It can also be used as an epidemiological and diagnostic tool in outbreak situations. Prior to coronavirus disease 2019 (COVID-19), informed fetal

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autopsy contributed to the growing body of information demonstrating a connection between the Zika virus and microcephaly.[4] One of the main reasons for our diminished understanding of the Middle East respiratory Syndrome compared with Severe Acute Respiratory Syndrome is the dearth of autopsy investigations.

It is impossible to overstate its educational advantages for medical professionals and trainees, and a quality assurance tool for diagnostic and therapeutic procedures is its added advantage. Compared with autopsy, pathology, and forensic science, this topic has been mentioned less frequently in the microbiological literature. In India, postmortem microbiology has not been widely used, and regulatory organizations have not issued any precise recommendations or standards.

In an effort to highlight the value of microbiological investigations postmortem, this narrative review focusses on suggestions made by a group of academicians from Europe in February 2016 and how they may be used in an Indian context. Based on the Indian Council of Medical Research (ICMR) and the Centers for Disease Control and Prevention (CDC) recommendations, the authors have also attempted to add a brief explanation of postmortem in COVID-19 instances.

Only 130 studies on sterile autopsy were found in a search of the PubMed database between the years of 1994 and 2022.[5] These investigations lacked standardized sample collection and culture procedures as well as a "gold standard" for identifying antemortem infectious diseases that were already present.

However, the aforementioned research did draw attention to a few theoretical and practical problems of postmortem microbiology. The pace of microbial multiplication, the availability of nutrients, and the concentration of oxygen available to the microbe all affect how quickly organisms multiply in the body after death. Some challenges to tracking pathogen cultures in postmortem samples are: in at least 50% of cases, saliva drains into the lung after death; about 12 to 15 hours after death, an experimental organism can pass through the human intestinal wall; and postmortem cooling of the body prevents bacterial development.

The Concept of Sterile Autopsy

O'Toole et al hypothesized this process in 1965, and Minckler et al confirmed it in 1966.[6,7] They started using "sterile mortuaries" with "surgical scrub and gowning" for the autopsy personnel, surgically cleaning the body, and performing dissection in the autopsy room with controlled air flow and using sterile surgical tools. Samples were cultured on common microbiological media during the postmortem interval, which was maintained at $<$20 hours. It was emphasized that single positive cultures for usual pathogens are likely to indicate actual infection and that these sterile techniques can prevent contamination.

Following these suggestions may help to reduce postmortem iatrogenic contamination[6,7]:

- Use of aseptic technique.
- Searing organ surface with hot spatula before sampling.
- Obtain blood/tissue samples prior to evisceration.
- Body should be moved to a 4 to 6°C refrigerated locker as soon after death as possible.
- Avoid making needless body movements.
- Clean autopsy room with good ventilation (at least 20 air changes per hour).
- Consider autopsy to be performed as a sterile procedure.
- All postmortem samples to be sent on ice to the microbiology laboratory without any delay in transport.

Postmortem blood cultures can be taken from the heart and spleen to investigate for bacteremia. By pushing a sterile cotton swab deeply through the burned region, tissue cultures can be obtained. Swabs can be cultured using trypticase soy broth, sheep blood agar, and MacConkey agar. Collection of five regular cultures can detect undiagnosed bacteremia, with the spleen being the most trustworthy organ. Due to contamination from upper respiratory microorganisms, lung cultures are unreliable for detecting pneumonia.[7]

Selection of Appropriate Type of Culture at Postmortem

Usually, standard bacteriological cultures are carried out. Nonetheless, as and when necessary, studies on viruses, mycobacteria, and fungi may be conducted.

Here, it's crucial to look at the four most typical scenarios —highlighted in recent recommendations made by a European Committee—where the use of microbiological methods at autopsy is necessary.[2]

These groups consist of:

- Sudden death.
- Bioterrorism.
- Tissue and cell transplantation.
- Paleomicrobiology.

Sudden Death

The cases of SD are further divided into four subgroups:

(a) Group 1: SD in infancy and childhood (0–16 y) without clinical symptoms. Appropriate samples to be collected: nasopharyngeal (NP) swab, blood, cerebrospinal fluid (CSF), feces, and portions of lung, spleen, myocardium.
(b) Group 2: SD in the young (17–35 y) without clinical symptoms. Blood, myocardium, and spleen are preferably collected.
(c) Group 3: SD at any age with clinical symptoms. Collection of spleen and blood are recommended while complementary samples can be collected according to the system involved in suspected underlying infection.
(d) Group 4: Trauma-related or iatrogenic deaths. Blood, tracheobronchial swabs, lung, spleen are recommended to be collected. According to medical and surgical procedures prior to SD, it is necessary to add tissue/fluid from the involved organ, 2 to 3 cm of distal end of central venous catheter tip and pus from deep wounds using a syringe or a sterile swab for bacteriology culture once the wound surroundings/skin surface are cleaned of debris.

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When doing virological studies, samples should be collected using sterile saline or an RNA stabilizing solution. An indication of an immunosuppressive condition should prompt the microbiologist to conduct mycological search as well. The choice to conduct molecular investigations depends on the clinical information obtained prior to death and the results of the autopsy.

Death due to Bioterrorism

The discovery of such biocrimes is a special area of microbial forensics where intentional or threatened use of bacteria, fungus, or poisons from live organisms is used to cause death or sickness in people, animals, or plants. Laboratory investigations are conducted in accordance with the patient's clinical background prior to death and the results of the autopsy.

Cell and Tissue Transplantation

To lower the risk of transmitting infectious diseases to the recipient during cell and tissue transplantation from the deceased, it is important to assess the possible donor's medical background and physical status, to exclude donor infection with *Treponema pallidum*, HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), and human T-cell lymphotropic virus. Serological and molecular assays are conducted for evaluation of cell/tissue transplant final product for yeast, filamentous fungus, aerobic and anaerobic bacterial presence. The Transplantation Society of Australia and New Zealand has issued guidelines (April 2016) regarding these viruses.[8] There are other recommended investigations in addition to these.

Paleomicrobiology

Paleomicrobiology is a growing subject dedicated to finding, identifying, and characterizing microorganisms in prehistoric remains where microbial DNA connected with the host can endure for approximately 20,000 years. In between sampling, instruments should always be decontaminated with 10% sodium hypochlorite. The samples should be put in sterile tubes or containers at once or kept at 4°C, shielded from light, and brought to the laboratory as soon as possible. Mummified bodies, skeletal remains, tooth powder, coprolites, and dental calculus can all be used to obtain samples. In Peru, the remains of humans were used to date a 9,000-year-old Chagas disease case.[9]

Specific Indications for Postmortem Microbiological Cultures

- Confirm presence of unproven infection.
- When cause of death is unknown.
- Holds relevance in public health—fatal notifiable diseases and emerging infections.
- Evaluation of efficacy of antimicrobial therapy.
- Investigation of malpractice related to treatment of infectious disease.
- Identification of infectious agents in human skeletal remains.
- Identification of microbial growth after death.
- Investigation of infectious agents in hospital acquired infections.[2]

Specimen Procurement for Postmortem Microbiology

Precautions to be taken in each autopsy are as follows[10]:

- Body should be placed in sealed body bag at 4°C as soon as possible until autopsy starts.
- Conducted within 24 hours following death, microbiological sample taken as soon as possible.
- Skin disinfected with 0.05% chlorhexidine with 0.5% cetrimonium bromide in water.
- Organ surfaces seared with red-hot spatula or soldering iron before sampling.
- Blood samples, body fluids, and NP exudates best collected at beginning.
- Tissue specimens obtained prior to evisceration.
- Tubes with heparin or oxalate or fluoride avoided, toxic to many microorganisms.
- Exudate collected using a syringe or flocked swab.
- Retrieved samples should arrive within 2 hours when stored at room temperature and within 48 hours when stored at 2 to 8°C in adequate transport media.

Recommendations for specimens to be collected are[10]:

- A total of 3 to 5 mL blood/fluid is to be collected from interior of heart/other organs by searing surface with hot spatula and withdrawing through syringe/pipette. Preferential order of places to take blood in situ: peripheral femoral → subclavian → carotid → jugular → left ventricle.
- More than 1 mL of CSF by lumbar puncture/cisternal puncture as in antemortem collection, indicated in sudden infant death.
- More than 1 to 2 cm^2 solid tissues to be cut with sterile scalpel/scissor through seared surface.
- Vegetations on cardiac valves cultured by picking small portion with sterile forceps.
- If heart opened aseptically vegetations to be washed in several batches of sterile saline, grinded, and plated immediately.
- Swabs if indicated, should be two in number.
- Smears to be prepared from all lesions subjected to culture.

Interpretation of Positive Bacterial Cultures

Positive bacterial cultures may be interpreted as one of the following[10]:

- Indicative of true infection.
- Culture contamination.
- Postmortem transmigration of bacteria.
- Agonal spread of bacteria.

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A typical pathogenic organism isolated in monomicrobial culture is likely to be a true pathogen. Polymicrobial growth is suggestive of contamination.

Postmortem transmigration of bacteria was first described by Gradwohl in 1904.[11] Bacteria migrate from mucosal surfaces and tissues into bloodstream after circulation has ceased. Reviews of clinical autopsy cases indicate:

- Blood culture positivity rates increased from 20 to 40% in correlation with length of postmortem interval.[12]
- Postmortem lung culture positivity correlates with increased length of hospital stay and postmortem interval.[12]
- Transmigration of bacteria through intact intestinal walls in humans occurs in 12 to 15 hours.[13]
- Comparison of conventional culture with real time polymerase chain reaction (PCR) for evaluation of transmigration is suggestive of:
  - Transmigration from intestine to blood, liver, portal vein, mesenteric lymph nodes, and pericardium,
  - Skin/oral cavity/respiratory tract also potential sources of transmigration,
  - Detection rates better by PCR.
  - Blood cultures unreliable (high rate of contamination/ polymicrobial/enteric organisms)
  - Liver/pericardium better samples (higher/stable rates of sterility)
  - Relative amounts of intestinal bacterial DNA (***Bifidobacteria***, *Bacteroides*, *Enterobacter*, *Clostridia*) increased with time.

The concept of agonal spread of bacteria, given by Fredette in 1916, states invasion of bacteria into the bloodstream when systemic circulation is artificially maintained during resuscitation efforts or is dropping during the agonal period.[14] On the basis of the relationship between antemortem and postmortem cultures, this has continued to be a contentious issue and has shed light on the potential existence of "terminal sepsis."

When compared with agonal spread, which has little support in the literature and is a theoretical idea, postmortem transmigration of bacteria is widely accepted by pathologists and microbiologists.

Importance of Postmortem Virology Including Severe Acute Respiratory Syndrome Coronavirus 2

In certain viral infections postmortem analysis is important:

- Postmortem testing in certain circumstances maybe the only option to confirm a diagnosis of novel influenza A virus infection. For immunohistochemistry staining, a minimum of eight blocks and preserved tissue specimens from pulmonary locations are advised. For culture and molecular analyses, fresh or frozen samples can be used as recommend by the CDC.[15]
- The World Health Organization advises using the fluorescent antibody technique to examine the impressions or smears of tissue samples taken from the brain stem and Ammon's horn.[16]
- For suspected Ebola virus infections, oral swabs can be collected following all standard safety precautions and placed in viral transport medium, along with postmortem tissue samples (liver, spleen, bone marrow, kidney, lung, and skin snips) and handled in Biosafety Level-4 laboratories.
- Siliguri saw an acute encephalitis outbreak between January 31 and February 23, 2001. A group of scientists from four prominent institutions collected necropsy samples for investigations. The results were later approved by the CDC.[17]

Severe Acute Respiratory Syndrome Coronavirus 2 (Indian Council of Medical Research)

Precautions for Packing and Transport of Body to the Mortuary

For COVID-19 positive or suspected instances, the Ministry of Health and Family Welfare of the Government of India has published instructions on dead body handling. At the mortuary, negative pressure must be maintained. Dissection of a body cavity should only be done one at a time. Use the proper recommended practices to reduce the production of aerosols during autopsies, particularly when handling lung tissue.[18,19]

Prior to transferring a cadaver to the mortuary, the NP swab for reverse transcription-PCR is advised by the ICMR in all suspect cases. All catheters, drains, and tubes should be taken out, and any wounds or holes created by their removal should be disinfected with 1% sodium hypochlorite solution (Hypo) and application of impermeable tape.

According to the Biomedical Waste Disposal recommendations, any associated sharp objects should be disposed of in sharp containers. The nasal and oral orifices should be sealed, and the body should be double-packed in a clear, leakproof body bag. Hypochlorite is to be used to clean the body bag's outside. Together with other information about the patient, the COVID status of the patient should be prominently displayed on the label. The health care worker moving the body to the mortuary should be outfitted in appropriate personal protective equipment (PPE).

Before and after the transfer, the trolley should be cleaned with hypochlorite solution. It is recommended that the cold chamber used to store bodies be divided into sections specifically designated for COVID-19 and non-COVID-19 bodies. With COVID-19 patient bodies, lower spaces should be chosen to prevent spilling of bodily fluids while handling the body. After touching a COVID-19-infected body, all surfaces, including high-risk touch sites, must be decontaminated with hypochlorite.

Considering the need for strict biosafety procedures, no forensic science or virology laboratory in India has been designated for the examination of specimens from COVID-19 infected patients. Nonetheless, the CDC has provided guidelines for completing autopsies in COVID suspect cases.

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Table 1 Specimens to be collected during postmortem in suspect/confirmed coronavirus disease 2019 patients

Covid status	Autopsy	Specimen to be collected
Suspect	Yes	PM swab for COVID: •URT: NP swab •LRT: Lung swab from each lung
		Separate swab specimens for testing other respiratory pathogens
		Formalin-fixed autopsy tissues from lungs, upper airway, and major organs
Suspect	No	NP swab for COVID Separate NP swab for other respiratory pathogens
Confirmed	Yes	NP swab specimens for testing of other respiratory pathogens •Other postmortem microbiologic and infectious disease testing, as indicated •Formalin-fixed autopsy tissues from lung, upper airway, and other major organs

Abbreviations: COVID, coronavirus disease; NP, nasopharyngeal; PM, post mortem ; URT, upper respiratory tract; LRT, lower respiratory tract.

►**Table 1** summarizes the specimens to be collected during postmortem in suspect/confirmed COVID-19 patients.[20] ►**Table 2** outlines the CDC recommendations for engineering control and PPE.[20] ►**Table 3** outlines the CDC guidelines for specimen collection.[20]

Swabs should be collected and put in a transport medium. To increase test sensitivity and reduce the consumption of testing resources, it is best to combine both NP and oropharyngeal swabs when they are collected in a single tube.

In addition to basic bacterial cultures, toxicological tests and other studies as necessary should be guided by the decedent's clinical and exposure history, scene investigation, and gross autopsy results.

Samples from these tissues should be taken if the clinical history or laboratory results acquired before to death indicate the involvement of other organs.

For the best fixation, it is advised to collect tissue samples that are around 5 mm thick and placed in 10% buffered formalin at 10 times greater than the volume of tissue.

Another option is to send original blocks of formalin-fixed, paraffin-embedded tissues from autopsies for examination.

Table 2 Engineering control and personal protective equipment recommendations (Centers for Disease Control and Prevention) for autopsy in suspect coronavirus disease 2019 patients

Specimen to be collected	Engineering control recommendations	PPE recommendations
Postmortem, NP swab only	A negative pressure room is not required	• Nonsterile, nitrile gloves. • Heavy-duty gloves over the nitrile gloves if there is a risk of cuts, puncture wounds, or other injuries that break the skin, • Clean, long-sleeved fluid-resistant or impermeable gown • 4. Plastic face shield or a face mask and goggles
Autopsy (COVID suspect/positive)	Conducted in airborne infection isolation rooms (AIIRs) • Are at negative pressure to surrounding areas • Have minimum of 6 air changes per hour (ACH) for existing structures and 12 ACH for renovated or new structures • Have air exhausted directly outside or through a high-efficiency particulate aerosol (HEPA) filter Doors should be kept closed except during entry and egress. If AIIR is not available, ensure the room is negative pressure with no air recirculation to adjacent spaces. A portable HEPA recirculation unit could also be placed in the room to provide further reduction in aerosols. Local airflow control (i.e., laminar flow systems) can be used to direct aerosols away from personnel. If use of an AIIR or HEPA unit is not possible, the procedure should be performed in the most protective environment possible. AIIR room air should never be recirculated in the building, but directly exhausted outdoors, away from windows, doors, areas of human traffic or gathering spaces, and from other building air intake systems	• Double surgical gloves interposed with a layer of cut-proof synthetic mesh gloves • Fluid-resistant or impermeable isolation gown • Waterproof apron • Goggles or face shield • NIOSH-certified disposable N-95 respirator or higher

Abbreviations: COVID, coronavirus disease; NIOSH, National Institute for Occupational Safety and Health; PPE, personal protective equipment.

