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Contents

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Original Article

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Purification and characterization of collagenase from Bacillus altitudinis

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Abstract

Microbial collagenases are secreted by anaerobic as well as aerobic pathogenic and non-pathogenic microorganisms to utilize collagen as a source of nutrition. The collagenase production from aerobic non-pathogenic strains can increase its application by decreasing the production time and avoiding pathogenicity. Keeping this in view, previously, collagenase had been isolated and partially purified by gel permeation chromatography (Sephadex G-200) from an aerobic non-pathogenetic microorganism, Bacillus altitudinis, in our laboratory. Thus, the present study was undertaken to purify further and characterize the collagenase. For this, collagenase was purified by anion exchange chromatography using a DEAE cellulose column. SDS-PAGE was carried out, where a single band of apparent homogeneity with the molecular mass of ~23 kDa was observed. Finally, the purified collagenase was characterized in terms of its stability at different temperature and pH ranges. It showed stability at temperature ranging from 4 to 45°C and pH ranging from 4 to 10 up to 60 minutes.

Keywords: Purification and Characterization; Bacillus altitudinis.

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Introduction

The use of chemicals in different industries has increased enormously, which is affecting life tremendously. So, present-day research is going on to replace these toxic chemicals with environmentally friendly products. Therefore, the focus is shifting to determine different enzymatic processes instead of chemical processes. Proteases are successfully considered an alternative to chemicals and an eco-friendly indicator for nature. They are one of the three largest industrial enzymes, and their global market is drastically increasing annually (Razzaq et al., 2019). Out of the various protease sources, microbial proteases have shown importance as they can be produced in large amounts rapidly and cost-effectively, thus extensively utilized in various fields (Nisha and Divakaran, 2014). Among these proteolytic enzymes' collagenases play an important role as they are the only enzymes that can hydrolyse the insoluble fibrous collagen, a major fibrous element of skin, bones, tendons, cartilage, and blood vessels. This enzymatic degradation of collagen results various peptides which have diverse industrial applications e.g., an immunotherapeutic agent, a moisturizer for cosmetics, a preservative, dietary material and significant applications in medical industries (Pal and Suresh, 2016).

The collagenases are mainly found in various animals, microorganisms, and plants but differ in substrate specificities (Bhagwat et al., 2018). Microbial collagenases have broad specificities. They can degrade both native and denatured collagens and attack various sites of the collagen chain in contrast to other collagenases that only cleave the native collagen at a specific site (Baehaki et al., 2012). These collagenases have been isolated from various organisms, most of them being pathogenic. This pathogenic nature of the microorganisms poses the possibility of an outbreak of microorganisms, leading to an increase

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in the cost of enzyme production and limiting the applications (Bhagwat et al., 2015). Hence, studies on microbial collagenase production and purification from non-pathogenic sources and further exploring their applications would be beneficial to improve the growth in various industrial sectors. Previously in our laboratory, collagenase was isolated and partially purified from an aerobic, non-pathogenic Bacillus altitudinis. Considering this, the present work was carried out to further purify and characterize the collagenase from Bacillus altitudinis.

Materials and Methods

Microorganism

Bacillus altitudinis, capable of producing collagenase, isolated previously in our laboratory was used in the present study (Chauhan and Prabha, 2017).

Isolation and partial purification of collagenase from Bacillus altitudinis

Collagenase was isolated and partially purified by gel permeation chromatography (Sephadex G-200) from 72 hours old cell culture of Bacillus altitudinis by the method earlier standardized in the laboratory (Chauhan and Prabha, 2017).

Purification of collagenase

The purification of collagenase was carried out by anion exchange chromatography. The pooled and concentrated fractions of collagenase after gel permeation chromatography (Sephadex G-200) were passedthrough DEAE cellulose column. 80 mL of elution buffer PBS (50 mM, pH 7.2) was allowed to run down the column. Elution of protein was carried out with PBS containing 0.05, 0.1, 0.2, 0.4 and 0.6 M NaCl. Fractions of 4 mL each were collected, and the absorbance was read at 280 nm on UV spectrophotometer. The fractions showing collagenase activity were pooled, concentrated and stored at -20 °C.

Molecular mass estimation of purified collagenase

The molecular mass of the purified collagenase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli (1970).A 10% gel was prepared and SDS was added accordingly. Purified collagenase was denatured and loaded onto the gel along with the standard molecular weight markers. After the gel was run, Coomassie blue staining was done and molecular weight was estimated.

Characterization of collagenase

Effect of temperature

The purified collagenase was incubated for 5, 15, 30, 45 and 60 minutes at various temperatures i.e., 4, 28, 37, 40, 45, 50 and 55 °C with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). The activity of collagenase was examined by gelatin plate method (Suphatharaprateep et al., 2011)in which wells were cut in gelatin agar plates with a sterile cork borer and loaded with the protein sample. The plates were flooded with 35% (w/v) Trichloroacetic acid and observed for clear zones around the wells after 24 hours of incubation at 37 °C.

Effect of pH

For studying the effect of pH, the purified collagenase was incubated for 5, 15, 30, 45 and 60 minutes at different pH i.e., 3, 4, 5, 6, 7, 8, 9 and 10 with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). and collagenase activity was examined by gelatin plate method(Suphatharaprateep et al., 2011).

Results

Purification of collagenase

For purification, the pooled and active fractions from Sephadex G-200 column (Figure 1) were applied on DEAE cellulose anion exchange column. It was found that collagenase bound in the column could be eluted with PBS. The fraction showing collagenase activity were 4-6 with peak value in fraction 5 (Figure2).

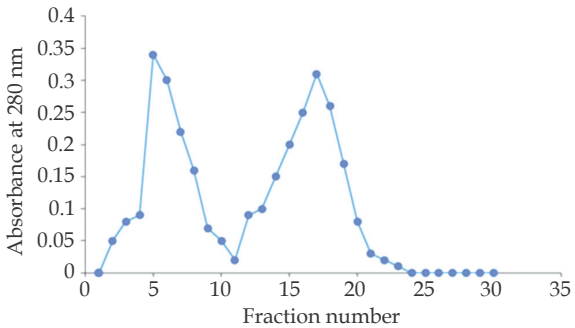

Fig. 1: Elution pattern of collagenase from B. altitudinis after gel permeation through sephadex G-200 column is showing the presence of collagenase in fractions 5-8 with peak value in fraction 5

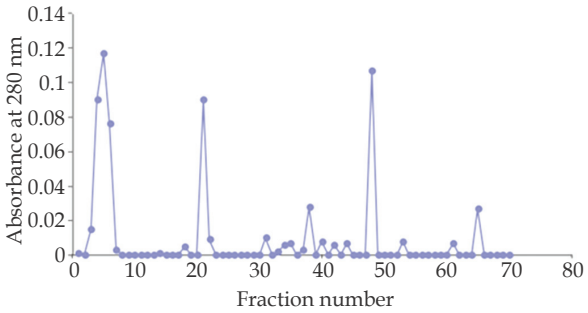

Fig. 2: Elution pattern of collagenase by DEAE cellulose ion exchange chromatography showing collagenase in fractions 4-6 with peak value in fraction 5

Estimation of molecular mass

SDS-PAGE was carried out to estimate the molecular mass of purified collagenase. The Coomassie blue stained gel showed that the purified collagenase was a ~23 kDa protein (Figure3).

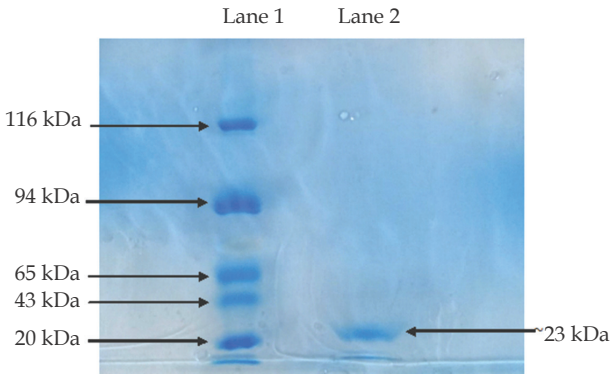

Fig. 3: SDS-PAGE of collagenase; Lane 1: Marker; Lane 2: DEAE-cellulose pooled and concentrated fraction

Characterization of collagenase

Characterization of enzymes is important to determine its applicatory use with respect to dierent sectors in which it is going to be exploited. Therefore, the purified collagenase has been characterized by their stability profiles corresponding to temperature and pH.

Effect of temperature

The purified collagenase was incubated for 5, 15, 30, 45, 60 minutes at various temperatures ranging between 4-55 °C with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). It was observed that collagenase was stable at temperatures 4 to 45 °C till 60 minutes (Figure 4).