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Table 3 Centers for Disease Control and Prevention guidelines for specimen collection in autopsy of suspect coronavirus disease 2019 patients

Specimen	Alternate specimen	Steps of collection	Storage
Nasopharyngeal (NP) swab	Oropharyngeal (OP) specimen nasal midturbinate (NMT) swab anterior nares (nasal swab; NS) specimenNP wash/aspirate or nasal aspirate (NA) specimen	Insert flexible wire shaft minitip swab through the nares parallel to the palate (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx. Swab should reach depth equal to distance from nostrils to outer opening of the ear. Gently rub and roll the swab. Leave swab in place for several seconds to absorb secretions. Slowly remove swab while rotating it. For NS, a single polyester swab with a plastic shaft should be used to sample both nares	2–8°C for up to 72 h after collection. If a delay in testing or shipping is expected, store specimens at −70°C or below
Lung swab		Collect one swab from each lung (left and right) by one of the two methods: During internal exam, after heart–lung block is removed, insert one swab as far down into the tracheobronchial tree as possible on either side (left and right) or first wipe the surface of each lung with an iodine-containing disinfectant clean and dry the surface, then use a sterile scalpel to cut a slit of the lung and insert the swab to collect sample on either side	2–8°C for up to 72 h after collection. If a delay in testing or shipping is expected, store specimens at −70°C or below
Autopsy tissue specimens		• A minimum of three representative sections of lung parenchyma, preferably from different locations • A minimum of two sections of airway, to include trachea, bronchi, or both airways	

Recently, a potential technique in forensic microbiology has been described: the use of the V4 hypervariable region of bacterial 16S rRNA gene sequences for taxonomic classification ("barcoding") and phylogenetic analysis of human postmortem microbiota.[21]

Microbiology in Minimally Invasive Autopsy

In nations where the body cavities are not accessed, minimally invasive autopsy (MIA) is an alternative to traditional autopsy. Needles are used to access the primary organs via the percutaneous route. The history leading up to the death and a thorough external examination are combined with the findings. It is believed that using needle aspirations and biopsies alongside multislice computed tomography or magnetic resonance imaging scans will boost the sensitivity of MIA.[22] In environments with limited resources, MIA can be used to determine the cause of death in order to improve public health in general. The MIA protocol calls for taking postmortem biopsies from important organs and doing in-depth histopathological and microbiological analyses. Blood, CSF, liver, brain, lungs, heart, spleen, kidneys, bone marrow, and uterus from women of childbearing age are among the specimens gathered.[23–25] Histopathological hints guide the selection of the necessary microbiological testing. Testing for HIV, HBV, HCV, malaria, and bacteriological and mycological culture are a few of the investigations conducted. The use of molecular methods is also an option depending on the circumstances. This method has been shown very effective at several institutions and is believed to be able to pinpoint an infectious cause of death in about 84% of cases.[22]

Underutilization of Postmortem Microbiology

Full diagnostic autopsies are rarely performed when they are not legally necessary, despite the fact that they might reveal crucial information concerning the cause of death. Postmortem microbiology thus continues to be a subject that is neglected, primarily due to issues with religious and local cultural beliefs, technical limitations, a lack of skilled labor, and infrastructure.[26,27] Postmortem cultures and MIB are extremely important in everyday settings but are underutilized as a result of doctors' lack of understanding of growing medicolegal concerns and clarity about consent. Organ retention, poor communication, and a lack of comprehension were some other problems related the inability to get consent that were brought to light by a review of the factors influencing the uptake of postmortem examination in the pediatric population.[26] This can be overcome by utilizing more modern methods, like MIA, which are more palatable to the staff, patients, and bereaved family members.

The specific purposes for which postmortem microbiology is used can explain its usefulness.

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The following are examples of its significance:

- Undiagnosed infection—A recent German case report detailed the detection of multidrug-resistant *Klebsiella pneumoniae* in a patient with sepsis superimposed on SLE (Systemic Lupus Erythematosis). This was not diagnosed antemortem, despite blood culture being collected 4 to 5 times.[28]
- New pathogen—A 16-year-old healthy child developed spontaneous nontraumatic lethal myonecrosis, which was caused by a new bacterium called *Clostridium fallax*.[29]
- Novel presentation—In Canada, a novel case of herpes simplex virus hepatitis that killed an immunocompetent man was reported.[30]
- A 53-year-old patient in Romania lost his life after having intestinal TB that was mistakenly identified as Crohn's disease.[31]
- Isolation of *Candida albicans* from heart blood at autopsy indicated disseminated disease.[32]
- *Exserohilum* in steroid solutions was discovered, which caused a fatal neurological illness outbreak in the United States.[33]
- Detection of coinfections with increased mortality in COVID-19. These include secondary disseminated mucormycosis in nine patients between March 1 and April 30, 2020.[34]
- A 58-year-old lady with flu-like symptoms with COVID-19 in her blood was found dead.[35]
- A pilot project was started in a tertiary care hospital in Delhi to find out about the perception of health care professionals regarding the role MIA played in identifying the causes of neonatal deaths.[36]

Medicolegal Aspects of Forensic Microbiology

Forensic microbiology still remains an underutilized entity in this era of modern medicine. It has a huge potential to be utilized for the purpose of administration of justice because of its medicolegal ramifications. The applied aspects of postmortem microbiology are immensely diversified including the estimation of postmortem interval, dwelling into the cause of death, especially in cases of sudden natural deaths, drowning, identification of emergent pathogens, novel presentation of known pathogens, biocrimes involving humans, agroterrorism, cases of sexual assault, medical malpractice, nosocomial infection control, estimation of antibiotic drug resistance, food safety, and environmental contamination, and investigation of food borne illnesses, etc.

Identifying the etiological agent as the origin of an infection that had previously gone undetected and validating the antemortem diagnosis are the two main conceptual justifications for obtaining postmortem blood and/or tissue cultures. In some cases, an infection may only be identified during the postmortem examination, as in the case of endocarditis and heart valve vegetations. Blood cultures acquired at the start of the autopsy may be extremely helpful in these situations to pinpoint the precise origin of the infection since cultures of the real tissue are inappropriate due to contamination concerns. In other instances, postmortem blood and tissue cultures may offer additional vital details on the extent and severity of a previously suspected or diagnosed antemortem infectious disease but caused the patient's condition to deteriorate rapidly and lead to death. However, in rare instances, postmortem cultures, particularly, blood cultures and spleen cultures, may be useful to determine the etiology of a fulminant infectious disease process when the patient's death occurred prior to obtaining adequate antemortem cultures. Lastly, the efficacy of antimicrobial therapy could be determined by the results from postmortem cultures. Differentiating between true-positive culture results and postmortem transmigration and/or contamination remains a major challenge to microbiologists and pathologists. Moreover, monomicrobial development of a typical opportunistic and/or pathogenic microbe seen in postmortem blood or tissue cultures seems to be a reliable sign of infection. However, it appears more likely that polymicrobial growth and/or the presence of typical contaminant organisms, like mixed intestinal flora and coagulase-negative Staphylococci, are the result of iatrogenic contamination during the collection of the specimen or because of postmortem bacterial transmigration.[10]

Conclusions

Postmortem cultures are indicated in a limited number of scenarios. It is of utmost important to safeguard cultures from contamination by following aseptic techniques. Cultures are spleen and heart blood are suggested as the best autopsy specimens for bacteriology. Whenever terminal infection is suspected, sample collection from at least two if not more different sited should be the standard practice as it increases the possibility to detect the causative agent of antemortem infection. Isolation of a single common organism is more suggestive of a true infection as compared with contamination in cases of rare polymicrobial isolation. Postmortem culture results must be interpreted along with clinical, other laboratory, and pathological findings to assess its actual weightage. Bacteriological examinations performed after death can be viewed as a consistent investigative device for postmortem quality check of the diagnostic and therapeutic procedures done antemortem and serve as an added supplement to health care–associated infections within a specific hospital environment. The recent pandemic of SARS-CoV-2 highlights the role postmortem microbiology will play in describing etiology and confirming the cause in SD. Techniques like MIA are promising tools to supplement application of this specialty.

Authors' Contribution
R.S.: Analyzed the data and revised the manuscript critically for important intellectual content (microbiology), final approval. S.K.: Analyzed the data and drafted the manuscript. A.K.: Revised the manuscript critically for important intellectual content (forensic medicine) and final approval. S.C.: Data collection and analysis of the data. All authors critically revised the manuscript,

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approved the final version to be published, and agree to be accountable for all aspects of the work.

Conflict of Interest
None declared.

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Molecular Genetic Analysis of Mycobacteria, Causing Female Genital Tuberculosis: Possibilities of Sexual Transmission—An Overview

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Abstract

Although tuberculosis (TB) is predominantly known to be a traditional air-borne disease, new modes of transmission have also come to light. While the lungs remain the main entry point, TB can spread to other regions of the body causing extrapulmonary tuberculosis (EPTB). Female genital tuberculosis is one such EPTB that can adversely affect females between the ages of 15 and 45 years and may cause hindrance in their ability of conception and successful pregnancy. Sexual transmission of TB is a lesser-known or poorly investigated route of spread that has recently been confirmed through molecular evidence. Targeted molecular-level studies by polymerase chain reaction (PCR), in addition to interim diagnostic techniques, have offered evidence for the sexual transmission of *Mycobacterium* subtypes. Recent studies conducted using multiplex PCR on both the male and female counterparts revealed that the male partners had *Mycobacterium* in their semen, while the female counterparts had it in their endometrium and products of conception resulting in miscarriage. These studies indicate that the mycobacterial infection/infestation in the females may have been brought on by contact with infected male semen. Therefore, it is necessary to identify the genetic loci that are responsible for the sexual transmission of mycobacteria. This can be done by whole-genome sequencing. It has also to be emphasized that screening of sexually active males for genital TB in endemic regions is necessary for the prevention of sexual transmission of mycobacteria.

Keywords

- ► mycobacteria
- ► genital tuberculosis
- ► infertility
- ► multiplex-PCR
- ► sexual transmission
- ► genome sequencing

Introduction

Tuberculosis (TB) is one of the aerosol-borne infections caused mainly due to *Mycobacterium tuberculosis* (MTB). During the past few centuries, dissemination of MTB was established as extrapulmonary tuberculosis (EPTB). Genital TB (GTB) is one of forms of EPTB.[1] It primarily reaches the genital areas via dissemination from foci outside the genitalia with the lungs being the original seeding site.[2] Males are

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affected with GTB mainly at a reproductive age between 29 and 32 years.[3] The semen (seminal fluid), in contact with the infected scrotal parts, gets infected and carries the nonmotile MTB along with it. Epididymis (45%) and prostate (22–49%) get mostly infected, whereas the chances of testes infection are comparatively much less (3%).[4] In the case of females, a high risk of infertility may result in recurrent miscarriage, ectopic pregnancies, and menstrual irregularities. MTB often damages the fallopian tube (90–100%), endometrium (50–60%), ovary (20–30%), cervix (5–15%), and vagina (1%). The disease is most prevalent between 15 and 45 years.[5] Infertility in females may be due to sexual transmission of MTB from an affected male through infected semen. The theoretical possibilities of sexual transmission of MTB from one mucosal surface to another have been described in animal models during intercourse.[6] But the sexual transfer of TB and nontuberculous mycobacteria (NTM) in humans has rarely been found and only anecdotally reported.[7] Here, we are going to review the possibilities of sexual transmission of both MTB and NTM from male to female.

Epidemiology

GTB is serious clinical condition that can often be asymptomatic. It can mostly go unnoticed or masquerade as other gynecological conditions.[8] Asia Pacific region has 58% of the global burden of TB.[9] EPTB accounts for 27% of TB cases worldwide, of which 9% is GTB. The global distribution of GTB varies according to geographical location: 15 to 20% in Asia, Africa, Eastern Europe and the Russian Federation; 2 to 10% in the United States and Western Europe.[10] Recent studies have shown an incidence of 3 to 16% female genital tuberculosis (FGTB) in Indian patients registered for infertility. Further studies with women inscribed for in vitro fertilization from North India reported the prevalence of FGTB in 48.5% of tubal factor infertility cases.[11] Indian Council of Medical Research has reported that the prevalence of FGTB in India has amplified from 19% of EPTB in 2011 to 30% in 2015.[12]

Diagnosis

Highly sensitive diagnostic modalities, such as polymerase chain reaction (PCR), molecular typing, enzyme-linked immunosorbent assay (ELISA), and whole-genome sequencing (WGS), are performed to detect mycobacterial DNA in male partners of female patients with suspected TB. PCR is a rapid, sensitive, and specific molecular method that targets various gene segments in the MTB DNA.[13] However, this technique has chances of giving false-positive reports.[14] Multiplex PCR can be performed on various TB samples using more than two primer pairs. This results in a highly specific and sensitive diagnosis of FGTB than the other conventional PCR technique.[15,16]

Molecular typing is one of the powerful tools used for identifying specific mycobacteria strains.[17] The identification and presence of the organism are confirmed by restriction fragment length polymorphism that is based on the insertion element.[18,19]

ELISA has shown to be significantly effective in detecting GTB compared to laparoscopy. Even in cases where the culture is found to be negative, ELISA can successfully detect the sensitivity of antimycobacterial antibodies immunoglobulin M and immunoglobulin G.[20] However, at present, it is not considered specific and sensitive. Hence, ELISA was banned by the World Health Organization and Government of India for diagnosis of GTB.[21] A summary of methods associated with detection of FGTB is illustrated in ►**Fig. 1**.

GTB is known to be one of the causes of female infertility with an incidence rate of 44-74% worldwide with more than 50% of cases reported in India. Due to the asymptomatic nature of latent genital tuberculosis, it often gets undiagnosed and/or misdiagnosed causing hindrance in the prediction of the accurate prevalence of the condition.[22] Because of its asymptomatic nature, it may mimic or coexist with other gynecological and abdominal disorders, making its diagnosis difficult.[12] In cases of fertility issues, the presence of tubercular bacilli in the genital tract is a critical factor because the endometrium becomes nonreceptive, restricting any implantation or rejection of implanted embryos in the initial months, resulting in recurrent pregnancy loss.[23] Several studies have shown that women who have TB are at greater risk of facing adverse pregnancy outcomes such as stillbirth, low-birth weight, and premature birth, than women who are not suffering from TB.[24,25] In clinical laboratories, traditionally, MTB is diagnosed either by acid-fast staining or can be grown on a Lowenstein–Jensen (LJ medium). But both cases have a set of disadvantages. In the first case, the microscopic observation of acid-fast bacilli is sensitive and requires a minimum of 10,000 organisms ml^{-1} in the sample. However, the growth on the LJ medium takes up to a week to develop.[26] Moreover, a considerable number of lesions found in the genital tract are seen to be bacteriologically mute. Owing to the drawbacks of the mycobacteriological and histopathological tests, their use in the diagnosis of GTB is restricted.[13] For analyzing and identifying both pulmonary TB (PTB) and EPTB, PCR-based studies have shown improved sensitivity, speed, and specificity. According to a blind study conducted by Bhanu et al on 61 female patients, use of amplification of the mpt64 gene segment demonstrated increased sensitivity in the diagnosis of FGTB.[13] Another infertility study related to FGTB has shown that even though hysterosalpingography and laparoscopy are used for the diagnosis of FGTB, a more molecular level technique such as PCR yields higher rates of specificity and sensitivity.[22]

Known Transmission Routes and Evidence of Sexual Transfer

From the early times of human civilization, TB was considered an exclusively air-borne pathogenic pulmonary infection. The lung is the main entry point of the disease; TB can also spread to other organs like the brain and bone, causing EPTB.[27] Lübeck disaster that occurred around 1929 to 1930 in Germany was one such example that pointed toward the transmission of *Mycobacterium* through ingestion. Among

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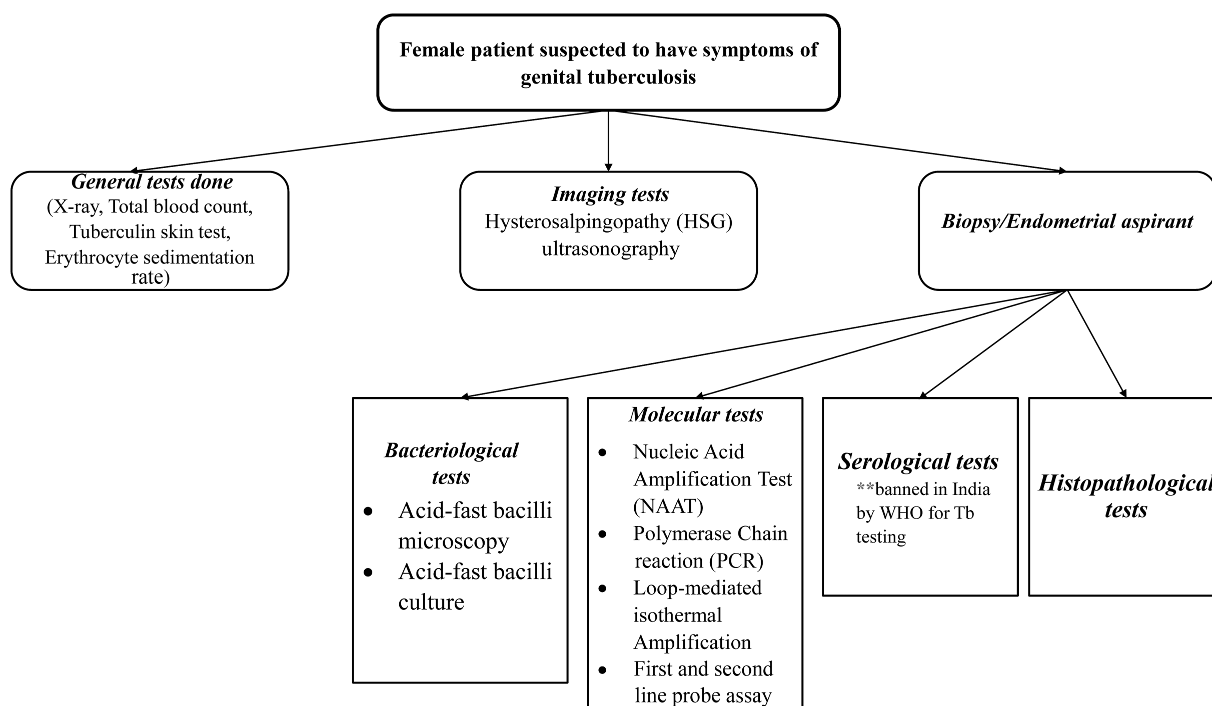

Fig. 1 Detection of female genital tuberculosis.

the 251 neonates who were given the oral Bacillus Calmette–Guérin vaccine contaminated with various inoculum of the virulent MTB Kiel strain, 90% developed clinical TB, whereas 13% developed PTB and 28.7% succumbed to death.[28] The lesser-known mode of transmission via the sexual route was not a popular hypothesis until meaningful molecular evidence was found in early 2000.[7,18] This was because usual pathogens that cause sexually transmitted diseases (STDs) are known to have the characteristic of being "fast and loose." They can usually change the surface antigen thus using this as a strategy to dodge the host immune response. They are also fast growing and hence can generate altered or mutated populations rapidly. This helps in colonization of the lower genital niches and subsequent upward migration.

Mycobacteria are the archetype of "slow and steady" and cannot change the surface quickly. Their genomes are relatively inert and, on the whole, they are slow growing. These properties do not fit well with the general characteristics of the STD-causing bacteria that colonize the lower niches of the genitalia.

Angus et al in 2001 observed that the MTB subtypes found in the female with endometrial TB were identical to the isolates found from the skin ulcers on the penis of the infected male counterpart.[18] In the study conducted by Kimura et al in 2018, it was discovered that organisms in both the male and female isolates were genotypically identical in one of the couples.[29] The study further demonstrated through WGS that the isolates from both the individuals had only one base pair difference. What was observed from both studies was that, the male counterpart was initially affected with TB. Then, after a period of time, the female was also infected. This strengthened the idea that the transmission was from male to female.

In 2021, a cohort study was done in mainland China with a total of 3,668,004 women along with their male partners. This study observed that women with partners who have TB were 2.13 times more likely to give stillbirth than women whose partners were not affected, making TB one of the risk factors causing stillbirth in pregnancy.[30]

A multiplex PCR-based study carried out by Datta et al in 2022 at the Calcutta Fertility Mission reported 165 couples with primary and secondary infertility between 2019 and 2020.[31] In this study design, the couples with known causes of infertility such as tubal factors (22 pairs), endometriosis (17), Polycystic Ovarian Disease (14), Pelvic Inflammatory Disease (11), Hormonal imbalance (10), history of pulmonary or EPTB (6), history of Azoospermia (2), and local bacterial infection in genitalia (2) were screened and excluded. The remaining 81 sexually active couples with unknown causes of infertility, normal functioning fallopian tube and hormonal profile, and absence of any local bacterial infection were included. All the couples were screened for mycobacterial DNA using the multiplex PCR. The couples were then segregated into group A (40), B (20), and C (21). In groups A and B, both the partners tested positive for GTB but were asymptomatic. However, group C was assigned as the negative control since all the couples in this group were tested negative for GTB. The main difference between the groups A and B was that, in group B, the female partner's endometrium was tested positive for GTB. On the contrary, in group A, the product of conception tested positive for GTB after miscarriage, but their endometria were tested negative. It was further observed that the couples were

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tested positive for three combinations of mycobacteria: two strains of MTB and one NTM strain. Whichever strain(s) were found in the male partner, they have shown a transfer rate of 100% to his female counterpart. Thus, for the first-time, a cohort-based reporting was done on large-scale sexual transmission of mycobacteria. A schematic diagram of sexual transfer of mycobacteria is illustrated in ►**Fig. 2**. This study also reported that the sperm quality or motility was not a factor in the failure of pregnancy.[31]

Conclusion

Several studies have offered substantial evidence for the likelihood of sexual transmission of TB over the years, but it is still an area that needs further exploration. Understanding the pathogenesis of FGTB is critical for establishing a link between female infertility and the transfer of *Mycobacterium* from male semen to female via sexual transmission. 16s rDNA sequencing, followed by Genome Wide Association

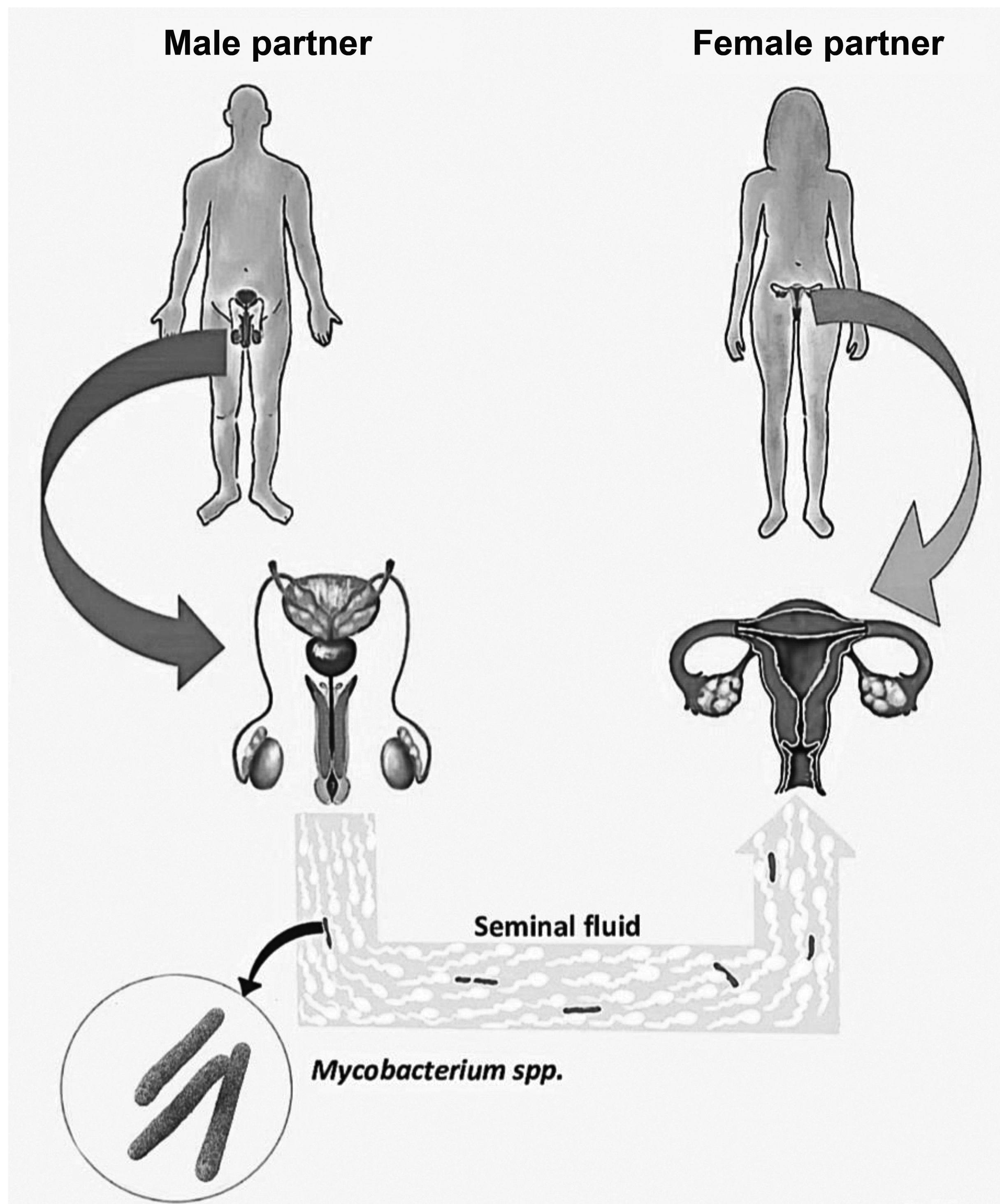

Fig. 2 Schematic illustration for sexual transmission of *Mycobacterial spp.*

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Studies of mycobacteria involved in FGTB, is needed to successfully identify the genetic determinants that are responsible for its sexual transfer. It also will reveal genetic factors behind lower genital colonization in women. Further, this will lead to the discovery of MTB/NTM types that are sexually transferred. Since GTB in male can often be asymptomatic, multiplex PCR using the semen samples can provide a tool for identifying the risk of infertility. Additionally, a public health clause for screening of sex partners in countries with a high incidence of TB can also be suggested.

Authors' Contribution

S.C., S.K., and S.G. were involved in manuscript writing, main draft and revised draft. P.G. contributed to manuscript planning, remodeling, and revision. P.S., S.N., A.D., and S.C. helped in manuscript writing, main draft. A.B. was involved in manuscript planning, writing, and revision; main draft and revised draft.

Conflict of Interest

None declared.

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Cystatin A Down-regulation in Head and Neck Squamous Cell Carcinoma Cell Lines Decreases Cancer Hallmark Signatures

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Abstract

Background Cystatin A (CSTA), an endogenous inhibitor of lysosomal cysteine protease, is expressed primarily in epithelial tissues. The expression of CSTA was found to be dysregulated in various cancers and associated with cancer pathogenesis, but its role is reported to be contradictory. Our previous preliminary study found CSTA to be upregulated in the saliva and tissues of patients with head and neck squamous cell carcinoma (HNSCC). In this current study, we have explored the role of CSTA in the pathophysiology of HNSCC.

Methods First, we confirmed the upregulation of CSTA in CAL 27 ($p = 0.0242$) and FaDu ($p = 0.0014$), two HNSCC cell lines, compared to the normal gingival epithelium. CSTA was then stably knocked down in CAL 27 and FaDu using the lentiviral short hairpin RNA pLKO vector transduction to study the effects of CSTA knockdown on various cancer hallmarks such as cell proliferation ability, invasion, migration, colony formation, and chemotherapy-induced apoptosis.

Results CSTA knockdown significantly decreased cell viability, cell migration, trans-well invasion, and colony formation in both cell lines. CSTA downregulation also enhanced cisplatin-induced apoptosis.

Conclusion Overall, this study suggests the protumorigenic role of CSTA in HNSCC.

Keywords
- cystatin A
- HNSCC
- shRNA pLKO vector transduction
- cancer hallmarks

Introduction

Head and neck squamous cell carcinoma (HNSCC) arises from the buccal cavity and upper aerodigestive tract carcinoma and is one of the most common cancers worldwide[1]. There have been significant advancements in diagnostic and treatment strategies for HNSCC in recent years, even though the 5-year survival rate remained abysmal.[2] This suggests that a better understanding of cancer cells' biological and molecular characteristics must be studied to design newer prevention and therapeutic strategies for HNSCC. Therefore, there is an urgent need to relook into the biological pathways/proteins

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dysregulated in a specific way that may cause the tumor progression in HNSCC.

The perturbation of various proteins due to the cancer cell's protective mechanisms or oncogenic changes in the genome and/or epigenome of the cancer cell is a known phenomenon in oncogenesis.[2] In both cases, detecting and quantifying specific proteins provide important information regarding the origin of oncogenic changes, disease staging, progression, and patient outcome. In our previous findings, various proteins in the saliva of HNSCC patients were found to be dysregulated using liquid chromatography-mass spectrometry analysis. We identified that levels of cystatin A (CSTA) were significantly raised in the saliva of HNSCC patients compared to controls (data not shown). We also found high cytoplasmic expression of CSTA in the tumor cells of HNSCC patients (►**Supplementary Material Fig. 1**, available in the online version).

In the literature, the contradictory role of CSTA has been reported in different normal tissues and cancers. CSTA belongs to type I cystatins, is a cytoplasmic inhibitor of cathepsins B, H, and L (cysteine proteases), and is primarily expressed in epithelial and lymphoid tissues.[3] CSTA is a cytoplasmic protein found in body fluids when upregulated.[4] In nasopharyngeal carcinoma, a high level of CSTA in patients' serum is related to poor prognosis, whereas prostate cancer with high CSTA expected to be less aggressive.[5,6] High extracellular CSTA levels in colorectal carcinoma were related to short patient survival, indicating CSTA's role in tumor progression.[7,8] There is a contradiction in the role of CSTA in HNSCC pathophysiology. In some studies, the level of CSTA was found to be downregulated, while in the human protein atlas, an enhanced level of CSTA has been reported in HNSCC.[9] In oesophagal squamous cell carcinoma, decreased levels of CSTA in tumor mucosa are associated with nodal involvement and overall bad prognosis.[10,11]

A few studies have explored CSTA's role in HNSCC pathophysiology. We hypothesized that CSTA might play a crucial role in HNSCC pathophysiology. Modulating CSTA in the HNSCC cell line enabled us to study its effect on cancer hallmarks like cell proliferation, apoptosis, migration, and invasion and generate preliminary information on its role in HNSCC pathophysiology.

Materials and Methods

Cell Lines

STR-matched cell lines—FaDu (ATCC HTB-43) and CAL 27 (ATCC CRL—2095) were used in this study. FaDu is a cell line of pharyngeal squamous cell carcinoma, while CAL 27 is a cell line of tongue squamous cell carcinoma. HEK 293T (ATCCCRL-3216) cell line was used to generate lentiviral particles.

Maintenance of Cell Line

The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) media (Lonza,12-604F) containing 10% fetal bovine serum (FBS) and 1X Antibiotic-Antimycotic (Gibco,15-240-062) in a humidified incubator with 5% CO_2 at 37°C. Passaging of cells was done once they reached approximately 60 to 80% confluency. The cell monolayer was given a wash with phosphate buffered saline (pH = 7.4) to take off the residual serum. Then, adherent cells were harvested with trypsin (0.25%) (Gibco, 25200056). The media was changed every 3rd day. All culture work was done strictly under aseptic conditions in the tissue culture hood with vertical laminar flow.

mRNA Expression Studies

For mRNA expression studies, total RNA was isolated using QIAzol Lysis Reagent (Qiagen,79306) and followed by synthesis of cDNA using a cDNA synthesis kit by Thermo Scientific (AB1453B). Quantitative polymerase chain reaction (qPCR) for CSTA was done using SYBR green chemistry in Bio-Rad CFX96 Real-Time qPCR System with *18S rRNA* as reference gene. The details of the primers and the amplification curves are given in ►**Supplementary Material Table 1** and ►**Supplementary Material Fig. 2** (available in the online version), respectively.