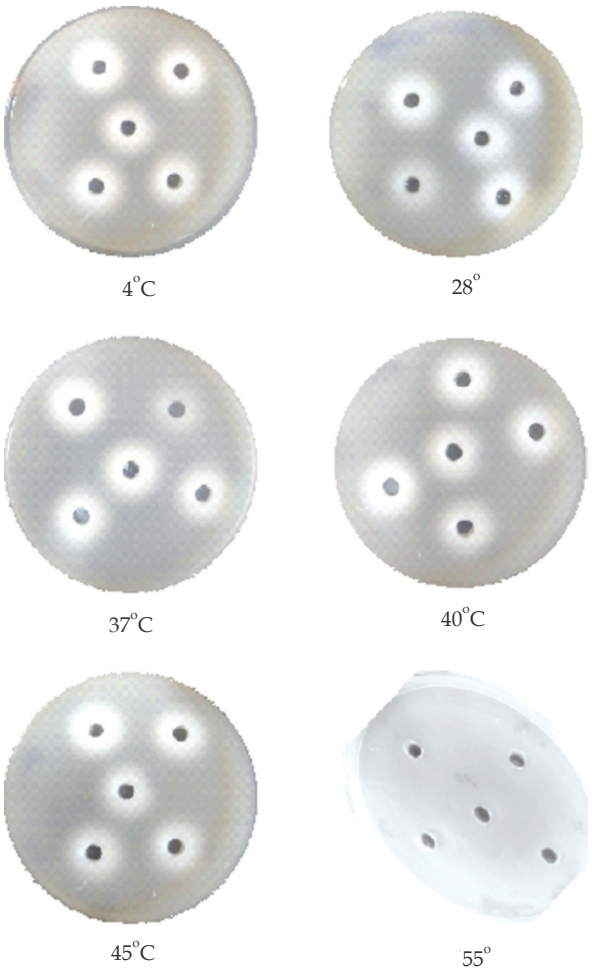

Fig. 4: Clear zones on gelatin agar medium after addition of TCA around wells loaded with purified protein after incubation for 5, 15. 30, 45 and 60 minutes at 4, 28, 37, 40, 45 °C and no clear zones at 55 °C

Effect of pH

The purified collagenase was incubated for 5, 15, 30, 45, 60 minutes at different pH ranging from 3-10 with 50 mM Tris–HCl buffer (50 mM CaCl2, pH 7.5). It showed collagenase was stable from pH 4 to pH 10 till 60 minutes (Figure 5).

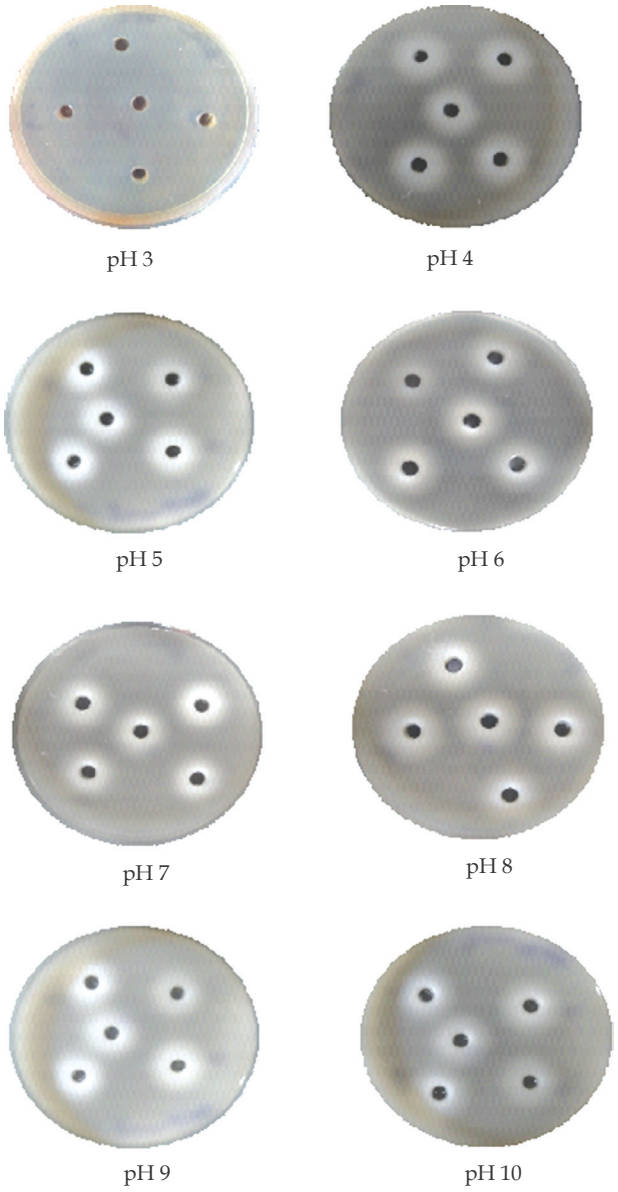

Fig. 5: Clear zones after addition of TCA on gelatin agar medium around wells loaded with purified protein after incubation for 5, 15, 30, 45 and 60 minutes at pH 4, 5, 6, 7, 8, 9, 10 and no clear zones at pH 3

Discussion

Collagens are the major proteins in the skin, tendons, teeth, blood vessels, bone, dentin, and other body structures. They determine the organ shape, tissue integrity, and cell attachment. Collagen molecules have a very rigid structure; therefore, only certain types of proteases, i.e., collagenases, can degrade them. Valuable collagen peptides, produced by the action of collagenases, have several biological applications in medicinal and industrial fields. Therefore, research is going on microbial collagenase purification where collagenases from Bacillus licheniformis, Pseudomonas sp. SUK, Pseudomonasmarinoglutinosa, Porphyromonasgingivali s, Rathayibacter sp., Bacillus cereus MBL13, Clostridium histolyticum, Vibrio vulnificus, Thermoactinomyces sp. E21, Alicyclobacillus sendaiensis, Bacillus pumilus Col-J are being purified and characterized (Baehaki et al., 2012; Bhagwat et al., 2016; Hamdy, 2008; Kato et al., 1992; Labadie and Hebraud, 1997; Liu et al., 2010; Matsushita et al., 1999; Miyoshi et al., 1998; Petrova et al., 2001; Tsuruoka et al., 2003; Wu et al., 2010). In this light, collagenase was isolated and partially purified in our laboratory. Hence, as an addendum to previous work, the present study was carried to further purify and characterize collagenase.

Numerous procedures have been used for the purification of microbial collagenases, such as gel permeation chromatography, ion-exchange chromatography, immobilized metal anity chromatography, amylose anity chromatography, and removal of N-terminal tag (Bhagwat et al., 2016; Ducka et al., 2009). Therefore, the fractions of gel permeation chromatography showing collagenase activity were pooled and subjected to anion exchange chromatography (DEAE cellulose). It is by far the most common ion-exchange technique that has been used by several researchers to purify collagenase and is based on diethylaminoethyl (DEAE) cellulose or agarose (Daboor et al., 2010).

The molecular mass of collagenases generally ranges from 20 to 120 kDa. Different molecular masses for different collagenases have been reported which include 125 kDa for B. subtilis FS-2 collagenase (Nagano and To, 2000); 120 and 29 kDa for B. licheniformis N22 (Asdornnithee et al., 1994); 42.8 kDa for Bacillus cereus (Sela et al., 1998); 58.64 kD for B. pumilus Col-J (Wu et al., 2010); 50 kDa for Thermoactinomyces sp. 21E (Petrova et al., 2006b); and 33 and 19.8 kDa for Pseudomonas sp. (Hisano et al., 1989). After ion-exchange chromatography, the fractions showing collagenase activity were pooled, and SDS-PAGE was carried out to determine the molecular mass of purified collagenase. A single band of apparent homogeneity with a molecular mass of ~23 kDa was observed.

A handful of purified microbial collagenases have been characterized by their activity and stability profiles corresponding to temperature and pH. These are considered to be the most important factors in retaining collagenolytic activity. Therefore, the purified collagenase was characterized in terms of its stability at different

temperatures and pH. The collagenase purified from Bacillus altitudinis showed stability up to 60 minutes at a temperature ranging from 4 to 45°C and pH ranging from 4 to 10. Earlier researchers have reported different optimum temperatures and pH for collagenases from various organisms, i.e., optimum temperature of 30–42°C for C. perfringens (Matsushita et al., 1994), 50°C for B. subtilis FS-2 (Nagano and To, 2000), 70–75°C for both Bacillus sp. MO-1 (Okamoto et al., 2001) and Thermoactinomyces sp. 21E (Petrova et al., 2006) and optimum pH of 8.5 for P. logei (Xu et al., 2004), 9.0 for B. subtilis FS2 (Nagano and To, 2000) and Bacillus sp. MO-1 (Okamoto et al., 2001), and 9.0–9.5 for Thermoactinomyces sp. 21E (Petrova et al., 2006),respectively.

Conclusion

From the observations, it can be concluded that collagenase from Bacillus altitudinis could be purified by gel permeation chromatography followed by ion-exchange chromatography, and the purified collagenase is stable at a temperature range of 4 to 45°C and a pH range of 4 to 10 up to 60 minutes.

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Review Article

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Covid 19: The first microorganism ever to cause global lockdown - A Microbiological review

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Abstract

Covid-19 pandemic has caused a public health crisis with millions of infected cases around the globe. The virus allegedly originated in bats in Wuhan city of China and then transmitted to humans. The common symptoms of Covid-19 are fever, cough, breathlessness, fatigue etc that may progress to pneumonia and multi organ dysfunction leading to death. Till March 24, 2021, a total of 128M people were infected including 2.81M deaths worldwide. The mortality rate varies from 2-3%. The molecular diagnosis (RT-PCR) remains the trusted method. The role of antiviral agents is uncertain. Moreover, the first vaccine for Covid-19 is yet to be developed and the impact of preventive measures like social distancing, lockdown etc. is yet to be analyzed.