CSTA Short Hairpin RNA Expression Vector Construction and Stable Transduction by Lentivirus

The *CSTA* gene was knockdown by stably expressing a short hairpin RNA (shRNA) against the CSTA mRNA, using lentiviral transduction. A 3rd-generation lentiviral negative control vector containing scrambled shRNA from add gene was used as a control vector. shRNA oligos against CSTA cloned in pLKO.1 vector was taken from the shRNA library of BROAD INSTITUTE (https://portals.broadinstitute.org/gpp/public/dir?dirpath=shrna_annot/legacy) (►**Supplementary Material Table 2**, available in the online version), and scrambled shRNA was taken from David Sabatini (Addgene plasmid # 184; http://n2t.net/addgene:1864; RRID: Addgene_1864) (►**Supplementary Material Table 3**, available in the online version). Both of the plasmids were already transformed into One Shot TOP10 Competent E. coli cells (C4040-10). These E. coli cells were cultured, and the plasmid DNA was isolated using Promega PureYield Plasmid Miniprep System (A1223).

Generation of Lentiviral Particles

HEK-293T was cultured in 6-well plates for each plasmid to be transfected using Lipofectamine 3000 Transfection Reagent (L3000001). The cells were regularly passaged in DMEM with 10% FBS and 1X Antibiotic-Antimycotic.

For transfection, a mix was prepared using the following for each plasmid:

- shRNA plasmid = 500 ng (pLKO.1)
- Packaging plasmid = 375 ng (psPAX2)
- Envelope plasmid = 125 ng (pMD2.G)
- 3 µL of P3000 Reagent
- 125 µL serum-free OPTI-MEM

This mix was then added to the diluted Lipofectamine 3000 Reagent (following the manufacturer's protocol) and then incubated at room temperature for 30 minutes. This mix

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was added to 60 to 70% confluent HEK 293T cells cultured in DMEM with 5% FBS. After 12 to 16 hours, the media was replaced by complete DMEM with 10% FBS and 1X Antibiotic-Antimycotic—3 mL each well. The viral titers were collected at 24 and 36 hours and stored at −80 degrees until further use. These lentiviral particles were used for the transduction of cell lines.

Transduction of Cell Lines

The cell lines, FaDu and CAL 27, were cultured in 6-well plates using complete DMEM with 10% FBS and 1X Gibco Antibiotic-Antimycotic. Transduction was done at 70% confluency of cells. The lentiviral titer was diluted with DMEM media (1:1) without any antibiotic and had 8 µg/mL polybrene. This mixture (2 mL) was added to each one of the wells and then incubated for 24 hours. After 24 hours, a fresh complete DMEM with 10 µg/mL puromycin was added as the selected antibiotic. These cells were then continuously grown in the puromycin-containing media (10 µg/mL). First, we used four CSTA shRNA clones (namely C7, C9, C10, and C11) for transduction, but we proceeded with the C11 clone as this showed maximum CSTA gene knockdown analyzed by mRNA expression.

Effect of Cystatin A Downregulation on Cancer Hallmarks in Head and Neck Squamous Cell Carcinoma Cell Line(s)

Various assays were performed to analyze cancer hallmarks like proliferation, migration, apoptosis, invasion and anchorage-independent growth in CSTA shRNA expressing vector, scrambled control (mock-treated cell lines), and the cell line having no transduction (parent or untreated cell lines). All of the experiments were repeated at least three times.

Cell Viability Assay

Cell viability was assessed by using MTT assay. The assay was performed 36 hours after seeding the cells in 6-well plates (detailed methodology added in supplementary data, available in the online version).

Cell Death Assay (Annexin V/PI Apoptotic Death Assay)

FITC Annexin V/Dead cell Apoptosis Kit (Invitrogen, V13242) was used. The cells were first treated with an LD50 dose of cisplatin for 24 hours to analyze the effect of CSTA knockdown on cisplatin-induced apoptosis—a widely used anticancer drug. The LD50 for CAL 27 was 7.5 µM and for FaDu was 3.72 µM used.[12] After 24 hours of the drug treatment, cells were incubated with annexin V and propidium iodide dye according to the manufacturer's protocol, and tubes were immediately analyzed by flow cytometry.

Cell Migration Assay

Scratch assay was done to analyze the migration of cancer cells. A scratch was made using a 10-µL pipette tip in the form of a cross through the cell monolayer at 80 to 90% confluency. Pictures of the scratch were taken at various time intervals using a bright field microscope. The time point at which the scratch was made, denoted as T0, and pictures were taken after 48 hours of scratch making (T48) (details in supplementary data, available in the online version).

Soft Agar Colony-Forming Assay

To determine the anchorage-independent proliferative capacity of cancer cells, soft agar colony formation was assessed. For this, cells were cultured in the agar of 0.3%, and another agar of 0.5% was layered beneath to cells to prevent adhesion to the culture plate. 5×10^3 cells of each type were counted, seeded in the top agar layer, and incubated for 15 days. The colonies were stained by crystal violet and counted under a 10x resolution bright field light microscope.

Cell Invasion Assay

The assay was carried out using Transwell Corning Matrigel matrix in the Boyden chamber with Matrigel in the upper chamber with low FBS (5%) media and lower chamber with high FBS (10%) media. Cells were counted, and 1×10^3 were resuspended in the upper chamber. After 24 hours of incubation, cells that invaded the transwell were stained using crystal violet and enumerated under a bright field light microscope at 10x resolution.

Statistical analysis: The results were analyzed by using GraphPad Prism V9. The nonparametric version of one-way ANOVA, that is, the Kruskal–Wallis test was used among different groups. $p \leq 0.05$ is taken as the level of significance.

Results

CSTA was upregulated in HNSCC cell lines: The gene expression of CSTA in two HNSCC cell lines, CAL 27 and FaDu, was analyzed by real-time quantitative reverse transcription polymerase chain reaction. CSTA was significantly upregulated in both HNSCC cell lines, CAL 27 ($p = 0.0248^{*}$) and FaDu ($p = 0.0015^{**}$), in comparison to normal gingival epithelial tissue in ►**Fig. 1a**.

CSTA was stably downregulated in HNSCC cell lines: The two HNSCC cell lines, that is, CAL 27 and FaDu, were transduced by lentiviral particles having CSTA shRNA and scrambled shRNA expressing vector (scrambled act as mock-treated cell lines). Initially, there were four clones of CSTA shRNA expressing vector. Still, CSTA was significantly downregulated in the C11 clone in both of the cell lines, CAL 27 ($p = 0.0054^{**}$) and FaDu ($p = 0.0067^{**}$), in comparison to respected wild-type cell lines (►**Supplementary Material Fig. 3**, available in the online version). Also, there was no significant change in the gene expression level of CSTA in scrambled shRNA-treated cell lines in ►**Fig. 1b**. A similar downregulation of CSTA protein expression was also found by immunocytochemistry in the shRNA-treated cells (►**Supplementary Material Fig. 4**, available in the online version)

CSTA downregulation is associated with a decrease in cell viability: To analyze the knockdown effect of CSTA on cell viability, an MTT assay was performed in the two cell lines. It was found that in the cell lines (both in CAL 27 and FaDu) in which CSTA was stably knockdown, the cell viability was

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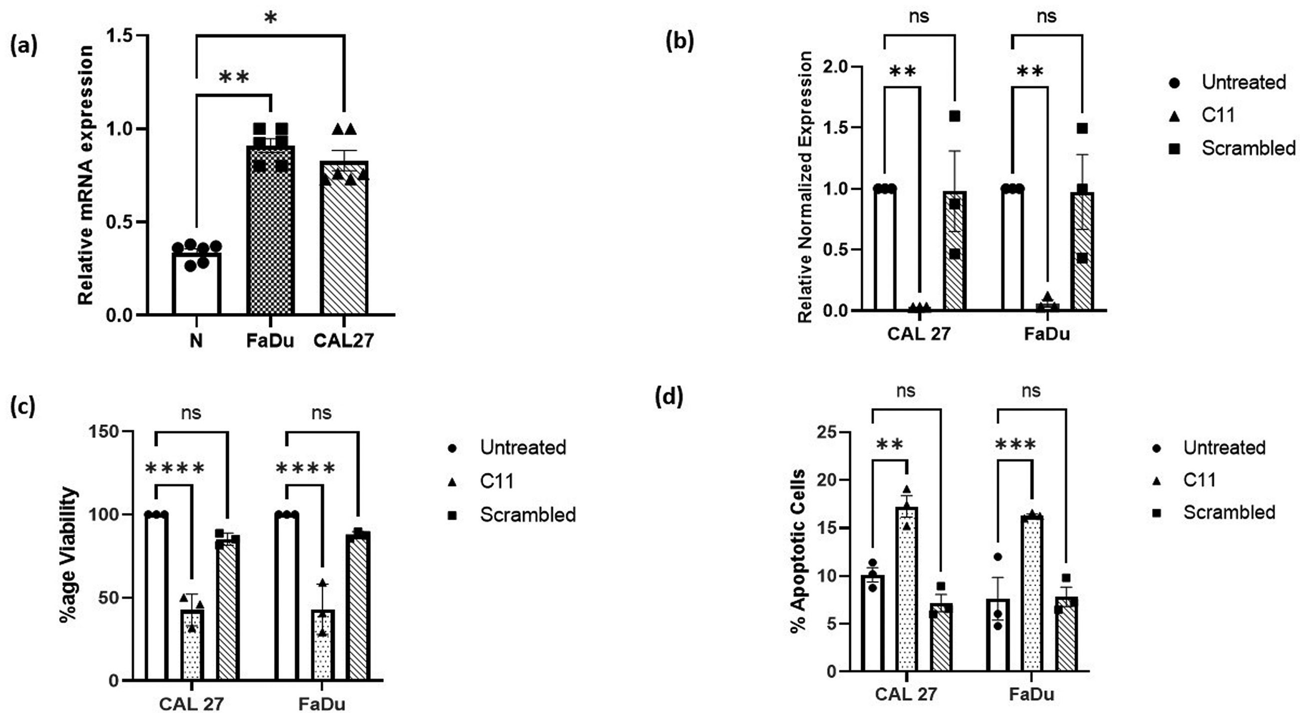

Fig. 1 (**a**) Cystatin A gene (CSTA) expression was analyzed by qRT-PCR. CSTA mRNA was significantly upregulated in CAL 27 ($p = 0.0248^{*}$) and in FaDu ($p = 0.0015^{**}$) cell lines as compared to normal gingival epithelium tissue (N). **Fig 1**(**b**) Cystatin A gene expression levels in CAL 27 and FaDu stable cell lines were analyzed by qRT-PCR. *CSTA* gene was significantly downregulated by C11 CSTA shRNA-expressing vector, both in CAL 27 ($p = 0.0054^{**}$) and FaDu ($p = 0.0067$ **) cell lines as compared to untreated cell lines. **Fig 1**(**c**) MTT assay revealed that the percentage viability of cells was significantly decreased both in C11 CAL 27 ($p < 0.0001^{****}$) and FaDu ($p < 0.0001^{****}$) in comparison with that of untreated. There was no significant change in the cell viability in case of scrambled cell lines. **Fig 1**(**d**) It was observed that the cisplatin-induced apoptosis was significantly increased in the CAL 27 ($p = 0.0025^{**}$) and FaDu ($p = 0.0005^{***}$) cell lines upon the knockdown of CSTA as compared to the untreated cell line. The induction of apoptosis in scrambled control was almost similar to that of untreated.

significantly reduced ($p < 0.0001^{****}$) as compared to untreated and scrambled shRNA-treated cell lines shown in ►**Fig. 1c**.

CSTA downregulation enhances cisplatin-induced apoptosis: Cisplatin is an important chemotherapeutic agent for treating HNSCC. To analyze the effect of CSTA downregulation on cisplatin-induced apoptosis, a flow cytometry-based annexin V/PI assay was performed. Cells treated with H_2O_2 were taken as a positive control of apoptosis. In both the cell lines CAL 27 and FaDu, in which CSTA was downregulated, the percentage of apoptotic cells was significantly increased ($p = 0.0025^{**}$ and $p = 0.0005^{***}$, respectively), as compared to the nontransduced (untreated) and scrambled shRNA-treated cells shown in ►**Fig. 1d**. This indicates that CSTA downregulation potentiates apoptosis induced by cisplatin.

Downregulation of CSTA is correlated with reduced cancer cell migration**:** Cell migration is one of the cancer hallmarks. To analyze the effect of CSTA downregulation on the migratory property of cancer cells, a monolayer wound healing/scratch assay was performed. The rate of scratch filling was observed at different time intervals. It was found that cells transduced with CSTA shRNA expressing vector spread much more slowly in comparison to the untreated group in CAL 27 ($p = 0.0002^{***}$) and FaDu ($p < 0.0001^{****}$) given in ►**Fig. 2a,b**.

CSTA knockdown significantly reduced transwell invasion of cancer cells: Boyden Chamber transwell invasion assay was done to analyze the impact of CSTA downregulation on cancer cell invasiveness. In this experiment, the invaded cells were quantified under a microscope, and the results were similar to the cell migration assay. It was found that the number of cells that have invaded was significantly reduced upon *CSTA* gene knockdown in CAL 27 ($p = 0.0491^{*}$) and FaDu ($p = 0.0001^{***}$) in comparison to the wild-type cell lines shown in ►**Fig. 2c, d**.

CSTA downregulation inhibits colony formation in HNSCC cell lines: To analyze the ability of transduced cells to form colonies in an anchorage-independent manner, colony-forming assay was done. The results revealed that the number of colonies established by the cells transduced with CSTA shRNA expressing vector was significantly low as compared with the parent both in Cal 27 ($p \leq 0.0013^{**}$) and FaDu ($p \leq 0.0003^{***}$) shown in ►**Fig. 2e,f**.

Discussion: HNSCC are the most common cancers globally. The 5-year survival rate in HNSCC patients is below 50% despite development in various cancer management strategies.[1] Therefore, it becomes crucial to understand the pathophysiology of HNSCC up to the molecular level.

Our group identified various dysregulated proteins in HNSCC patients' saliva using the liquid chromatography with tandem mass spectrometry approach. Among them,

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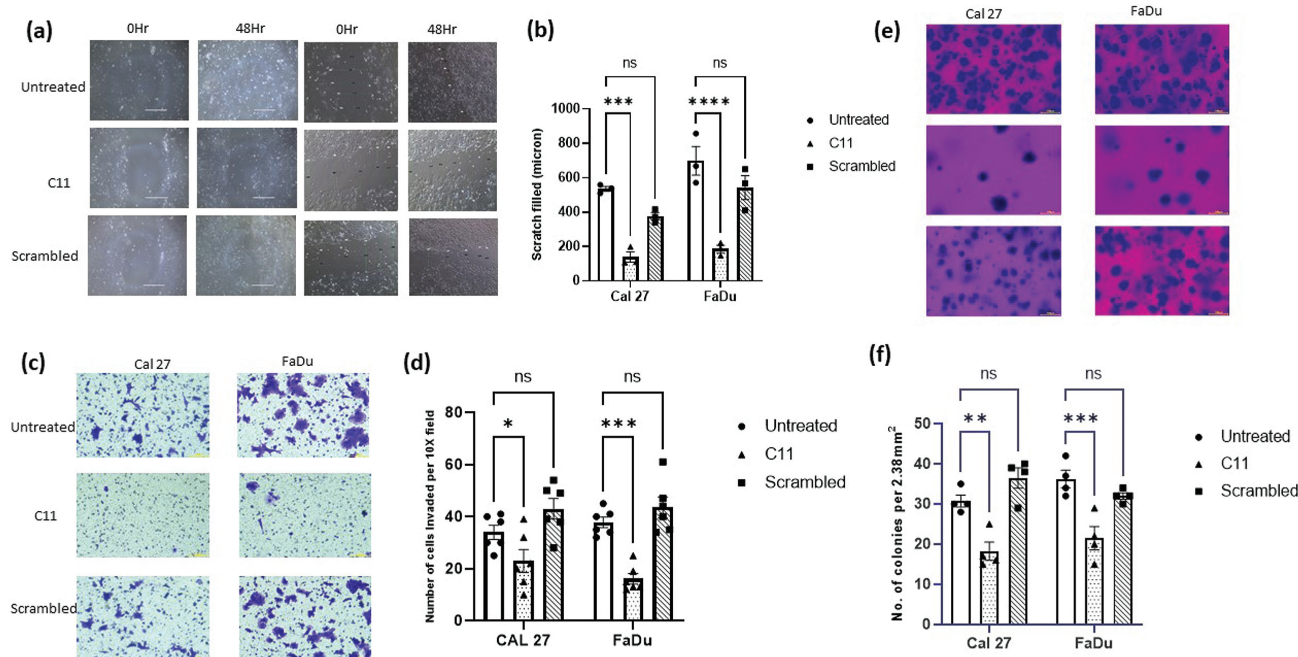

Fig. 2 (A) In wound healing/scratch assay, a scratch was made across the monolyer and images were take at 0 hour and at 48 hours after the scratch formation in the phase microscope under 10X resolution. (B) In monolayer wound healing/scratch assay, the rate of scratch filling was significantly reduced in CSTA knockdown CAL 27 ($p = 0.0002^{***}$) and FaDu ($p < 0.0001^{****}$) cell lines in comparison to the untreated group. (C) The invaded cells in the Boyden Chamber transwell invasion assay were quantified under a brightfield microscope at 10x resolution. (D) The effect of CSTA knockdown on invasion of cancer cells was analyzed by the Boyden Chamber transwell invasion assay. The invaded cells were quantified after 24 hours of seeding by microscope under 10X resolution. The number of invaded cells were significantly reduced upon CSTA gene knockdown in CAL 27 ($p = 0.0491^{*}$)and FaDu ($p = 0.0001^{***}$) as compared to the untreated cell lines. (E) Colony-forming assay, the cells were grown in an agar layer and incubated for 15 days. The number of colonies formed were stained by crystal violet and visualized under a bright field microscope. (F) In colony-forming assay, the number of colonies formed by the cells transduced with CSTA shRNA expressing vector was significantly low as compared with the both parent cell lines, Cal 27 ($p \leq 0.0013^{**}$) and FaDu ($p \leq 0.0003^{***}$).

CSTA was significantly elevated in the saliva of patients with HNSCC compared to the controls.

CSTA is a cytoplasmic protein and an inhibitor of cysteine proteases, specifically lysosomal cysteine proteases (cathepsin B, H, K, and L). Lysosomal cathepsins promote cancer metastasis and invasion by degrading the extracellular matrix (ECM). CSTA is a tumor suppressor in some cancers, inhibiting some cathepsins' activities and preventing cancer metastasis.[9–11,13–18]

Luo et al found that CSTA levels in esophageal squamous cell carcinoma (ESCC) patients decreased in tumor tissue compared to normal tissue and were related to tumor progression and poor prognosis.[10] In contrast to the above study, a more recent study by Shiba et al demonstrated that increased levels of CSTA in ESCC were associated with locoregional metastasis and tumor progression with the advanced cancer stage.[11] Similar contradictory results were also found in other cancers and HNSCC. Also, higher levels of CSTA were associated with the increased survival probability of HNSCC patients (Human Protein Atlas accessed on 02/03/2023).

Studies have shown that CSTA may play a dual role in the pathogenesis of HNSCC. It can be inferred from the literature that CSTA may play an additional role other than cysteine protease inhibitor.[18] However, the exact role of CSTA in the pathophysiology of HNSCC is still not clear.

In our study, CSTA was knocked down in two HNSCC cell lines, and its effect on various cancer hallmarks was elucidated in vitro.

We found significantly higher levels of CSTA in FaDu and CAL 27 cell lines compared to normal healthy oral gingival tissue. CSTA mRNA expression was successfully downregulated by lentiviral transduction of CSTA shRNA in FaDu and CAL 27 cell lines. Interestingly, it was found that cell viability after CSTA downregulation was significantly reduced. One possible mechanism behind this reduced viability may be apoptotic death, so we performed an annexin V/propidium iodide cell death assay. CSTA's knockdown significantly increased cisplatin-induced apoptosis in both HSNCC cell lines, that is, CAL 27 and FaDu. Also, in the literature, a study reported that high expression of CSTA in tumor cells attenuates tumor necrosis factor -induced apoptosis along with cathepsin activity.[11] This suggests that an increased expression of CSTA prevents cancer cells from apoptosis. Hence, the downregulation of CSTA may potentiate apoptosis through chemotherapeutic agents like cisplatin.

Cancer cells grow anchorage independent, while normal epithelial cells undergo anoikis following detachment from the surrounding ECM. By performing a soft agar colony formation assay, it was observed that cells with downregulated CSTA showed a reduction in the number of colonies

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formed in the agar layer, indicating that CSTA downregulation reduces anchorage-independent growth in cancer cells.

Cancer cell migration and metastasis are important steps in cancer progression. The scratch assay and transwell invasion assay were performed to analyze the effect of CSTA downregulation on cancer cells' migration and invasion properties. The results of both assays showed that the property of migration and invasion was significantly reduced after CSTA knockdown, showing that CSTA may be involved in tumor progression as its downregulation reverse the processes.

From our study, it can be suggested that CSTA downregulation reduces various cancer hallmarks, viz. cell viability, cell proliferation, cell migration, anchorage-independent growth, and significantly increased cell death by apoptosis in the in vitro model.

A study by Shiba et al on ESCC showed that the proliferation marker, Ki67, was highly expressed in the CSTA-expressing cancer cells. In normal mucosa, the Ki67-expressing cells do not express CSTA, suggesting that CSTA up-regulation may be advantageous for cancer cells in terms of an increase in proliferation, thus supporting our results[11].

Our results have shown the protumorigenic role of CSTA in HNSCC pathophysiology, suggesting the possibility of CSTA targeting in cancer treatment. However, the results must be further validated in more HNSCC cell lines, primary culture, and in-vivo models. Also, the mechanistic studies for elucidating the pathways affected by CSTA in the progression of HNSCC need to be well explored.

Ethics Approval Statement

This study was performed after getting the approval of the Institute Ethics Committee, PGIMER, Chandigarh.

Data Availability Statement

Detailed data and protocols shall be made available by the corresponding author on reasoned request.

Authors' Contribution

G.S.B., A.C., and A.P. conceived and planned the experiments. G.S.B. carried out the experiments. G.S.B. wrote the manuscript with input from all authors. A.B. analyzed the IHC and ICC slides. G.S.B., A.C., A.B, R.K.V., and A.P. contributed to the analysis and interpretation of the results. A.C. and A.P. edited the manuscript

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Conflict of Interest

None declared.

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Second Wave of SARS-CoV-2: Impact on Pregnant Women and Newborns—A Tertiary Care Experience in North India

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Abstract

Objectives The aim of this study was to evaluate the clinical presentation, course of disease, and management of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in pregnant women. We also aimed to evaluate the fetomaternal outcomes in these women.

Material and Methodology This was a single-center, retrospective study performed in a tertiary care hospital for pregnant women with coronavirus disease 2019 (COVID-19) in India. The medical records of all antenatal or postnatal women who were admitted to COVID-19 facility from April 1 to June 30, 2021, were reviewed. The demographic characteristics, obstetric parameters, presence of comorbidities, disease severity, investigations, management, and fetal outcome were recorded.

Statistical Analysis The data were entered in MS Excel spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

Result A total of 94 women were admitted to the COVID-19 facility; 54 (57.45%) were antenatal and 40 (42.55%) were postnatal, 75.53% of them were between 20 and 30 years of age, and 62.96% were multigravida. In addition, 42.55% were asymptomatic and 32.98, 9.58, and 14.89% had mild, moderate, and severe disease, respectively. Also, 42.59% of women had cesarean delivery. Among these, 14 (14.89%) required intensive care unit (ICU) admission, and 24.46% needed oxygen therapy. Comorbidities were present in 48.94%, with hypertensive disorder being the most common (14.89%). Common residual complaints were malaise, body ache, and cough. Among women admitted in the ICU, eight (57%) had comorbidities such as preeclampsia, diabetes, heart disease, and anemia. All these women required oxygen therapy, antibiotics, and thromboprophylaxis. Six among them received steroid (methylprednisolone) and four received antiviral drug (remdesivir). Three women succumbed to death. The mortality rate was 3.19%. Among the neonates, six babies were affected with SARS-CoV-2 and all recovered. On follow-up after discharge, one woman with rheumatic heart disease expired after 2 days of discharge and one woman had a spontaneous abortion.

Keywords

- COVID-19
- SARS-COV-2
- second wave of COVID-19
- pregnancy and COVID-19
- Delta variant
- oxygen therapy
- remdesivir

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Conclusion During the second wave, caused by the Delta variant, maximum requirement was for oxygen therapy, antiviral drugs, and steroids. We conclude that women with comorbidities and advanced period of gestation had a severe course and required critical care. The optimum care and counselling regarding possible outcome in such a vulnerable population is needed.

Introduction

More than a year after its first appearance in Wuhan, China, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic is raging in recurrent waves in countries around the world. The World Health Organization (WHO) had declared it a pandemic in March 2020.[1] Various countries adapted and applied many strategies to cut down the cases, imposing various restrictions and lockdowns. However, most of them experienced a second wave eventually and small but recurrent spurge of cases continues in clusters.

Delhi experienced a second wave with a surge of new cases since the first week of March 2021. A new variant was reported to be more contagious and virulent, and reached a peak daily active cases of more than 400,000 during the end of the first week of May 2021 and average daily deaths of 4,000 in India.[2]

During the first wave of SARS-CoV-2, the maternal mortality rate had been lower than the influenza (H1N1 virus) pandemic in 2009 and severe acute respiratory syndrome or Middle East respiratory syndrome in 2012. A recent study suggested that pregnant and peripartum women are experiencing more severe illness in the second wave of the coronavirus disease 2019 (COVID-19) pandemic than was observed during the first wave. However, the true cause of this change is currently unclear.[3]

Pregnant women are prone to infections as pregnancy is an immunocompromised state and risk of pneumonia is higher than in nonpregnant woman.[4] The possibility of vertical transmission is still a case of debate and research. Women with severe acute respiratory illness in pregnancy are more prone to develop complications such as high incidence of pneumonia, renal failure, thrombosis, and disseminated intravascular coagulation and need for intensive care. Fetal complications can be miscarriage, preterm delivery, growth restriction, and perinatal death.[5]

Few studies have been done during the second wave in India to evaluate the effect of the new variant (B.1.617.2). This has been labeled as Delta variant by WHO and was designated as variant of concern on May 11, 2021.[6] The differences in infectivity, clinical symptoms, and complications have not yet been established fully.

We aim to evaluate the fetomaternal outcomes in women who were admitted to the COVID-19 facility during the second wave. This will add to the evidence and research armamentarium on COVID-19.

Methodology

Study Design and Participants

This was a single-center, retrospective study performed in a tertiary care hospital of North India with 24,000 deliveries per year. The hospital has a separate COVID-19 facility for Obstetrics and Gynecology and a level II intensive care unit (ICU). A total of 98 women were admitted from April 1, 2021, to June 30, 2021, among whom 4 women were admitted with gynecological conditions and hence were excluded from the study. Finally, 94 antenatal and postnatal women comprised the study population.

The testing strategy was according to Indian Council of Medical Research (ICMR) guidelines. All women were tested with rapid antigen test (RAT) kit on admission. Antenatal women who tested negative with RAT underwent quantitative reverse transcriptase polymerase chain reaction (qRTPCR). All RAT- and qRTPCR-positive women were admitted to COVID-19 block unless they were fit for home isolation. The guidelines of Ministry of Health and Family Welfare (MoHFW), Government of India (GOI), were followed for classification and management of these women.[7] Women with mild and moderate symptoms were admitted to the isolation ward, whereas those with severe disease or those who deteriorated during hospital stay were transferred to the ICU. ICU care comprised oxygen therapy, antibiotics, antivirals, steroid, and thromboprophylaxis. In those women in whom chest imaging was indicated, chest X-ray (posteroanterior view) with abdominal shield for pregnant women was the modality of choice. Neither any pregnant female nor any postpartum female required computed tomography of chest. More of noninvasive oxygen therapy was used during ICU stay such as non-rebreathing mask and high-flow nasal cannula (which can deliver oxygen up to 6 0L/min). In antenatal women with early gestation prone positioning was implemented and in advanced gestation lateral tilt was implemented. Antibiotics (oral antibiotics: azithromycin; parenteral antibiotics: penicillin [ampicillin, piperacillin/tazobactam], cephalosporin [ceftriaxone], clindamycin to prevent secondary bacterial infection) were administered according to hospital policy. Antivirals such as remdesivir were used in severe cases after appropriate counselling and written consent due to unproven teratogenicity. Methylprednisolone (40 mg 12 hourly) was used during the second wave instead of dexamethasone. Low-molecular-weight heparin, 0.4 mL/day by subcutaneous route in women with body weight < 60 kg and 0.6 mL/day subcutaneous route in women with body weight > 60 kg, was used for

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thromboprophylaxis in all women. Women who were asymptomatic for 10 days after diagnosis, including 3 days after resolution of symptoms, were discharged. The test for SARS-CoV-2 was not repeated prior to discharge. All medical records regarding demographic characteristics, obstetric parameters, presence of comorbidities, disease severity, investigations, management, and fetal outcome were reviewed. The study was approved by the institutional ethics committee.

Statistical Analysis

Categorical variables are presented in number and percentage (%) and continuous variables are presented as mean ± standard deviation and median. The data were entered in MS Excel spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

Result

Study Population Characteristics

Among 94 women, 54 (57.45%) were antenatal and 40 (42.55%) were postnatal. A majority (75.53%) of them were between 20 and 30 years of age. Among 54 antenatal women, 34 (62.96%) were multigravida, 9 (20.37%) women were discharged undelivered and in subsequent follow-up 1 woman had miscarriage within 2 weeks of discharge, 5 continued their pregnancy without any adverse outcome, and the remaining 3 women delivered healthy baby at follow-up (1 had term and the other 2 had preterm deliveries).

Severity of Infection and Comorbidity

A large number of women was asymptomatic (42.55%) during the second wave. Among the symptomatic population, most (32.98%) had mild symptoms such as fever, cough, loose stool, ageusia and anosmia, with fever being the most common symptom. Moderate disease was present in 9.58%, such as fever with either dyspnea (respiratory rate ≥ 24/min) or $SpO_2 \leq 93\%$ on room air. Severe disease was present in 14.89% of women with breathlessness (respiratory rate > 30/min) or $SpO_2 < 90\%$ on room air; 14.89% required ICU admission and 24.46% needed oxygen therapy. Among the study population, 48.94% had some medical comorbidity, with hypertensive disorders being the most common (14.89%), followed by anemia (8.51%), diabetes, hypothyroidism, and intrahepatic cholestasis of pregnancy (6.38% each) (►**Table 1**). Three women succumbed to the disease: two became symptomatic in the postnatal period and were tested positive, and the third woman had severe COVID-19 disease at 34 weeks of gestation. One of them was overtly diabetic and another had rheumatic heart disease (►**Table 2**).

Women were discharged based on the government's (MoHFW) discharge policy. The average hospital stay was 14 ± 2 days.

Pregnancy Outcome

Among 43 antenatal women admitted to the facility, 20 (37%) had vaginal delivery and 23 (42.6%) underwent cesarean delivery while remaining 17 (20.4%) discharged undelivered. The indications for cesarean section in almost all cases were purely obstetric indications, with fetal distress with meconium-stained liquor being the most common indication. In one case, the mother presented with acute-onset desaturation and emergency cesarean section was performed to improve the outcome.