Keywords: SARS-CoV-2, Pandemic, Respiratory Illness; RT-PCR

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Introduction

Coronavirus belongs to a large family (Coronaviridae) of positive sense, single stranded RNA viruses. The Coronaviridiaefamily is divided into Torovirinae and Coronavirinae subfamilies, which is further divided into alpha, beta, gamma and delta Coronaviruses. Alpha- and beta-coronaviruses usually infect mammals, while gamma and delta coronaviruses usually infect birds and fish. Coronaviruses are named for the crown-like spikes on their surface.[1],[2]

Human coronaviruses were first discovered in the mid-1960s. However, till date, seven coronaviruses that can infect humans have been documented. These are 229E (alpha coronavirus), NL63 (alpha coronavirus), OC43 (beta coronavirus), HKU1 (beta coronavirus), MERS-CoV (beta coronavirus), SARS-CoV (beta coronavirus), SARS-CoV-2 (beta coronavirus).[3]

Most probably,SARS-CoV-2 has its ecological reservoir in bats, and transmission of the virus to humans has likely occurred through an intermediate animal host – a domestic animal, a wild animal or a domesticated wild animal which has not yet been identified. Moreover, coronaviruses that infect animals gets genetically evolved and pose a new threat to public health.[4]

Pathogenesis

Viral entry and Receptors

Viral entry to host cell is an indispensable step to initiate a viral life cycle. Covid-19 spike proteins bind with the great affinity to ACE-2 (Angiotensin converting enzyme 2) Receptor, an enzyme that play a key role to regulate functions in the cell by cutting large protein angiotensionogen.[5] ACE-2 is present in humans, bats, pigs, civet cats etc and may justify the facile transmissibility of this virus.[6]

Nevertheless, additional host interactions may also associate with transmission of Covid-19. It has been noted that a distinct (N-terminal) domain of SARS-

CoV-2spikeproteinsmaybindtoalternativehost-cell receptors.[7],[8] Alternative host cell communications by N-terminal domains permit consideration, as it is known that analogous domains on several human CoVs have significant assisting cell-binding functions.[9] SARS-CoV-2 spike proteins have also acquired several basic residues, forming a furin protease cleavage site. Time-based development of proteolyticscissorsprimes and activate the SARS-CoV-2 proteins to catalyze virus– cell membrane fusion.[10] Thus, the SARS-CoV-2 furin substrate site is probable to facilitate the grooming cleavage step, which sensitizes spike proteins to the subsequent triggering cleavages occurring on susceptible target cells, and enables virus entry and infection.[11]

Pathogenesis of Covid-19 is largely dependent on its genomic structure. Covid-19 is +ssRNA of roughly 30 kb in size with a 5′-cap structure and 3′-poly-A tail.[12] Beginning from the viral RNA, the fusion of polyprotein 1a/1ab (pp1a/pp1ab) in the host is realized.[13] The transcription drives (Figure 1) all the way through replication-transcriptioncomplex

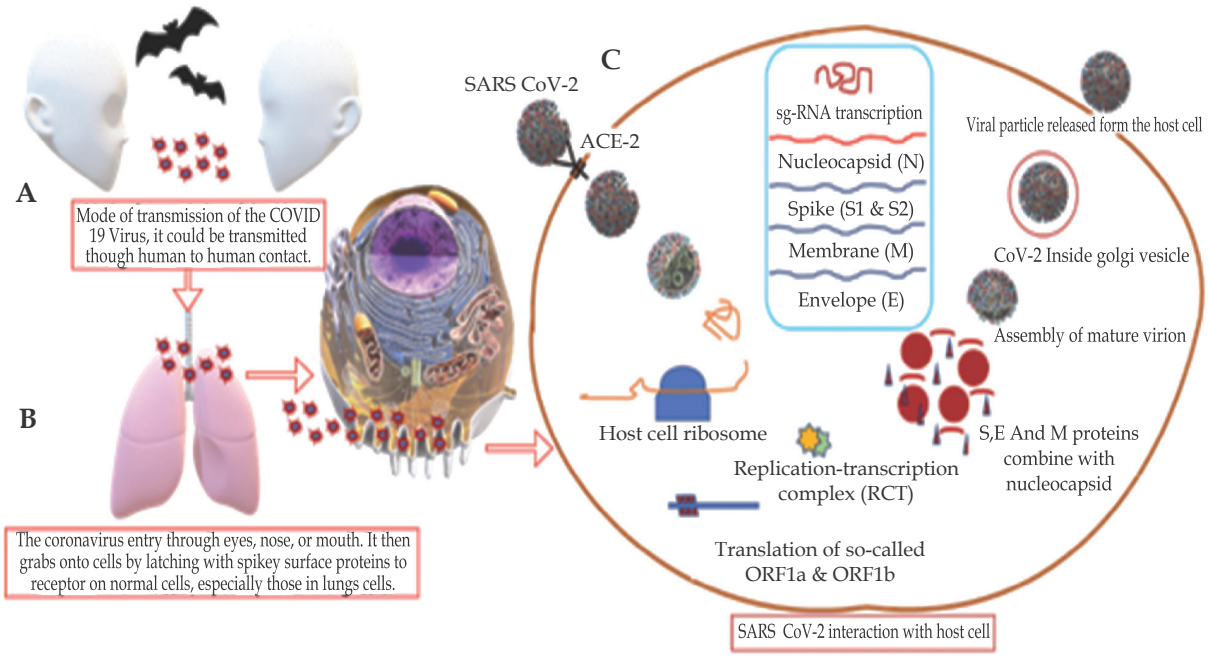

Fig. 1: A. Transmission of the COVID-19 virus by animals or Human to Human close contact. B- Entry of SARS CoV-2 into the lungs, virus attaches with host cell receptors which is mainly present on lungs cells. C- Covid-19 interaction with host cell.

(RCT) organized in double-membrane vesicles and via the manufacture of sub-genomic RNAs structures. Apart from ORF1a and ORF1b, other ORFs encode for structural proteins, including spike, membrane, envelope, and nucleocapsid proteins.[14],[15] Although the pathogenesis of Covid-19 is poorly known, the similar mechanisms of SARS-CoV and MERS-CoV still can give us a lot of knowledge on the pathogenesis of new coronavirus disease to expedite our recognition of Covid-19. It has been analyzed that trans-membrane helical segments in the ORF1ab encoded 2 (nsp2) and nsp3, position 723 presents a serine instead of a glycine residue, while the position 1010 is occupied by proline instead of isoleucine[16]. The matter of viral mutations is key for explaining potential disease relapses.

The virus has high affinity towards the targeting organs that express ACE2, such as the lungs, heart, kidney, liver and gastrointestinal tract.[17],[18] The SARS-CoV-2 found in the fecal samples[19] is more likely for the reason that the virus enters the blood from the lungs and then travels from the blood to the intestines.

Clinical manifestation

Covid-19 is associated with the clinical symptoms like fever, cough, myalgia or fatigue, pneumonia, and complicated dyspnea (Table 1), whereas less common reported symptoms include headache, diarrhea, hemoptysis, runny nose, and phlegm-producing cough[12],[20] or decreased leukocyte counts, and radiographic evidence of pneumonia, which are comparable to the symptoms of SARS-CoV and MERS-CoV infections (Figure 2).[21] Patients with mild symptoms are reported to d

Table 1: Clinical complications due to COVID-19 infection

S.no	Fever	Dry cough	myalgia	headache	Fatigue	Diarrhoea	Dyspnoea/ chest distress	Pharyngeal pain	Haemoptysis	Sputum production	Ref.
1	98.60%				69.60%						[28]
2	87%	60%	11%	13%	39%	14%	16%	13%			[31]
3	81.80%	48.20%	32.10%	9.50%	32.10%	8%	19%				[54]
4	98%	76%	44%	8%	44%	3%	55%		5%	28%	[55]
5	59.40%	34.80%	6.50%	69.60%	10.10%	31.20%	17.40%		26.80%		[56]
6	88.70%	67.80%	14.90%	13.60%	38.10%	3.80%	18.70%	13.90%	0.90%	33.70%	[57]
7	83%	82%	11%	8%		2%	31%	5%			[58]
8	98%	77%	11.50%	6%			63.50%				[36]
range	81.80-98.6%	48.20-82%	11-44%	6-13.8%	32.1-69.6%	2-14%	16-63.50%	9	0.9-5%	26.80-33.7%	

recover within 1 week while severe cases are prone to suffer progressive respiratory failure due to alveolar damage and subsequently leading to death. Mortality rate is comparatively high in elderly patients and all patients with pre-existing conditions (tumor surgery, cirrhosis, hypertension, coronary heart disease, diabetes, and Parkinson's disease etc).[22]

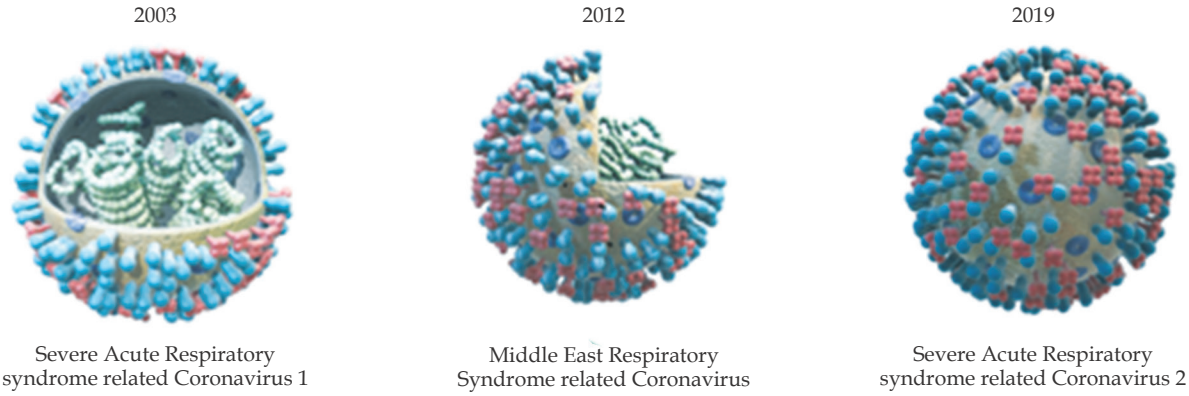

Fig. 2: The diagram showing clinical sing and symptom of COVID-19 and other similar virus (MERS-CoV, SARS-CoV-1)