Among 20 women who delivered vaginally, 12 (60%) had term delivery and 8 (40%) women had preterm vaginal delivery. Among 23 cesarean deliveries, 16 (69.56%) were

Table 1 Demographic parameters of the study population

Parameters	Number ($n = 94$)	Percentage (%)
Antenatal women	54	57.45
Postnatal women	40	42.55
Age (y)		
< 20	2	2.13
20–30	71	75.53
> 30	21	22.34
Gravidity/parity ($n = 54$)		
Primigravida	20	37.04
Multigravida	34	62.96
Mode of delivery ($n = 54$)		
Vaginal delivery	20	37.04
LSCS	23	42.59
Discharged undelivered	11	20.37
RAT positive	48	51.06
RTPCR positive	46	48.94
Type of infection		
Asymptomatic	40	42.55
Mild	31	32.98
Moderate	9	09.58
Severe	14	14.89
Comorbidity		
Diabetes	6	6.38
Hypertension	14	14.89
Anemia	8	8.51
Heart disease	1	1.06
Chronic kidney disease	0	0
IHCP	6	6.38
Hypothyroidism	6	6.38
Thrombocytopenia	2	2.12
Bronchial asthma	1	1.06
Others (seizure disorder)	2	2.12
ICU admission	14	14.89
Oxygen requirement	23	24.46
Mortality	3	3.19

Abbreviations: ICU, intensive care unit; IHCP, intrahepatic cholestasis of pregnancy; LSCS, lower segment cesarean section; RAT, rapid antigen test; RTPCR, reverse transcriptase polymerase chain reaction.

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Table 2 Details of maternal mortality

Parameters	Case 1	Case 2	Case 3
Presentation	Postnatal	Postnatal	Antenatal
Age (y)	30	24	30
Parity	P2L2	P2L1 (IUD)	G2A1
Comorbid condition	k/c/o RHD	Overt DM (on insulin)	None
Presenting symptom	Fever	Fever	Breathlessness with $SpO_2 < 90\%$
Pregnancy complications	None	None	IUD
Mode of delivery	Emergency LSCS	Vaginal delivery	Emergency LSCS
Baby	Live, COVID-19 negative	Macerated still birth	Twin, both fresh stillbirth
Length of stay (d)	11	9	14
Place of death	Home (2 d after discharge from hospital)	Hospital	Hospital
Cause of death	Not known	COVID pneumonia with sepsis	ARDS

Abbreviations: ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; DM, diabetes mellitus; IUD, intrauterine death; LSCS, lower segment cesarean section; RHD, rheumatic heart disease.

done for fetal distress and 7 (30.43%) had preterm emergency cesarean, with multiple pregnancy and antepartum hemorrhage being the common indications. Overall, the preterm delivery rate was 34.88%. Seven elective cesarean deliveries were done on maternal demand in term women with previous cesarean section as they did not give consent for trial of vaginal delivery after being explained regarding the risk of scar dehiscence or scar rupture. Health care workers are at additional risk and all appropriate COVID-19 protocols were followed.

Maternal Mortality

Neonatal Outcome

A total of 40 women were admitted postpartum and another 43 antenatal women delivered during their stay. Among 83 women, 1 woman was admitted with sepsis. Also, 34.88% had preterm delivery and 13.95% (6 women) had intrauterine demise (IUD) and delivered a stillborn baby. Among the women who had IUD, two had severe COVID-19, resulting in hypoxia. Four of these had preterm vaginal delivery and two had diabetes, which may be the cause of intrauterine fetal demise. Two women with singleton pregnancy had an early neonatal death. One woman with twin pregnancy delivered by emergency preterm lower segment cesarean section at 32 weeks in view of both twin fetal growth restriction with one twin having reversed end diastolic flow but ultimately resulted in early neonatal death of both the twin babies. Two women with twin pregnancy had early neonatal death of one twin and another twin required nursery admission. Total neonatal deaths were 5 (11.62%). Among these, four had preterm vaginal delivery and three had twin pregnancy. Among 76 newborns, 6 (7.89%) babies had positive RTPCR report, but no mortality among them was observed. All babies were discharged in healthy state.

Follow-up

All women were contacted telephonically after 2 weeks of discharge; eight were lost to follow-up as they could not be contacted. One woman with rheumatic heart disease expired after 2 days of discharge at home; the cause was not known. Among 11 women who were antenatal during discharge, 3 had vaginal delivery within the next 2 weeks, 1 had spontaneous abortion, 2 could not be contacted, and the rest were continuing their pregnancy well at the time of writing this paper. Common complaints after discharge were malaise, body ache, and cough. Few had complaints of persistent two or three spikes of fever. Another group complained of palpitation and breathlessness on exertion.

Discussion

Pregnancy causes suppression of immune system and predisposes the woman to a higher risk of acquiring contagious diseases.[8] Reports from China and Europe corroborate that asymptomatic and mildly symptomatic infected pregnant women were more in number than women with severe symptoms. A report from New York City stated that 86% of COVID-19 pregnant patients presented with mild or no viral-associated symptoms.[9] In the current study, 42.55% of women were asymptomatic, 9.58% had moderate symptoms, and 14.89% had severe disease.

A meta-analysis of 11 studies in 2020, involving 9,032 pregnant women with COVID-19 and 338 infants, reported that the most prevalent symptoms was fatigue (54.5%), followed by cough (50.1%) and fever (27.6%). Other common symptoms such as dyspnea, myalgia, and sore throat were present in ~21, 16, and 11% of pregnant women with COVID-19, respectively. In less than 10%, atypical symptoms such as diarrhea were present.[10] In the current study, among the symptomatic population, most (32.98%) had mild symptoms

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such as fever, cough, loose stool, ageusia, and anosmia. Fever was the commonest symptom. In the above-mentioned study, 30% of pregnant women with COVID-19 had preterm delivery, whereas premature rupture of membranes and fetal distress were observed in ∼2% of women. Fetal death and neonatal death rate were reported to be 2 and 0.4%, respectively.[10] In the current study, 40% of women who delivered vaginally had a preterm delivery. Among cesarean deliveries, 69.56% were done for fetal distress and 30% had preterm emergency cesarean, with multiple pregnancy and antepartum hemorrhage being the common indications. Intrauterine death occurred in 13.95% of women, and in 11.62% of women, their babies had an early neonatal death. Preterm birth and stillbirth rate were higher than the contemporary non-COVID-19 population in the current study.

A systematic review and meta-analysis of 13 studies on pregnant women during the first wave of SAR-CoV-2 reported fever and cough as the most prevalent symptoms and clinical signs.[11] Pooled proportion of pregnant women having fever and cough was 76.0 and 38.0%, respectively. The most frequent complications during pregnancy, as reported in the study, were nonreassuring fetal heart (38.5%), premature rupture of membranes (38.5%), placenta previa (23.1%), preeclampsia (15.4%), gestational diabetes (15.4%), hypertension (7.7%), cholecystitis (7.7%), abnormal amniotic fluid (7.7%), umbilical cord abnormalities (7.7%), fetal asphyxia (7.7%), meconium staining (7.7%), and stillbirth (7.7%) from various published studies.[4,12–20] Although women were heterogeneously treated (antibiotics, antivirals, and corticosteroids, in different combinations), 45% of infected pooled proportion of women developed pregnancy-related complications. A relatively high number of pregnant women (13.0%) were admitted to the ICU, although there was no maternal mortality.[4,12–22] A low probability of vertical transmission was evident as the proportion of infected neonates was low (6%).[10] In the current study, maternal and fetal clinical profile was similar.

Mahajan et al in 2021 conducted a retrospective observational study in a dedicated COVID-19 hospital in Mumbai, and analyzed the data of 1,530 pregnant and postpartum women admitted during the first (1,143) and second waves (387) of the COVID-19 pandemic regarding the maternal and fetal outcomes. They reported that admission to the ICU or high-dependency unit, case fatality rate, and maternal mortality ratio were significantly higher during the second wave ($p < 0.001$). The majority of maternal deaths (93%) were due to COVID-19 pneumonia and respiratory failure. They also reported the genome sequencing data to correlate the direct association of B.1.617 to adverse outcomes were not available, so definitive conclusions regarding the effect of the B.1.617 variant could not be made.[23] In the current study, the case fatality rate was 3.8% and both the maternal deaths were COVID-19 pneumonia and respiratory failure.

Strength of the Study

It is a single-center study done in a tertiary referral hospital with COVID-19 facility and a fairly large cohort of women. Guidelines of ICMR, WHO, and MoHFW, Government of India, were judiciously followed during admission, classification the disease severity, and management protocol and when deciding the discharge criteria.

Limitation of the Study

A limitation of this study is the lack of long-term follow-up. No histopathological correlation could be done to determine possible vertical transmission.

Conclusion

The course of this disease in pregnant and postpartum women can be rapid and aggressive, leading to death. During the second wave of SARS-CoV-2, women with comorbidities and advanced gestation were prone to severe disease and death, thus requiring more ICU admission, oxygen therapy, and antiviral therapy. There was a higher rate of preterm delivery and stillbirth. Operative deliveries were mostly done for fetal distress. It is thus recommended that all women planning a conception or currently pregnant must also complete the vaccination against COVID-19. This study will help in further evaluation regarding natural course of the disease and fetomaternal outcome of SARS-CoV-2 infection in pregnancy.

Funding
None.

Conflict of Interest
None declared.

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Customized Elastics Fabricated from Foley's Catheter for Traction in Maxillofacial Trauma

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Abstract

Like in orthopaedics, many maxillofacial fracture cases require elastic traction to control the muscle spasms, pain and aid in reduction of the fractured segments to achieve acceptable occlusion before the definitive management. In trauma and emergency hospital setup, sometimes conventional stock elastic bands are not readily available for the traction (non-rigid maxillo–mandibular fixation). In such cases, customized elastic bands, made from Foley's catheter, can be a better alternative. The current technique is hassle free and has ease of customization as per individual case due to easy availability of Foley's catheter in hospital setup.

Keywords
- ► elastic band
- ► maxillomandibular fixation
- ► intermaxillary fixation
- ► traction
- ► Foley's catheter
- ► fracture

Introduction

Like in orthopaedics,[1] many maxillofacial fracture cases require elastic traction to control the muscle spasms, pain and aid in reduction of the fractured segments to achieve acceptable occlusion before the definitive management. Elastic traction given during maxillofacial injury is called as nonrigid maxillomandibular fixation (MMF).

Stock elastic bands like orthodontic chain elastics or orthodontic elastic bands are commonly used for nonrigid MMF as they are easily available in dental clincs.[2] However, in trauma and emergency hospital setup, conventional stock elastic bands are not readily available. In addition, conventional elastics are made up of weak elastic material, hence not suitable in old case where heavy traction is required. In such cases, customized elastic bands made from Foley's catheter can be a better alternative.

Technique

In the present technique, elastic bands were fabricated from the central portion of Foley's catheter. The catheter (20 Fr) was cut such that the width of the bands was 1 mm (►**Fig. 1**). Additionally, different size catheters cut out in different widths can be used to provide varied elastic traction. We suggest 1-mm width band from 20-Fr catheters for routine (fresh) trauma cases.

After placement of stable arch bars or MMF screws on both jaws, we placed the customized elastics (1 mm width, 20 Fr) in a case of anterior mandible fracture with deranged occlusion. Acceptable occlusion was achieved within 12 hours (►**Fig. 2**).

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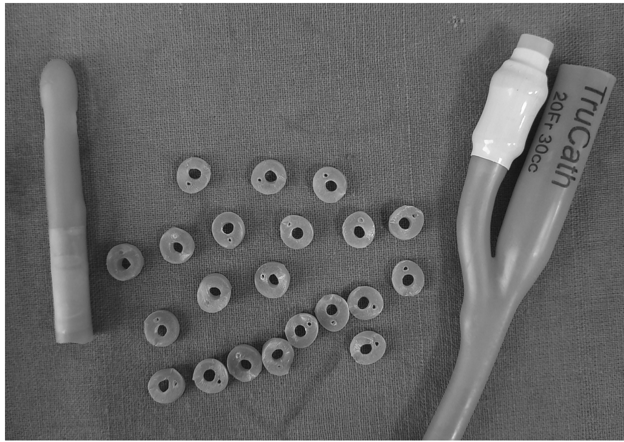

Fig. 1 Customized 1-mm width elastic bands cut out from central part of Foley's catheter (20 Fr).

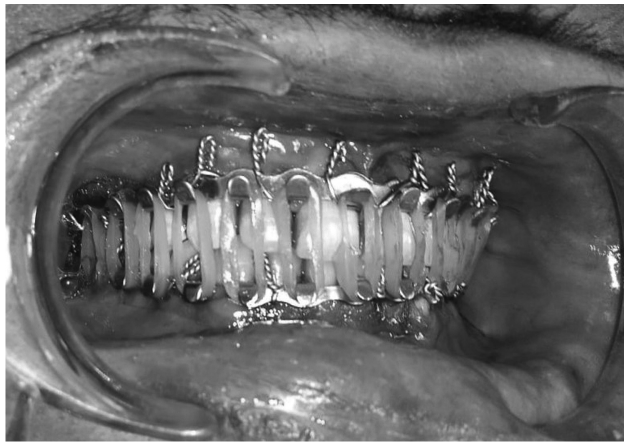

Fig. 2 Acceptable occlusion achieved with customized elastic bands in parasymphysis fracture of mandible.

The present technique is hassle free, does not require special expertise, and can be customized as per individual case requirement. Use of such customized elastics can be extrapolated to old fracture cases where heavy and prolonged traction forces are required to achieve proper reduction. In our opinion, these bands can withstand without rupture during the heavy traction forces.

Discussion

The present technique has multiple advantages as follows:

- Foley's catheter is easily available in hospital setup.
- The bands dimension can be easily customizable as per case requirements.
- This technique does not require special skills or expertise to master.
- These bands are easy to apply (loop-to-loop manner).
- Time-efficient.
- Requires less number of elastic bands.

Disadvantages of using Foley's catheter customized elastics:

- Replacing of elastic by patient's is difficult as size of band hole relatively small.
- Risk of supraeruption of teeth in long-duration MMF.

Authors have a long experience in using such elastics for traction in routine practice and recommend for various conditions like short-term MMF during postoperative period, conservative condylar fracture management, and maxillofacial rehabilitative procedures. However, further observational study of larger sample size is recommended to interpret the ideal dimension of elastic bands as per individual case.

Patient Consent

Written informed consent was obtained from the patient for publication of this article and accompanying images.

Authors' Contribution

J.K. and B.L. participated in data collection and wrote the manuscript. J.K., B.L., A.J. R., and D.K.V. participated in the study design and helped to draft the manuscript. J.K., B.L., A.J.R., D.K.V., R.A., P.S. read and approved the final manuscript

Conflict of Interest

None declared.

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Percutaneous Snaring and Retrieval of Cephalad Migrated Double J Stents in Pediatric Patients—A Bailout Technique Urologists Should Know

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Abstract

Double J (DJ) stents are placed in patients with obstructive uropathy to relieve the obstruction. Curved ends prevent slippage or migration of the stent. Antegrade migration into the urinary bladder or retrograde migration into the ureter are common complications. Mode of retrieval of migrated stents depends upon the location of the stent. We present a case series of four cases with percutaneous snaring of cephalad migrated DJ stents in pediatric patients.

Keywords
- migrated DJ stents
- obstructive uropathy
- snaring

Introduction

Double J (DJ) stents are commonly deployed in cases with post-pyeloplasty internal urinary diversion. Cystoscopy is a commonly used method of DJ stent removal. Caudal migration of the stent into urinary bladder is amenable to endoscopic retrieval. However, proximally migrated stents in the renal pelvis cannot be retrieved using cystoscopy or ureteroscopy. Ureteroscope-guided retrieval is further difficult in postoperative cases and in pediatric population due to difficult anatomy.[1,2]

We report four cases of percutaneous snaring of stents migrated cephalad into the renal pelvis.