Clinical symptoms	SARS-CoV-1 (2003)	MERS-CoV (2012)	SARS-CoV-2 (2019)
Fever or chills	YES	YES	YES
Dyspnea	YES	YES	YES
Muscle pain	YES	YES	YES
Headache	YES	YES	YES
Cough	Dry	Dry or productive	Dry
Diarrhea	YES	YES	Uncommon
Nausea or vomiting	YES	YES	Uncommon
Sore throat	Y	Uncommon	Uncommon
Arthralgia	YES	Uncommon	Unknown

Diagnosis

Several diagnostic methods have been developed for the detection of Covid-19. Reverse Transcriptase Polymerase Chain Reaction (RT PCR) remains the most trusted method as it directly detects the viral RNA in nasopharyngeal & oropharyngeal swabs, tracheal aspirate or bronchoalveolar lavage (BAL) samples. Several studies have shown that SARS CoV2 RNA can also be detected in blood and stool

specimens.[23],[24],[25][26] Charité Berlin, from Germany, was the first to develop the assay and standardize the protocol for real time RT-PCR.[27] The test detects the presence of three genes- E, S, RdRp and N.RT-PCR shows very high sensitivity and specificity. However, the time and type of specimen collected for RT-PCR play an important role in the diagnosis of Covid-19. It has also been suggested that in the early days of infection, patients have high levels of virus in spite of the mild symptoms.[28]

The few molecular based sample-result devices have been developed for the detection of Covid-19 target gene directly in the samples (Table 2).[29],[30]

Table 2: Performance of molecular based closed system for the detection of Covid-19

Manufacturer	Device name	Clinical Sample	Target gene	TAT	Ref
Cepheid	Xpert Xpress SARS-CoV-2	Nasopharyngeal & Oropharyngeal swab	NG	45 minutes	[59]
Qiagen	QIAstat-Dx Respiratory SARS-CoV-2	Nasopharyngeal swab	Orf1b and RdRp genes	1 hour	[60]
Roche	Cobas SARS-CoV-2	Nasopharyngeal & Oropharyngeal swab	ORF1ab gene	3 hours	[61]
Hologic	Panther Fusion SARS-CoV-2	Nasopharyngeal & Oropharyngeal swab	ORF1ab gene	<3 hours	[62]
Abott	Abott Real Time SARS-CoV-2	Nasopharyngeal & Oropharyngeal swab	RdRp and N genes	470 samples in 24 hours	[63]
	Abott ID NOW Covid-19	Nasopharyngeal & Oropharyngeal swab	RdRp gene	5-13 minutes	[64]
Becton, Dickinson (BD)	BioGX SARS-CoV-2	Nasopharyngeal & Oropharyngeal swab	N gene	<3 hour	[29]

Several studies have documented other diagnostic method like Isothermal nucleic acid amplification that detects viral RNA much faster than PCR as there is no requirement of repeated heating and cooling cycles.[31],[32] ELISA and rapid immunochromatographic tests have also been developed for the detection of Covid-19 spikes protein antigen and Covid -19 IgG/IgM antibodies in various clinical samples[33],[34] These tests are cheaper, easy to perform and require no expertise. However, the accuracy of these tests remains uncertain. A study revealed that the sensitivity of SARS-CoV N-based IgG ELISA (94.7%) was significantly higher than that of SARS-CoV S-based IgG ELISA (58.9%).[35]

The radiographic imaging also plays a key role in diagnosis of Covid-19. The typical CT images show bilateral pulmonary parenchymal ground-glass and consolidative pulmonary opacities, sometimes with a rounded morphology and a peripheral lung distribution. Lung involvement with a peripheral predominance is also seen in patients with SARS-CoV and MERS-CoV infections. Thus, a combination of CT scan and RT PCR are found to be helpful. [36],[37],[38]

Prevention and treatment

There is, no specializedtreatment for COVID-19 is available till date. Some antiviral drugs such as ribavirin, lopinavir-ritonavir have been used based on the knowledge with SARS and MERS, whereas, the role of corticosteroids is unverified[39],[40],[41] In a historical control study in patients with SARS, patients treated with lopinavir-ritonavir with ribavirin had better consequences as compared to those given ribavirin alone.[42] Supportive treatment for complicated patients has included continuous renal replacement therapy (CRRT), invasive mechanical ventilation, and even extracorporeal membrane oxygenation (ECMO). The first reported patient with 2019-nCoV infection in the USA was treated with remdesivir[43], and others have used antiretrovirals like ritonavir, with trials of both in progress.[44] A recent study conducted by the "front-line" health care providers combating COVID-19 in Wuhan indicated that systemic corticosteroid treatment did not show significant benefit.[45] Baricitinib has been suggested as a potential drug for the treatment in the hope that it might reduce the process of both virus invasion and inflammation.[46] One recent study shows, that monoclonal

antibody (CR3022) binds with the spike RBD of SARS-CoV-2. This is likely due to the antibody's epitope not overlapping with the divergent ACE2 receptor-binding motif. CR3022 has the potential to be developed as a therapeutic candidate, alone or in combination with other neutralizing antibodies for the prevention and treatment of COVID-19 infection.[47],[48] Several studies have also reported Hydroxychloroquine (antimalarial drug) as a potent anti Covid-19 agent.[49],[50],[51],[52]

The guidelines focusing on the preventive measures have been published by every country depending on the number of active cases, mortality rate and their health facilities. However, the preventive measures like social distancing, lockdown, quarantine etc. were implemented by most of the countries. Lockdown was used as the biggest preventive experiment against covid-19. Till June 16, 2020, a total of 78 Countries have put lockdown with an average of 51 days and maximum 90 days[53] The effect of lockdown on Covid-19 cases are mainly affected by the environmental factors, health facilities, Federal and state government policies, public awareness, country economy etc. Thus the global data with and without lockdown has still to be analyzed.

Conclusion

Covid-19 has become a global issue ever since it had first emerged and caused the outbreak in China. Now, it has turned to pandemic, infecting more than 7.82 million people worldwide so far. Antiviral agents like Lopinavir, Ritonavir etc do not appear to be effective. However, some studies shows that the antimalarial drug, Hydroxychloroquine (HCQ), has some therapeutic effect against this deadly virus but more scientific research need to be conducted to prove its efficacy. it is a highly pathogenic human virus, possibly a zoonotic agent, it is critical that countries around the world are taking preventive steps like lockdown and home-quarantine to stop transmission and save lives. However, some universal public health guidelines, focusing on combating Covid-19, are urgently needed to be developed.

Compliance with Ethical Statement: This work does not require institutional ethical approval.

Conflict of interest: The authors declare no conflicts of interest.

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Review Article

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Next generation sequencing technologies: Methods and applications in animal virology

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Abstract

Recent advancement in next-generation sequencing (NGS) technologies hasrevolutionized the sequencing technology for research and diagnostic applications by virtue of its high throughput and accuracy of data generation. In animal virology, NGS has been successfully used formetagenomicsbased discovery of previously unknown viruses. Moreover, NGS technologies are alsoemployed for study of viral dynamics and genetic characterization of viral genomes.The absenceof proof reading during genome replication inseveral viruses along with high replication rate, results in formation of several genetically related viral variants known as quasi-species which in turn, got selected and established itself as new virus variant by antiviral drugs or immune system itself. The identification ofviral quasi species havingbiological significance is difficult using conventional sequencing approach. However, NGS may provide exact sequence information about these virus clones. NGS is a powerful tool to investigate deeper insights ofvirus activities such as viral genetic diversity, vaccine candidate selection, identification of viral reservoirs in nature, re-emergence of viral disease after treatment interruptions, development of drug resistance etc.

Keywords: Virus, NGS, Ion Torrent, Illumina sequencing

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Introduction

Viruses are omnipresentcreatures and responsible for severe diseasesin humans, animals and plants. Several animal viruses are of zoonoticin nature and important frompublic health point of view. Livestock is directly or indirectly remain in contact with humans and they are also part of food sourcesfor humans. Therefore, the specificdiagnoses of the viral diseases and their etiological agents especially in livestock are of prime importance(Lambe et al., 2016). The early diagnosis ofthe viral pathogen at the primary level of infectionthrough molecular diagnostics techniques such as Real-Time PCR and Next-generation sequencing (NGS) techniques, etc.is essential to control the infection before the maximum population is affected which may result in decreased losses to human health and livestock industry (Vishwaradhya et al., 2013; Stephen et al., 2015).