Materials and Technique

Retrospective analysis of the cases was done after approval from Institute Medical Records Section. Cases with cephalad migrated ureteric DJ stents were searched in archive of old cases. Keywords used were "migrated ureteric stent" and "snare retrieval." Four cases were selected which underwent stent retrieval with percutaneous snaring technique. All the cases were post-pyeloplasty patients of pelviureteric junction obstruction. Patients were referred for stent retrieval within first week of placement after failed ureteroscopic retrieval. Procedures were performed after an informed consent, under sedation as all the patients were young children. Time for administration and recovery from sedation were included in the procedure time. Fluoroscopic time and radiation dose were recorded in each case. All procedures were done using single plane frontal arm of Philips Allura HD (Philips, Amsterdam, the Netherlands). Procedural steps included ultrasound-guided calyceal puncture of affected kidney with 18G Chiba needle (Blueneem, Indiana, United States). 8F angiographic sheath (Arrow, Teleflex, North Carolina, United States) was placed to establish access. 5F Kumpe multipurpose catheter (Cook Medical, Indiana, United States) and J-tip 0.035" hydrophilic guidewire (Terumo, New Jersey, United States) were navigated into pelvicalyceal system. 5 mm diameter Amplatz Goose-Neck snare (Medtronic, Minnesota, United States) was used to capture and retrieve migrated stents. Snare was advanced over the snare sheath. The most important and challenging step of the procedure was stent engagement by

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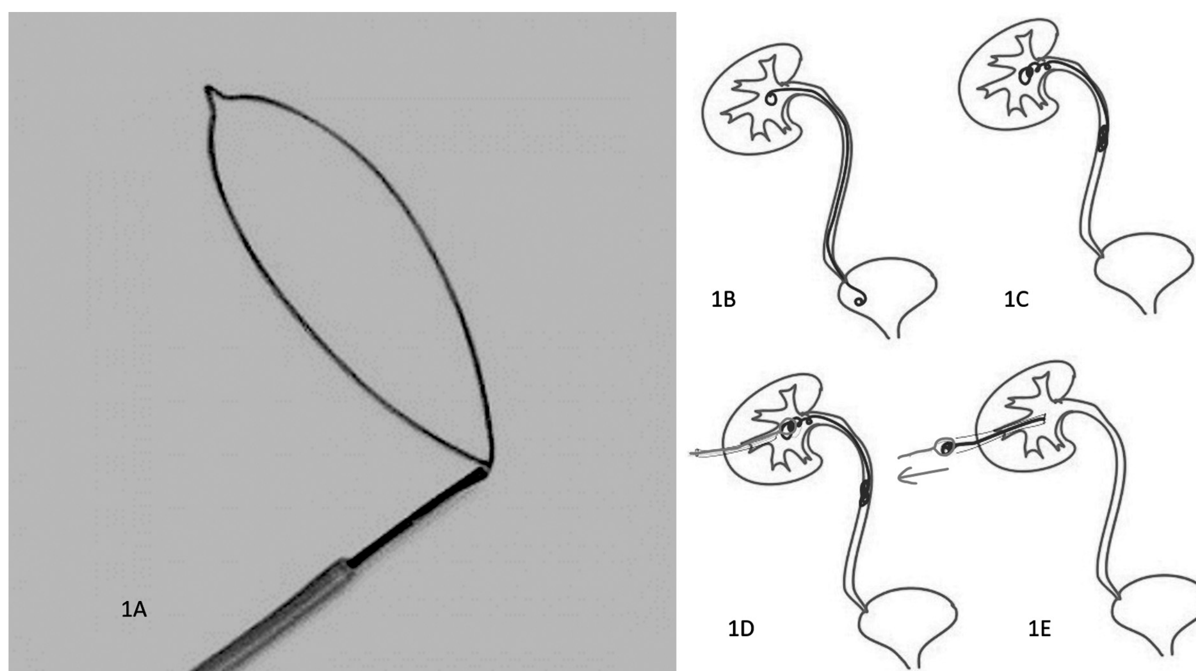

Fig. 1 Photograph of a snare (**A**). Schematic line diagrams showing a normal stent (drawn in blue) position (**B**) and a migrated double J stent (**C**), introduction of a snare with sheath (drawn in black and green) (**D**), engagement of the stent (**D**), and retrieval by the snare (**E**).

snare (►**Fig. 1A–E**). Engagement was confirmed in fluoroscopy by movement of the stent upon snare manipulation. Coiled stents overlap with the snare. So, oblique views were employed to ensure adequate engagement and capture. Tightly looped stents were difficult to deal with. Fluoroscopic time increased in such cases.

Stents were pulled out along with the catheter once engaged. Post-retrieval nephrostogram was obtained in all the cases to ascertain contrast leakage or any signs of injury. Patients were observed for post-procedural hematuria and other complications.

All stents were retrieved in first or second attempt. Dilated renal pelvis was favorable for maneuvering of snare and capturing the stent. Minimal dilated collecting system poses technical difficulty as space for maneuvering of the stent is less. Manipulation in such cases would damage the urothelium.

Case 1

A 3-year-old child was operated with Anderson-Hynes pyeloplasty for right-sided pelviureteric junction obstruction. A DJ stent was placed to relieve the obstruction. Post-procedure radiograph showed complete proximal migration and coiling of the stent in the renal pelvis. Ureteroscopic retrieval was attempted, and was unsuccessful as no part of the stent was accessible in urinary bladder or in the ureter. Patient was planned for percutaneous snaring of migrated stent. Stent was retrieved with a 5 mm (►**Fig. 2A–D**). No immediate or late post-procedure complications were observed. Total procedure time was 20 minutes and fluoroscopic time was 60 seconds. Dose area product was 505 mGy cm2.

Case 2

A 12-year-old boy with pelviureteric junction obstruction on right side underwent Anderson-Hynes pyeloplasty for pelviureteric obstruction. While placing the DJ stent, whole length of the stent migrated and coiled in the renal pelvis. Ureteroscope-guided retrieval was attempted, but was unsuccessful. So, to keep the diversion patent, another DJ stent was placed to avoid obstruction. Patient was planned for percutaneous snaring and retrieval of the migrated DJ stent. This case was technically challenging as the retrieval of the migrated stent was to be performed without displacing the second functioning stent (►**Fig. 3A–D**). Oblique fluoroscopic projections were used to deal with overlapping of two stents and capturing the migrated stent. Snare was maneuvered to separate the migrated stent. Migrated stent was caught by the snare and was later pulled out. No immediate or late post-procedure complications were seen. The second stent remained functioning. Total procedure time was 25 minutes and fluoroscopic time was 50 seconds. Dose area product was 490 mGy cm2.

Case 3

An 8-year-old boy with left-sided pelviureteric junction obstruction underwent Anderson-Hynes pyeloplasty for pelviureteric obstruction. During placement, upper end of the stent migrated into the renal pelvis and lower end migrated into the distal ureter. Patient was planned for percutaneous snaring and retrieval of the migrated DJ stent. Stent was engaged by the snare and was later pulled out along with (►**Fig. 4A–C**). No immediate or late post-procedure complications were seen. Dilated renal pelvis allowed maneuvering

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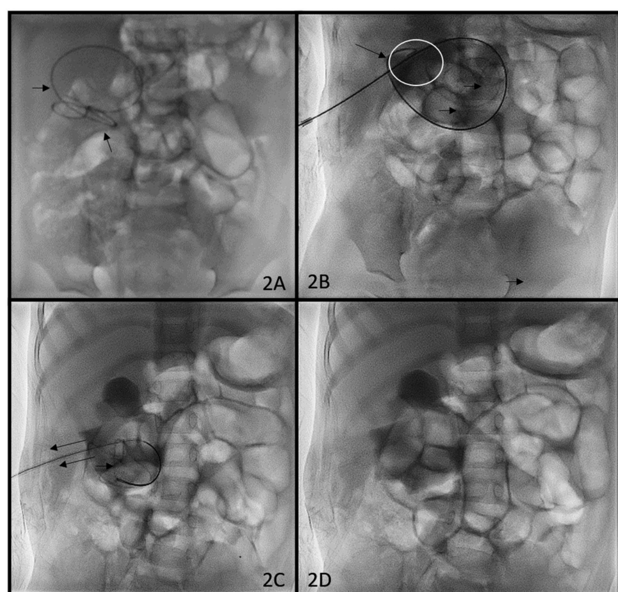

Fig. 2 Fluoroscopic spot image (**A**) of the upper abdomen region shows coiled migrated double J stent (short arrows) in the right renal area. Loop of snare (arrows) engages the stent (circle) in the lower pole region (**B**). Stent is being pulled with snare (arrows) (**C**). Post-procedure nephrostogram spot image (**D**) shows completely removed stent and no leakage of contrast from pelvicalyceal system.

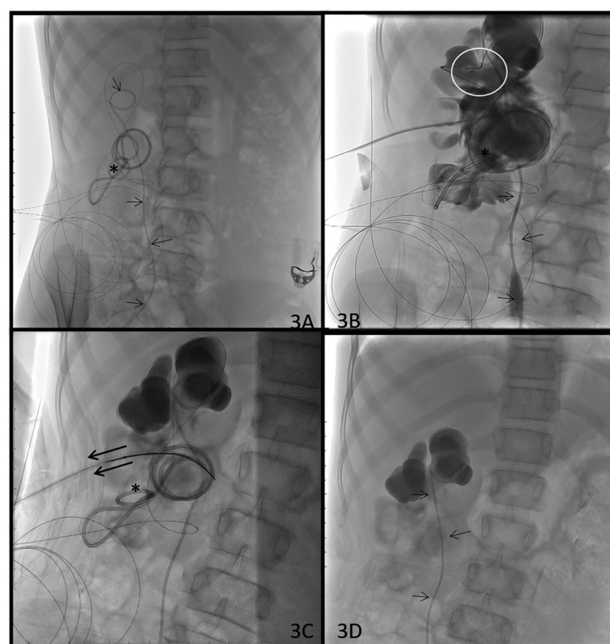

Fig. 3 Fluoroscopic spot image (**A**) of the KUB region shows coiled up migrated double J (DJ) stent in right renal area (asterisk) along with normally positioned another DJ stent (short arrows). Nephrostogram images (**B** and **C**) show loop of snare engaging migrated DJ stent (circle) and stent is being pulled out (long arrows) through sheath without displacing normally placed DJ stent (short arrows). Post-procedure spot image (**D**) shows intact normally placed DJ stent. No leakage of contrast seen from pelvicalyceal system after snaring.

of the snare and engagement of the stent without much technical difficulty. Total procedure time was 30 minutes and fluoroscopic time was 80 seconds. Dose area product was 750 mGy cm2.

Case 4

A 10-year-old girl with right-sided pelviureteric junction obstruction underwent Anderson-Hynes pyeloplasty for pelviureteric obstruction. During the procedure, upper end of the stent migrated into the renal pelvis and lower end into the distal ureter. Stent formed a tight loop within the renal pelvis. Dilatation of the pelvis was minimal. This allowed less manipulation with the snare. Stent was engaged by the snare and was later pulled out along with. No immediate or late

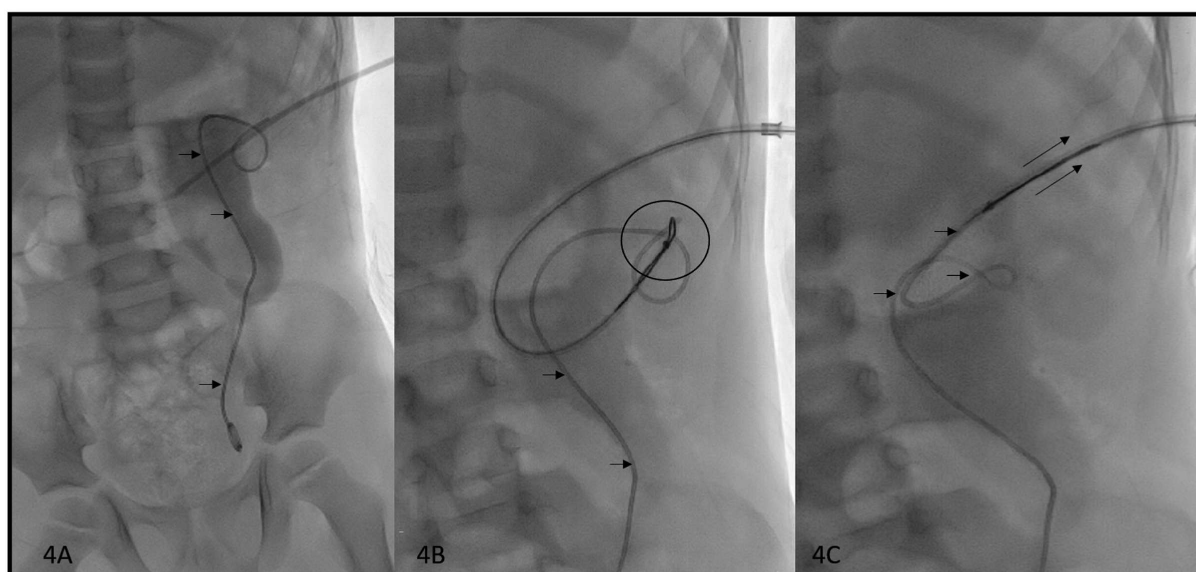

Fig. 4 Nephrostogram image (**A**) of the KUB region shows coiled up migrated double J (DJ) stent in left renal area (short arrows). Renal pelvis is dilated. Upper end of stent is caudally migrated. Images (**B** and **C**) show loop of snare engaging migrated DJ stent (circle) and the migrated DJ stent being pulled with snare (long arrows) through sheath.

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post-procedure complications were seen. Total procedure time was 35 minutes and fluoroscopic time was 120 seconds. Dose area product was 980 mGy cm2.

Discussion

Since first use in 1967, DJ stents are commonly used in to decompress the obstructed pelvicalyceal system. Post-pyeloplasty DJ stent placement is done to avoid obstruction at anastomotic site secondary to postoperative edema, clots, or fibrotic stricture formation. Migration of the stents is common in caudal direction into the urinary bladder.[1] Cephalad migration into renal pelvis or collecting system is uncommon. Reported incidence of cephalad migration is 0.6 to 3.5%.[2]

Common technique for the removal of cephalad migrated stents is ureteroscopy-guided retrieval. Other methods used are ureteric balloon dilators, percutaneous forceps, and basket.[2,3] Shin et al performed percutaneous antegrade removal or exchange of DJ stents with the help of a snare or a basket catheter. They treated 27 patients (39 stents) with a technical success rate of 95%.[4] They used 9-Fr sheath to access the collecting system and used 6 mm angioplasty balloon for retrieval and manipulation. Use of larger sheath and balloon may injure the urothelium. In our case series, angioplasty balloon was not used. Manipulation with snare did not result in post-procedure hematuria. Percutaneous retrieval was done with 8F sheath and 5F catheter, which are more compliant and less traumatic than forceps or balloon dilators.

Liang et al treated 24 patients (26 stents) with 100% technical success rate[5] using forceps or snare. All patients without a pre-existing percutaneous nephrostomy (8 patients) had undergone retrieval of their DJ stents by forceps. In this case series, without a pre-existing nephrostomy tube, retrieval of the migrated stent by snare was successful.

Yeung et al treated 20 patients with fluoroscopically-guided stent retrieval with a success rate of 85%.[6] Out of the 17 procedures, two procedures (12%) were performed with a snare.

Conclusion

Percutaneous snaring is a safe and effective technique in appropriate setting and favorable anatomy. It can retrieve stents migrated into renal pelvis, which are inaccessible with ureteroscopy. Use of oblique fluoroscopic projections facilitates effective capture by the snare. Limited and careful maneuvering and manipulation of the snare avoid urothelial injury and subsequent complications.

Details of Earlier Presentation
None.

Authors' Contributions
Khanak Nandolia was involved in data collection, data analysis and interpretation, and drafting of the article. Udit Chauhan contributed to conception or design of the work, data analysis and interpretation, and critical revision of the article.

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Conflict of Interest
None.

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Cathelicidin Antimicrobial Peptide (CAMP) and Its Correlation with Serum 25 Hydroxy Vitamin D in Under-Five Children with Pneumonia

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Abstract

Cathelicidins are human peptides involved in innate immunity, and are believed to be mediators of vitamin D-related immune benefits. We aimed to evaluate the correlation between serum levels of 25 hydroxy vitamin D (25(OH)D) and serum cathelicidin antimicrobial peptide (CAMP) in children hospitalized with severe pneumonia. This was a secondary analysis from a larger randomized double-blind placebo-controlled trial on vitamin D supplementation in severe pneumonia. Children aged 6 months to 5 years with the World Health Organization (WHO) defined severe pneumonia were included. Any child with rickets, immunodeficiency, or chronic illness was excluded. Baseline serum CAMP, serum 25(OH)D, and immunoglobulins were measured and analyzed. A total of 163 under-five children were recruited with mean (standard deviation) age 16.4 (13.0) months. The median (interquartile range [IQR]) serum CAMP was 12.5(8.0–20.0) ng/mL and serum 25(OH)D was 14.4 (8.4–24.2) ng/mL. There was no significant correlation between serum cathelicidin and serum 25(OH)D ($r = -0.064$, $p = 0.415$). Serum 25(OH)D was low (<20 ng/mL) in 105(64.4%) participants. Median (IQR) serum CAMP was comparable between children with low (<20 ng/mL) and normal serum 25 (OH)D levels (13.0 [8.2, 22.1] and 12.0 [8.0, 17.4] pg/mL; $p = 0.2$). Serum cathelicidin showed significant positive correlation with serum immunoglobulin M levels ($r = 0.456$, $p < 0.001$). There was no significant correlation between serum cathelicidin and serum vitamin D in under-five children hospitalized with severe pneumonia.