Recent advancement in sequencing technologies hasinitiated a revolution and new perspectives for research and diagnostic in field of virology (Minakshi et al., 2012). The NGS technologies have hallmark features of high throughput sequencing at a modest cost, lesser time, and huge amount of sequence data (in Gigabases)generation in a single run of reaction. The first commercially NGS platform was made available by 454 Life Sciencesin 2005. Subsequently, several NGS technologies such as HiSeq sequencers (Illumina, USA), Genome Sequencer (GS) FLX (454 Life Sciences,

USA),Heliscope platform (HelicosBioSciences, USA),Ion Torrent (Applied Biosystems),SOLiD technology (Applied Biosystems, USA), and PacBio RS (Pacific Biosciences, USA) came in the market for commercial use (Capobianchi et al., 2013). All these NGS platforms have their specific advantages and disadvantages. However, with the decreasing costs of sequencing, the NGS techniques have allowed several achievements in virology researchsuch as diagnosis of emerging viral infections, the study of molecular epidemiology of viruses, the studyof viral drug-resistance, and basic and clinical research (Radford et al., 2012). In the current review paper, we have discussed the various NGS techniques and their applications in the field of animal virology.

Virus asa potent pathogen

Viruses constitute serious form of pathogens found in diverse form of ecosystems and hosts such as human, animal, birds, plant and marine ecosystems. Several metagenomics studies have shown that viruses are the dominant species of our living system (Vibin et al., 2018; Schulz et al., 2020). The deep sequencing of natural samples shows that approximately 90% of the sequences obtaineddid not encode any known proteins, which are already reported in other organisms, including viruses that have been characterizedrecently. Thisclearly indicates that the actual viral diversity has not been explored so far (Chalkias et al., 2018). Recently, the major emphasis has been given to study on economic as well aszoonoticimportant viral infections.

World Health Organization reported that communicable diseases (including major culprit as viral diseases) is approximately 15 million annually (Dye et al., 2013).

Viruses play a central role among infectious diseases due to its smallest size, short generation time, large population sizeand high mutation rates. Moreover, variation in nature of genome (DNA/ RNA),genome size, assembly of virion particles make viruses an ideal subject for evolutionary study of living system (Koonin and Dolja, 2013). It is well known that viruses use all the known replication and expression strategies dynamically to adapt the continuously changing environments. Viruses possess several molecular mechanisms to escape from host defense mechanismwhich can be deciphered throughNGS based genome sequencing and subsequent bioinformaticsanalyses.

Viralgene and genome sequencing

Sanger sequencing

Sanger sequencing is first-generation DNA sequencing protocol. This technique is based on principle ofselective incorporation of chain-terminating dideoxynucleotides (ddNTPs) during in vitro DNA replication in a DNA strand (Sanger et al., 1977a). Once these ddNTPsare incorporated in DNA, the chain elongation gets termination. The radio-labeled(32P)nucleotide bases and addition of one out of four modified nucleotide (ddNTP) bases per reaction allows the determination of nucleic acid sequence on polyacrylamide gel by X-ray films based autoradiography. The introduction of thermostable DNA polymerases (Saiki et al., 1985) in molecular biology, florescent labeleddideoxynucleotidesfollowed by capillary sequencing technique (Marsh et al., 1997) allowed the automation of Sanger's method of sequencing. This led to major breakthrough in unraveling of genomes of several model organisms. Later on it was commercialized by Applied Biosystems(ABI) in 1986 (Adams, 2008).The ABI system (3030xL genetic analyzer) was used for molecular characterization several Bluetongue virus serotypes in India (Ranjan et al., 2013; 2014; 2015a;Dadawala et al., 2013; Kumar et al., 2013).

Next-Generation Sequencing

The story of complete genome sequencing of viruses starts from complete genome sequencing of bacteriophage MS2 having RNA genome of 3,569 nucleotides long (Fiers et al., 1976). Next year complete genome sequence of a DNA bacteriophage ΦX174 of approximately 5,375 nucleotides was sequenced using Sanger's shotgun sequencing technique (Sanger et al., 1977b).The major objective of early days genome sequencing was to characterize the genomic content of an organism in terms of its amino acid coding. In last decades, the sequencing technologies havegrown tremendously and apart from viral genome several other eukaryotic species genomes are also sequenced. Later on, several platforms of next generation sequencing technologies such as Ion torrent, Illumina etc. have been developed to generate complete genome sequence data of any organisms. The huge scale of generating the genome sequence data became a reality today, which was unimaginable previously. The major advantages of NGS technique over the conventional capillary sequencing are the rapid generation of complete genome sequencing data on a very massive scale and at relatively lower cost.NGS also provides tools for several types of molecular studies includingsingle-nucleotide polymorphism, RNA profiling, transcriptomics, gene expression and regulation etc.Viral genome

sequencing has important role in development of newer vaccines, to understand and predict the spread of viral epidemics (Kasibhatla et al., 2016). Several NGS based techniques such as Ion Torrent (https://www.lifetechnologies.com), Illumina(http://www.illumina.com/),Roche 454 (http://www.454.com/), and recently developed fourth-generation sequencing methodologies such as single-cell sequencing, including Oxford Nanopore (https://www.nanoporetech.com/) and Pacific Biosciences (http://www.pacificbiosciences.com/) are available for complete genome sequencing.

Different platforms for NGS technology

Although chemistry of different NGS technologies varies but their basic workflow is similar (Figure 1). Based on chemistry involved, NGS technologies can be classified into sequencing by ligation, sequencing by synthesis and single molecule sequencing. The basic principles, advantages and disadvantages of various sequencing platforms are summarized in Table 1.

Table 1: Comparisons of different sequencing platforms (Minakshi et al., 2014; https://nanoporetech.com/products/comparison)

Sequencing Platforms Parameters	Ion Torrent (Ion semi-conductor)	Roche 454 (Pyrosequencing)	Illumina (Sequencing by synthesis)	SOLiD (Sequencing by ligation)	Pacific Bio (Single-molecule real-time sequencing)	Helicos (True Single Molecule Sequencing)	Oxford Nanopore Technology (Real-time sequencing)	Sanger method (Chain termination)
Sequencing chemistry	Detection of released H+	Pyrosequencing	Reversible terminators	Ligation	Fluorescently labelled dNTPs	Reversible terminators	Nanopore sequencing	Di- deoxy Chain termination
Amplification method	Emulsion PCR	Emulsion PCR	Bridge amplification in situ	Emulsion PCR	Linear amplification	No amplification	Amplification free approach	Sequencing PCR
Separation method	Ion Spheres and high density array	Microbeads and 'picotitre' plate	Glass slide hybridization	Beads on glass slide	Captured by DNA polymerase in microcell	Flow-cell hybridization	Changes to electrical current as nucleic acids passed through protein nanopore	Electro-phoresis
Read length	200 -400bp	700 bp	50 to 250 bp	50-75 bp	1000 bp	25bp	Up to 2Mb	400 to 900 bp
Reads per run	up to 5 million	1 million	up to 3 billion	1.2 to 1.4 billion	35-75 thousand	1 billion	7-12 million	Not available
Maximum data output per run	1 Gb	700 Mb	600 Gb	20 Gb	Not available	35 Gb	2 Gb to 5.2 Tb	Not available
Accuracy	98%	99.9%	98%	99.9%	99%	99%	98-87%	99.9%
Advantages	• Equipment relatively less expensive •Fast reaction	•Long read size. • Fast reaction.	•High sequence yield	•Low cost per base of sequencin-g	•Longest read length. •Less time consuming	•No PCR induced bias and errors •Tolerates degraded samples	•Portable machine •Less time consuming •Real-time result	•Long individual reads. •Applied in many sequence based research.
Disadvantages	Homopolymer error.	•Homopolymer error. •Runs relatively expensive.	. •High DNA concentratio-n required •Very expensive equipment.	• Slower than other sequencin-g methods.	•Low yield at high accuracy. • Equipment very expensive.	•More time to sequence a single nucleotide •High error rate	•High error rate	•Higher cost per base of sequencing. •Impractical inwhole genome sequencing

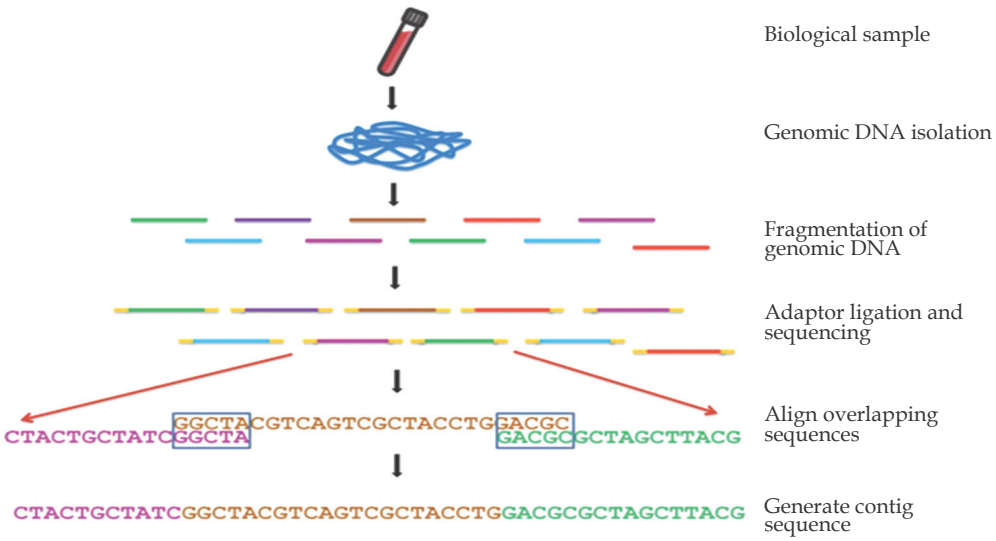

Fig. 1: Basic workflow of next generation sequencing technologies (Ranjan et al., 2015b)

Sequencing by ligation

This method of DNA sequencing uses DNA ligase enzyme to identify the position of a particular nucleotide in a DNA sequence. It does not require DNA polymerase enzyme to create a second strand. The target DNA sequence is determined by the mismatch sensitivity of DNA ligase enzyme.The SOLiD (Sequencing by Oligonucleotide Ligation and Detection) sequencing platform is based on the principle of sequencing by ligation (Valouev et al., 2008).

In this method of sequencing, clonal magnetic bead based library of DNA fragments is prepared from the sample in such a way that only one fragment will be present on surface of each bead. Emulsion PCR is allowed to run using primers against the P1 adopter attached to terminal end of each fragment on beads. The resulting PCR products attached to the beads are allowed to covalently bind to glass slide.

Later on, primers hybridize to P1 adapter in library template. A set of four fluorescentdyeslabeled di-base probes compete for ligation to sequencing primer. Specificity of the di-base probe is achieved by searching every 1st and 2nd nucleotide base in each ligation reaction. For complete sequencing of nucleic acid strand, multiple cycles of ligation, detection and cleavage are required. In subsequent ligation cycle, the extension product is removed and the nucleic acid template is reset with primer complementary to the n-1 position and second round of ligation cycle starts. Although this technique got popularity in early days but later on short read length of 75 bp only reduces its use over other NGS techniques.

Sequencing by synthesis

In this technique library is prepared form nucleic acid (DNA) fragment by fragmentation of DNA strand,adopter ligation and clonal amplification called as library preparation. Later on, clonally amplified products are purified and allowed for sequencing. During sequencing reaction,newnucleotides are added by the polymerase enzyme and generate signals which are detected and read by the NGS machine.

Sequencing by synthesis can be of two type viz., singlenucleotide addition and cyclic reversible termination. Single nucleotide addition approach is used inIon Torrent and 454 pyrosequencingtechniques. The Ion Torrent is a unique technique for sequencing because it is based on detection of pH change during newer dNTP incorporation which releases of H+ ions (Rothberg et al., 2011). It utilizesanion semiconductor sensor to identify the H+ ions released.The Ion-Torrent platform was used for complete genome sequencing of Bluetongue virus (BTV) serotype 16 from India (Minakshi et al., 2012). However, thePyrosequencing method is based on detection of bioluminescence signal which isgenerated due to release of pyrophosphate upon fresh nucleotide incorporation (Margulies et al., 2005).

In cyclic reversible termination method cleavable fluorescent terminator molecule blocks the chain elongation (Guo et al., 2008). GeneReaderand Illuminaplatform utilize this technique with certain modifications. In this technique the mixture of all the four nucleotide bases along with di-deoxydNTP's are added each time of reaction. The unbound dNTP's are removed by washing.

The detection of fluorescent signal determines the specificity incorporated dNTP.

Single-molecule sequencing

The recent advancement in sequencing technology allows real-time sequencing of single molecule of nucleic acid. The Oxford Nanopore Technology and Pacific Biosciences utilizethis technique. Both the sequencing platforms can read 10 to 100 kb of ssDNA (single-stranded DNA) strand. The single-stranded DNA molecule is made to pass through a protein pore in presence of electric current. Nanopore sequencing technique does not utilizethe labeleddNTP. The DNA strand translocation into the pore causes a significantchange in voltage which can be measured. The change in voltage is characteristic of specific DNA sequence. This technique can be used for sequencing of about 70 bp per second. The latest modification to nanopore sequencing is CsgG bacterial amyloid secretion pore based sequencer which can achieve DNA translocation rate upto 250 bases per second (Carter and Hussain, 2017) with much higher sequencing accuracy (Brown and Clarke, 2016). Nanopore platform was successfully used in monitoring of Ebola hemorrhagic fever outbreaks (Quick et al., 2016). Oxford Nanopore Technologies Limited Company has developed MinION machine (a portable DNA sequencer) for the direct analysis of single DNA molecules even in spacecraft and space (Spaceref, 2016).However, the Pacific Biosciences platform uses thephospho-linked fluorescent nucleotides. The signal produced by incorporation of such nucleotide is monitored by a zeromodewaveguide detector (Eid et al., 2009). Although the sequencing errors are higher than othertechniques, the nanopore sequencer has several additional benefits such asits low cost of equipment, portablesizeand real-time data generation.

Application of NGS in Animal Virology

The high-throughput sequencing methods can be used for sequencing of all the organisms available in a sample. It can assists in metagenomics level of study. It has several applications in animal virology which are mentioned below.

Diagnosis of viral diseases

Conventionally, diagnosis of viral disease is done by symptomatic study of disease, virological assays or immunological assays etc. Compared to conventional methods, molecular assays have higher sensitivity and specificity. However, the knowledge of complete genome sequence of the virus is a prerequisite for molecular assays such as PCR, RT-PCR or nucleic acid sequencing etc. Moreover, conventional diagnosis depends on availability of agent-specific diagnostics reagents whereas, NGS can be used for metagenomics study of a biological sample and can identify several types of infectious agentssuch asbacteria, viruses, fungus etc. NGS can also be used diagnosis of mixed infections especially of those that are of immunosuppressive in nature with no clear clinical symptoms. Pyrosequencingtechnique was used to identify torque teno virus and a novel boca-like parvovirus along with causative agent porcine circovirus2 in pig lymph node (Blomstrom et al., 2010). Furthermore, NGS can also be used for molecular identification andepidemiological characterization of non-cultivable viral infectious agents such as retrovirus infection leading to Jaagsiektein sheep (Spencer and Palmarini, 2012).

Bluetongue viruses (BTV) from field samples are regularly genotypedusing conventional cell culture, vp2 gene specific RT-PCR followed by nucleic acid sequencing (Ranjan et al., 2013; 2014; 2015; Dadawala et al., 2013; Kumar et al., 2013). Now a day, NGS techniques are regularly used for typing of newer BTV isolates to identify new serotypes and genomic reassortants (Minakshi et al., 2012). Different NGS platforms had also been used for diagnosis of several animal viruses in India such as Bluetongue virus (Minakshi et al., 2012), foot and mouth disease virus (Mahapatra et al., 2016)etc.

Vaccine development

The NGS data could be of help at different levels in vaccine industry.NGS has shown its importance inselection of vaccine candidate andpreparation as well as testing of viral vaccines. Apart from identification of candidate vaccine stains, NGS can also establish the vaccine contaminating agents. Live attenuated vaccines have major problem with reversion to virulence strains. Nucleic acid based assays including NGS can be used to identify the virulent markers in candidate vaccine stains. The genetic mutations in poliovirus, necessary for attenuation have been identified by complete genome sequence based study using NGS (Victoria et al., 2010). Similarly, NGS can also be used for detection of virulent markers in vaccine viruses. It will improve the safety of viral vaccines.

The vaccine should be devoid of anything other than the vaccine specificantigenic material (Kumar et al., 2012).The vaccine materials must be free from contaminating agents such as bacteria, virus, fungi, rickettsia, protozoa etc. Several contaminating virusesin vaccines have been reported (Table 2).

Table 2: Major viral contamination to human and animal vaccines.

S.n.	Vaccine against	Contamination	Reference(s)
1	Canine vaccines	Bluetongue virus	Akita et al., 1994
2	Rotavirus	Porcine circovirus 1	Victoria et al., 2010
3	Yellow fever	Avian retroviruses	Hussain et al., 2003
4	Lumpy skin disease and sheep pox	Bluetongue virus	Bumbarov et al., 2016
5	Marek's disease	Reticuloendotheliosis virus	Takagi et al., 1996
6	Measles, mumps and Rubella (MMR)	Bovine viral diarrhea virus	Studer et al., 2002
7	Poliovirus and adenovirus	Simian virus-40	Lewis, 1998
8	Poliovirus and MMR	Bacteriophage (φV−1)	Haselkorn et al., 1978
9	Measles and mumps	Avian leucosis virus	Tsang et al., 1999
10	Swine fever	Bovine viral diarrhoea virus	Wensvoort and Terpstra, 1988

Identification of exotic viral pathogens

Many of the animal diseases are spread from territory of one country to another through import of live animals, their products and live attenuated vaccines. To control trans-boundary movement of diseases, various serological and molecular assays along with post-import quarantine measure is being practiced by most of the nations. In India, imported animals are quarantined for 30 days to develop disease symptoms. Several viruses such as RNA virus where, where nucleic acid diversity is high (e.g. BTV), primers designed for diagnosis specific to one territory may not diagnose the viruses from other territory (Kumar et al., 2013; Shafiq etal., 2013). Apart from that, the test needs to be done against pathogens. Despite strict measure of animal import, several exotic viral pathogens have already been entered to India such asBTV (Gollapalli et al., 2012). Introduction of exotic pathogens to previously unexposed population lead to severe disease outbreak, mortality and economic losses. These exotic pathogens can be easily diagnosed by NGS technique and complete genome sequencing based analysis.

Transcriptome analysis

The transcriptome analysis refers to study of complete set of expressed RNA which are produced by a genome of specific cells, microbes etc under specific condition. This technique can be employed to study the loss and gain of specific function of a mutant strain of pathogens, disease diagnosis, functional characterization of genes, detection of molecular pathways inside a cell which may improve the environmental stress tolerance capability, in biomedical research field such as biomarker discovery, risk assessment related to newer medicines etc.Moreover, RNA-Seq analysis can also be used to detect single nucleotide polymorphisms in pathogens and host, allele specific gene expression etc.