Keywords
- cathelicidin
- vitamin D
- infection
- children
- pediatric

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Introduction

Cathelicidin antimicrobial peptides (CAMP) are a group of antimicrobial peptides that are released on leukocyte activation and cause disintegration of cell membranes of microorganisms. They act through multiple receptors including toll-like receptors and chemokine receptor type 4 on neutrophils to chiefly affect apoptosis, phagocytosis, cellular migration, and cytokine release.[1] Cathelicidins maintain equilibrium during an inflammatory response by its proinflammatory and anti-inflammatory properties and potential for angiogenesis and epithelial wound repair. They contribute to innate immunity and defense against inhaled organisms in the lungs.[2] Poor CAMP response is reported in various diseases like tuberculosis, sarcoidosis,[3]and asthma.[4] Few adult studies have documented defensive role of CAMP in bacterial infections of respiratory tract caused by intracellular microorganisms.[5]

The transcription of cathelicidin gene is postulated to be affected by active form of vitamin D (1,25 dihydroxy vitamin D). Therefore, low CAMP levels have been reported in vitamin D deficiency states.[6] Vitamin D supplementation has shown to increase cathelicidin levels in experimental studies.[7,8] However, data from these studies is limited by lack of clinical data for inference, small sample size, and differences in populations that were assessed. The validity of this correlation, if any, has not been tested in clinical setting of infections in populations that are vitamin D-deficient per se.

We, thus, undertook this study to evaluate the levels of serum CAMP in under-five children with pneumonia from Northern India as per their vitamin D status. We also wanted to ascertain if there existed any correlation between CAMP and serum 25 hydroxy vitamin D (25(OH)D) in children with active infection.

Methods

These data were collected as part of a randomized double-blind placebo-controlled trial that evaluated efficacy of single oral mega-dose of vitamin D3 for treatment and prevention of pneumonia in hospitalized under-five children.[9] The original trial was approved by Institutional Ethical Committee (including collection of blood for the parameters analyzed in this study; Trial Registration: CTRI/2013/01/003317). Children aged 6 months to 5 years with a clinical diagnosis of severe pneumonia (defined as presence of lower chest indrawing in children presenting with cough or difficult breathing) were assessed for enrolment after hospitalization.[10] Children with rickets, chronic illness including immunodeficiency, and those with a recent consumption history of vitamin D or calcium supplements were excluded.

A baseline serum sample was collected for estimation of serum calcium, phosphate, alkaline phosphatase (ALP), serum 25(OH)D, intact parathyroid hormone (iPTH), and immunological markers (serum immunoglobulin IgA, IgG, IgM, and CAMP. Blood samples for 25(OH)D and PTH were collected and transported in ice immediately to an in-house testing laboratory. Serum PTH and serum 25(OH)D were estimated by radioimmunoassay using commercially available kit manufactured by Immunotech SAS, Marseille, France (interassay variation: below or equal to 10·3%; intra-assay variation: below or equal to 7·7%; sensitivity: 2 pg/mL) and DiaSorin, United States (interassay variation: 11%; intraassay variation: 12·5%; sensitivity: at or below 1·5 ng/mL), respectively. Vitamin D sufficiency, insufficiency, and deficiency (VDD) were diagnosed at serum 25(OH)D more than 20 ng/mL, 12 to less than or equal to 20 ng/mL and less than 12 ng/mL, respectively.[11] Systematic sampling method was followed to measure immunological markers in every alternate child in view of available logistic support. Serum CAMP was estimated using a standard commercial kit (Human LL-37, HK 321 Hycult Biotech, the Netherlands [sensitivity: 0·1 ng/mL], based on sandwich enzyme immunoassay (enzyme-linked immunosorbent assay [ELISA]). Serum immunoglobulins (IgA, IgG, and IgM) were measured quantitatively with immunoenzymatic colorimetric method using ELISA-based kits (Xema Co Ltd, Russia) having a sensitivity of 0·12 g/L.

All children were managed as per standard protocol[10] and were monitored 8 hourly during hospitalization. The child was discharged when fever and fast breathing were absent for at least 24 hours. The duration of hospitalization (hours) and time to resolution of pneumonia (hours) defined as abatement of fever and fast breathing were recorded.

Statistical Analysis

Data were analyzed using SPSS version 20.0. Quantitative variables like biochemical and immunological markers were depicted as median and interquartile range (IQR). The strength of correlation between serum CAMP and 25(OH)D and immunological markers (nonparametric data) was measured using Spearman correlation (r). Statistical significance was at p-value less than 0.05

Results

Total 324 children were recruited in the original trial. Baseline data on immunological markers was available for 163 children with mean (standard deviation [SD]) age of 16.4 (13.0) months. The median (IQR) cathelicidin levels were 12.5(8.0–20.0) ng/mL. The median (IQR) levels of serum 25 (OH)D, iPTH, and ALP were 14.43 (8.42–24.17) ng/mL, 25.71 (13.85–58.54) pg/mL, and 208 (158–291.5)IU/L, respectively. The median (IQR) levels of serum immunoglobulins IgA, IgG, and IgM were 0.84 (0.59–1.21), 7.36 (5.39–9.66), and 2.59 (1.65–4.11) mg/dL, respectively. The proportion of vitamin D sufficiency, insufficiency, and VDD were 58 (35.6%), 39 (23.9%), and 66 (40.5%), respectively.

Serum CAMP showed an inverse but insignificant correlation with serum 25(OH)D ($r = -0.064$, $p = 0.415$; ►**Fig. 1**) and iPTH ($r = -0.03$, $p = 0.710$). Among immunoglobulins, serum CAMP showed significant positive correlation with serum IgM ($r = 0.456$, $p < 0.001$, ►**Fig. 2**) unlike IgA ($r = -0.05$, $p = 0.49$) and IgG ($r = 0.05$, $p = 0.54$). Serum CAMP levels were not related to age ($r = 0.03$), severity of pneumonia ($r = 0.13$), duration of hospitalization ($r = 0.05$), and time to resolution of pneumonia ($r = 0.10$; $p > 0.05$). Weight for

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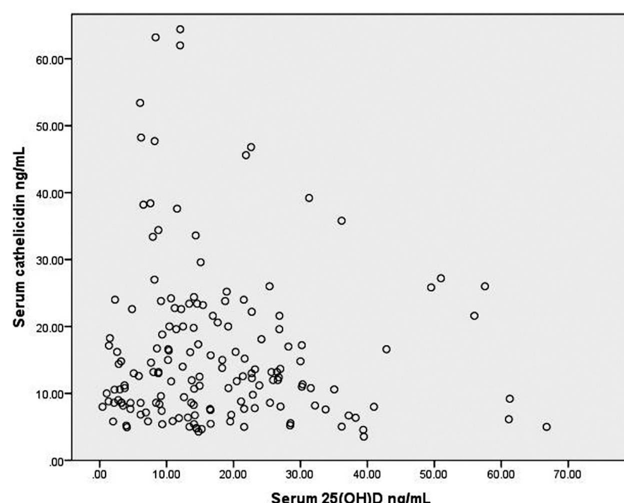

Fig. 1 Correlation between serum cathelicidin and serum 25-hydroxy vitamin D (25 (OH)D) levels ($n = 162$).

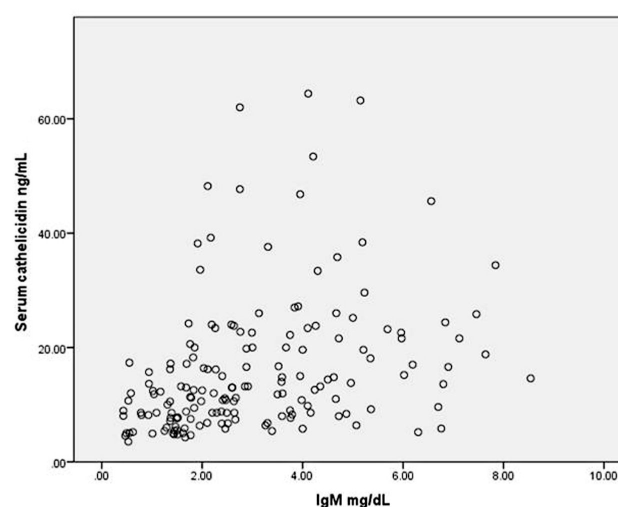

Fig. 2 Correlation between serum cathelicidin and serum immunoglobulin M (IgM) levels ($n = 162$).

height had an inverse correlation with serum CAMP ($r = -0.09$; $p = 0.67$).

►**Table 1** compares the laboratory parameters between those with and without vitamin D sufficiency. There was no significant correlation between serum CAMP and serum 25(OH) D in either of the groups ($r = -0.235$, $p = 0.08$ and $r = 0.028$, $p = 0.77$), respectively. Serum CAMP correlated significantly with serum IgM ($r = 0.36$, $p < 0.001$) in those with vitamin D insufficiency ($\leq$20 ng/mL). Serum CAMP correlated significantly with both IgM and IgG ($r = 0.6$, $p < 0.001$ and $r = 0.27$, $p = 0.040$), respectively, in those with vitamin D sufficiency.

Discussion

In this study, under-five children hospitalized with pneumonia were evaluated and found to have an insignificant inverse correlation between serum cathelicidins with serum 25(OH) D. Low serum vitamin D levels were present in majority of these children.

Cathelicidins exhibit a dual role in systemic inflammation by acting as proinflammatory or anti-inflammatory molecules.[12] Its levels may be beneficial in infections like pneumonia, meningitis, and skin infection but detrimental in severe sepsis.[12] A significant positive correlation was seen between cathelicidin and serum IgM levels reiterating the proinflammatory role in acute pneumonia. This correlation remained significant irrespective of the vitamin D levels.

Data on quantitative level of serum cathelicidin and its association with 25(OH)D in infection has been contentious. Low cathelicidin levels were reported earlier among healthy adults with low (<32ng/mL) serum 25(OH)D levels.[13,14] Lower serum cathelicidins with low vitamin D levels were associated with poorer lung function among adults with pneumonia.[15] Conversely, higher cathelicidin values with low serum 25(OH)D levels were reported among 30 neonates with congenital pneumonia.[16] Vitamin D modulates the immune response by its effect on CD4+ T cells and attenuation of proinflammatory

Table 1 Comparison of biochemical parameters according to serum 25 hydroxy vitamin D (25(OH)D) levels

Parameter	Serum 25(OH)D <20ng/mL ($n = 105$)	Serum 25(OH)D ≥20ng/mL ($n = 58$)	*p*-Value
Age, mo	12 (7, 25)	11 (7.8, 18)	0.752
Serum cathelicidin, ng/mL	13.0 (8.2, 22.1)	12.0 (8.0, 17.4)	0.447
Serum 25 (OH) D, ng/mL	10.2 (6, 14.2)	27.6 (23.1, 37.5)	<0.001
Serum parathyroid hormone, pg/mL	34.2 (14.3, 74.6)	19.7 (13.3, 31.8)	0.002
Serum calcium,[a] mg/dL	8.6 (8.0, 9.1)	8.7 (8.2, 9.2)	0.878
Serum phosphorus,[a] mg/dL	5.0 (4.0, 5.7)	4.5 (4.0, 5.6)	0.062
Serum Alkaline phosphatase, IU/L	237 (164, 335)	184.5 (148.8, 213.8)	0.005
Serum IgA, mg/dL	0.86 (0.55, 1.23)	0.81 (0.62, 1.0)	0.752
Serum IgG, mg/dL	7.0 (5.2, 9.0)	7.5 (6.7, 10.2)	0.583
Serum IgM, mg/dL	2.6 (1.8, 4.1)	2.6 (1.4, 4.6)	0.916

Abbreviations: 25 (OH D, 25 hydroxy vitamin D; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IQR, interquartile range. Data represented as median (IQR).
[a]$n = 60$ and 30 in first and second group, respectively.

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response.[17] The higher median levels of cathelicidins in those with lower 25(OH)D levels and negative correlation between the two were seen in this study. This is similar to a recent study in hospitalized adults with coronavirus disease 2019 pneumonia, where CAMP LL-37 levels were higher in those with pneumonia than healthy controls. The cathelicidin levels further decreased after vitamin D supplementation at day 7 and day 14.[18] This suggests the proinflammatory role of cathelicidins in infection and a declining trend with vitamin D supplementation. The vitamin D levels were reported as lower in critically sick children[19] that can independently affect the clinical outcomes in an adverse manner. An understanding of the association with other antimicrobial peptides can help to decipher the role of vitamin D supplementation in sick children.

The results of our study should be interpreted considering insufficient vitamin D levels in most children. Second, the cohort comprised of children with acute respiratory infection. Inclusion of a comparative healthy control group could have improved understanding of these biochemical associations. This study was not statistically powered to conclude a significant association between cathelicidin and serum vitamin D levels as it was a secondary analysis of collected data.

To conclude, serum cathelicidins showed poor correlation with serum 25(OH)D levels in hospitalized children with acute pneumonia, but correlated with immunoglobulin levels suggesting their role in inflammation. Future studies are required to ascertain the role of vitamin D in systemic inflammatory response in childhood pneumonia.

Any Previous Presentation of the Manuscript
We declare that manuscript has not been previously published nor is not being considered for publication elsewhere.

Authors' Contribution
PG, DS, PD, and AKB contributed to study design and conceptualization. AD and NK were involved in data acquisition and analysis. Initial draft was written by AD and NK. The manuscript was revised with critical inputs from DS, PD, AKB, and PG. All authors have read and approve the final content of the manuscript.

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Conflict of Interest
Zuventus Healthcare Ltd. India was involved in procurement of vitamin D administered in the original trial. This manuscript does not bear any conflict of interest with the firm. The pharmaceutical company did not influence data acquisition, analysis, or drafting of the manuscript.

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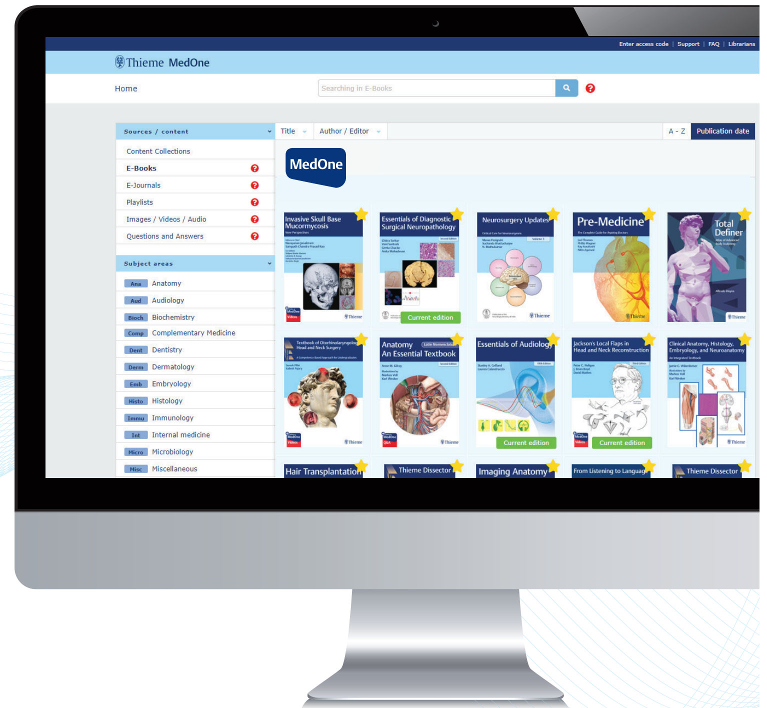

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