In one of the study, RNA-Seq based transcriptomeanalysis using various tools such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) in cattle vaccinated with killed Bovine viral diarrhea virus I (BVDV-I) vaccine was performed to identify the immune response related differentially expressed genes (DEGs) (Lopez et al., 2020). The study identified the severalgenes related with immune response, interferon-γ production and MHC class I genes with up and downregulated activitiesindicating the immune response against the BVDV-I in cattle which potentiates the application of RNA-Seqin animal improvement programs by selection of specific animals with improvedefficacy of the vaccine.

Common tools for NGS data analysis

The complex genomic research demand deep insight of information which is beyond the capacity of traditional sequencing platforms. The NGS has filled the gap and became a regular research tool to address specific problems. However, NGS machines generates huge amount of data which again needs high computing power and specific dedicated software tools for data analysis. For NGS data analysis several bioinformatics tools are available. Some of these tools are commercially available for various computer operating systems.

Conclusion

The current revolution in field of virology is primarily driven by advancement in sequencing technology especiallyby development of massive parallel sequencing technology or NGS. NGS

has led to high throughput genome sequencing with accuracy at reduced cost in comparison to conventional Sanger's sequencingtechnology.In Animal virology, NGS may play a crucial role in earlydisease diagnosis and control. However, in current scenario, NGS techniques are too expensive to use in animal disease diagnosis. The high cost of NGS machine and its reagents along with need of skilled molecular biology andbioinformatics staffs limits its application in routine veterinary applications.However, the recent advancement in NGS technologies such as fast and portable Nanoporesequencing platform may replace currently used other molecular diagnostic techniques in animal virology.

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A Review On Respiratory Tract Infections

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Abstract

Bacteria, fungi and viruses are the major microbes which can enter both nasal and lung region. The present article gives an outline of different bacteria, viruses and fungi which causes respiratory tract infection (RTI). Streptococcus pyogenes, Bacterial rhinosinusitis, Diphtheria, Bacterial pneumonia, Pneumococcal pneumonia, Haemophilic pneumonia and Mycoplasma pneumonia, Tuberculosis, Pertussis (Whooping Cough) Legionnaires Disease and Q disease are the RTI caused by the bacteria. Infections like Histoplasmosis, Blastomycosis, Coccidioidomycosis, Aspergillosis, Candidiasis, and Mucormycosis are some of the examples of respiratory tract caused by fungi. RTI caused by viruses included influenza, common cold, measles, mumps, rubella, chickenpox, and syndromes like SARS and MERS. The present article discusses about the various organisms that causes respiratory tract infection.

Keywords: Upper respiratory tract infection, lower respiratory tract infection, bacteria, Streptococcus pneumonia, Staphylococcus, Influenza, opportunistic infection, Aspergillosis, Histoplasmosis, Mucoromycosis

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Introduction

Respiratory tract infections are caused by bacteria (Gram positive (G +Ve) and Gram negative (G-ve)), fungi, fungal spores, and viruses. They lodge in the upper and lower respiratory tract causing common cold and pneumonia. Some of these are opportunistic in nature. In some cases the major symptoms will be associated with skin but they also infect the upper respiratory and lower respiratory tract as their route of entry to human body is through nasal region. Some of these infections can spread to other parts of the body leading to fatality.

Bacterial infection of respiratory tract

Gram +ve and Gram –ve bacteria causes RTI and in some cases it can affect other parts of the body.

Streptococcus pyogenes

Streptococcus pyogenes transmitted through air droplets enters the respiratory tract and forms a condition called Streptococcal pharyngitis. They are G+ve cocci in chain. Connective tissues are degraded by using hyaluronidase, collagenase and streptokinase. Streptokinase causes cleavage of blood clots, which assists in the spread of the pathogen.

The classic symptoms of streptococcal pharyngitis include fever, pain redness, swelling of palatine tonsils and affect the soft and hard palatine regions. The mode of transmission is by droplet and direct contact.

Serological diagnosis with group A antigen and culture methods are used for diagnosis of S.pyogens[1]

Bacterial rhinosinusitis

Nasopharynx contains diverse microbes and most of them are opportunistic in nature. Bacterial rhino sinusitis is often seen as secondary infection after the onset of a viral infection. As the bacteria affect the nose and paranasal sinuses it is also called rhinosinusitits. Bacterial rhinosinusitis are caused by Streptococcus pneumonia, Haemophilus influenza, and Moraxella catarrhalis.

Diphtheria

The causative agent of diphtheria, Corynebacterium

diphtheria, is a G+ve rod that belongs to the phylum Actinobacteria. Diphtheroids are commensals but, some strains of C. diphtheriae has a temperate bacteriophage-encoded protein—the diphtheria toxin giving them pathogenic nature. It can also cause impetigo-like lesions on the skin. Children are more affected than adults older than fourty and is transmitted through droplets and aerosols produced by coughing. After colonizing the throat, the bacterium remains in the oral cavity and begins producing the diphtheria toxin. Toxin has two subunit A(effector) and B(binding unit) and blocks host-cell protein synthesis by inactivating elongation factor (EF) and leads to death of cell. Dead cells accumulate and form psuedomembrane (classical sign of diphtheria). The pseudo membrane enlarges to obstruct the fauces of the pharynx or trachea and can lead to suffocation and death. Toxin spreads throughout the body; it can damage other tissues as well damaging the heart and nerve cells. The disease is diagnosed by bacterial culture using throat swabs, toxin detected by amplifying tox gene using polymerase chain reaction and antigen detection like radial immunodiffusion or Elek'simmunodiffusion test.

Penicillin and erythromycin effectively control C. diphtheria infections and toxins are nullified using antitoxins (preformed antibodies against the toxin).

Pneumococcal Pneumonia

The most common cause of community-acquired bacterial pneumonia is Streptococcus pneumonia. This Gram-positive, lancet shaped, alpha haemolytic Streptococcus is commonly found a normal microbiota of the human respiratory tract and appears as pairs[2]. The pneumococcal initially colonize the bronchioles of the lungs and spread to all part of lungs. The polysaccharide capsule prevents the phagocytic clearance. Pneumolysin O is a protein helps in the attachment of bacteria to the host cells and induces cytokine production. The cytokine induces inflammation and accumulation of neutrophils and red blood cells inside the alveoli. Bloody sputum is the main symptom of this disease. The infection is identified by microscopic observation and blood culture. Alpha haemolysis is observed on blood agar medium. All clinical pneumococcal isolates are stereotyped using the quelling reaction with typing antisera produced by the CDC. S. pneumonia is extremely sensitive to optochin and colonies are rapidly destroyed by the addition of 10% solution of sodium deoxycholate.

Haemophilus Pneumonia

They are Gram negative and cocobacillus in nature, Aerosols containing H. influenza are the main agents for transmitting disease. These are fastidious in nature as they grow well on media containing factor X (hemin) and factor V (NAD), like chocolate agar. The organisms are confirmed by antigen antibody interaction and isolation methods.

Mycoplasma Pneumonia (Walking pneumonia)

Mycoplasma pneumonia a wall less slow growing pleomorphic bacteria causes Pneumonia also called Walking pneumonia is spreaded through aerosols. Fever and cough are the main symptom of walking pneumonia. The pathogenesis is by using specialized attachment organelle which bind to ciliated cells and damaging the epidermal cells. Mycoplasma grows very slowly when cultured. To prevent the growth of other fast growing organism penicillin and thallium acetate are added to agar. The recovery from M. pneumonia infections are faster and can be cured by macrolide antibiotic therapy.

Chlamydial Pneumonias and Psittacosis

Chlamydial pneumonia and Psittacosis are caused by obligate intracellular pathogens. Chlamydophila pneumoniae (formerly known as Chlamydia pneumoniae), Chlamydophila psittaci (formerly known as Chlamydia psittaci), and Chlamydia trachomatis. Of the three, Chlamydophila pneumoniae is the most common and is transmitted via respiratory droplets or aerosols. Chlamydia trachomatis, the causative agent of the sexually transmitted chlamydia, can also cause congenital chlamydia

Diagnosis of chlamydia by culturing tends to be difficult and slow. Because they are intracellular pathogens, they require multiple passages through tissue culture. PCR and serologically based tests are used for easier identification of these pathogens. Tetracycline and macrolide antibiotics are typically prescribed for treatment.

Pseudomonas Pneumonia

Pseudomonas aeruginosa is a nosocomial infection leading to bacterial pneumonia in patients with cystic fibrosis (CF). P aeruginosa secretes exotoxins and acts as virulence factor. Defective Cystic fibrosis transmembrane receptor (CFTR) results in accumulation of mucus in the alveoli. Mucocilary escalator is inhibited and defensins produced by the host will not be effective. Exopolysaccharide secreted by the organism helps them to escape the phagocytosis process and enable them to multiply inside the lungs. The major cause of death in

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patients with CF is due to lung damage[3].

Tuberculosis

M. tuberculosis is an acid-fast, high G + C, Gram-positive, nonspore-forming rod and slow growing. Mycolic acid present on the cell wall of bacteria determines the permeability. M. tuberculosis can enter any part of the body and form tubercles. The mode of transmission of the bacteria is by inhalation of respiratory droplets or aerosols containing mycobacteria[4]. The bacterium enters the lungs through inhalation and inside the lungs the alveolar macrophages engulf the bacteria. The bacterium has the ability to prevent the fusion of the phagosome with the lysosome. The presence of the bacteria inside the alveoli leads to activation of neutrophils and macrophages and migration of cells to that area. Activated immune cells damage the infected and normal cells causing liquefaction. The cells during this stage will soft and caseous. Due to calcium deposition inside the alveoli cells and accumulation of dead immune cells, the area become thick and form a complex called Ghon complex[5]. Initial stages of infection it is called as primary tuberculosis and later on the reactivation of infection is called secondary tuberculosis. The bacteria can enter the blood and enter different parts of the body and this condition is called disseminated tuberculosis. The formation of tubercle and Ghon complex can be identified by X-ray diagnosis of the infected lung. The disease can also be diagnosed by Acid fast staining of the patient's sputum. As this is a slow growing organism the culturing the organism is difficult. The identification of organism is done by PCR methods.

The treatment modality for TB is using antibiotics. Due to misuse of antibiotics more organisms become resistant to these drugs and based on drug resistance M. tuberculosis are named as multidrug resistance tuberculosis (MDR-TB) and extensively drug-resistant (XDR-TB) tuberculosis strains. So for treatment combination of different antibiotics like isoniazid, rifampin, ethambutol, and pyrazinamide are given for one month. The prevention of the disease can be done by vaccination using Bacillus Calmette-Guérin (BCG) strain of M. bovis commonly found in cattle.

Pertussis (Whooping Cough)

Whoophing cough is caused by Bordetella pertussis, a Gram-negative Coccobacillus. Due to the accumulation of mucus in the respiratory tract, the blockage of air passage will occur and the patient produce a whoop sound during coughing. The cough lasts for more than two weeks. Infants and children are more affected with the disease. Following inhalation, B. pertussis specifically attaches to epithelial cells using an adhesin, filamentous hemagglutinin. The organism produces A-B exotoxin also called the pertussis toxin (PT). The mechanism of action of PT is by increasing the expression of the cyclic adenosine monophosphate (cAMP) levels and disrupts cellular signaling. The exotoxin increases the level of inflammatory mediators like histamine and serotonin. Tracheal toxin damages the ciliated epithelial cells and accumulation of mucus in the lungs. The CDC reported 20 pertussis-related deaths in 2012, but that number had declined to five by 2015[5].

Specimen collected directly from a nasopharyngeal (NP) is streaked onto Bordet-Gengou medium within twenty four hours of collection of sample. B. pertussis infection is diagnosed using PCR techniques and ELISA methods.

Legionnaires Disease

An aerobic Gram-negative bacillus, Legionella pneumophila is commonly seen in cooler region like air-conditioning cooling towers, humidifiers, misting systems, and fountains. The mechanism of pathogenesis is by preventing the fusion of phagosome with the lysosome by the protein secreted by the microbes which can bind to endosomal membrane. The disease can range from mild to severe pneumonia, depending on the status of the host's immune defences. Although this disease primarily affects the lungs, it can also cause fever, nausea, vomiting, confusion, and other neurological effects.

Culturing of L. pneumophila is difficult and is fastidious bacterium. Warthin-Starry silver-precipitate is used to visualize this pathogen. Detection of Legionella antigen in a patient's urine is specific and selective and takes less than one hour.

Legionnaire disease can be effectively treated with fluoroquinolone and macrolide antibiotics. However, the disease is sometimes fatal; about 10% of patient's die of complications [6].

Q Fever

The zoonotic disease Q fever is caused by a rickettsia, Coxiella burnetii. The primary reservoirs for this bacterium are domesticated livestock such as cattle, sheep, and goats. The bacterium may be transmitted by ticks or through exposure to the urine, feces, milk, or amniotic fluid of an infected animal. In humans, the primary route of infection

is through inhalation of contaminated farmyard aerosols. It is, therefore, largely an occupational disease of farmers. Humans are acutely sensitive to C. burnetii—the infective dose is estimated to be just a few cells[7]. Symptoms associated with acute Q fever include high fever, headache, coughing, pneumonia, and general malaise. In a small number of patients (less than 5%)[8]. The condition may become chronic, often leading to endocarditis, which may be fatal. Diagnosing rickettsial infection by cultivation in the laboratory is both difficult and hazardous because of the easy aerosolization of the bacteria, so PCR and ELISA are commonly used.

Doxycycline is the first-line drug to treat acute Q fever. In chronic Q fever, doxycycline is often paired with hydroxychloroquine.

Fungal infections of respiratory tract

Invasive fungal infections occur in both immunocompetent and immunocompromised patients.

Histoplasmosis:

Histoplasmosis is an intracellular infection of the reticuloendothelial system caused by the dimorphic fungus Histoplasma capsulatum. Infection is acquired by inhalation. The large majority infections are asymptomatic and as in tuberculosis, heal, leaving behind an area of miliary calcification. The fungus H capsulatum is endemic to the Ohio and Mississippi River valleys, Central American and Southeast Asian rivers, and the Mediterranean[9]. H capsulatum grows optimally in caves and bird roosting areas with rich nitrogen soil. Itraconazole (mild and chronic pulmonary disease) and combination of Amphotericin B (AmB) with itraconazole (moderate-to-severe) are used for treatment of histoplasmosis[10].

Blastomycosis:

Blastomyces dermatitidis is endemic to the Great Lakes, the Mississippi and Valleys of Ohio River, the South eastern United States, and the African Mediterranean[11, 12]. The fungus grows in dead or decaying wood and acidic soil and near to bodies of water. Blastomycosis occurs with mold inhalation into the alveoli, where further dissemination may ensue[10,11]. Extrapulmonary dissemination involving the skin occurs in up to 40% of cases[13] Treatment includes itraconazole for mild-to-moderate disease and liposomal AmB (L-AmB) followed by itraconazole for life-threatening pulmonary infections[10].

Sporotrichosis

Sporothrix schenckii is globally located and not endemic to certain regions[13]. This fungus may be found in soil, decaying material, moss, hay, and infected animals. Infection results primarily from cutaneous contact with sporotrichosis[11,13]. Pulmonary sporotrichosis and nodular lesions result from inhaling S schenckii. Mild-to-moderate pulmonary disease requires Iitraconazole, whereas AmB followed by itraconazole is used for treating severe disease [10].

Coccidioidomycosis:

Coccidioidomycosis is endemic to South America, Central America, northern Mexico, and the western U.S., fungal growth occurs in nitrogen-enriched soil from rodent and bat droppings[10,14]. Coccidioides immitis and Coccidioide sposadasii are indistinguishable fungi, with C immitis being more common. Inhalation of a few inocula may cause pulmonary disease, with presentation as community-acquired pneumonia in endemic areas. Immunocompromised patients are treated with fluconazole or itraconazole. In serious pulmonary disease, treatment with AmB is initiated, followed by an azole[10.14].

Aspergillosis

Aspergilla fungi isolated from soil, plant debris, and indoor environments—are the most common cause of mortality due to invasive fungal infections[10, 15]. Severely immunocompromised patients the spores entered in to lungs cause pulmonary aspergillosis (IPA) and chronic necrotizing aspergillosis in patients with chronic lung diseases. Aspergilloma and allergic bronchopulmonary aspergillosis (identified in patients with a hypersensitivity to aspergillus antigens) are noninvasive manifestations[16]. The primary treatment for IPA is voriconazole and lipid-based AmB formulations, echinocandins, and posaconazole[10].

Cryptococcosis

Cryptococcosis is an opportunistic infection seen in immunocompromised individuals, including HIV or AIDS patients and organ-transplant recipients. Most of these show no symptoms, which results in a dormant infection[10,17, and 18]. Found in soil contaminated with pigeon droppings, cryptococcosis commonly presents as cryptococcal meningoencephalitis; it also occurs as an isolated primary infection in the lungs after spore inhalation [19] The spores can disseminate in to the central nervous system.

The treatment strategy of severe symptomatic pulmonary cryptococcosis is AmB formulation with or without flucytosine, followed by oral fluconazole, For immunosuppressed or immunocompetent patients exhibiting mild-to-moderate symptoms, fluconazole therapy is recommended[10,17].

Candida pneumonia

Infection is in the pulmonary area is rare and difficult to diagnosis. Primary Candida pneumonia refers to an invasive infection in the lungs, while secondary pneumonia refers to dissemination of invasive candidiasis[10,20]. Colonization of the lung parenchyma with Candida species is common; in critically ill patients, however, and defence mechanisms are rendered ineffective, thus enabling penetration of lung tissue. Triazole antifungals and echinocandins, AmB formulations are effective for treating pulmonary candidiasis [20, 21].

Mucormycosis

Pulmonary mucormycosis is primarily observed in patients with a predisposing condition of neutropenia or corticosteroid use[22]. Fungal attachment and damage of endothelial cells and invasion of vessel thrombosis, and successive tissue necrosis can lead to disseminated mucormycosis infections. These complications make for poor penetration of antifungal agents.

Treatment should include control of the predisposing problem, debridement of necrotic tissue, and antifungal therapy. Current recommendations for efficacious treatment of mucormycosis include AmB formulations, posaconazole, and iron chelation therapy. Although echinocandins as monotherapy do not act against mucormycosis, a few studies have found improved outcomes when AmB and an echinocandin are used[10, 14].

Pneumocystis jirovecii

Pneumocystis pneumonia (PCP)

PCP mostly seen in patients with HIV/ AIDS, hematologic and solid malignancies, organ transplant, and diseases requiring immunosuppressive agents. Infection occurs through the inhalation of airborne spores, with further maturation occurring in the lungs[23].

PCP is extremely resistant to common antifungal therapy, including AmB formulations and triazole antifungal. Trimethoprim / sulfamethoxazole remains the mainstay for PCP treatment and prophylaxis. Drugs like primaquine plus clindamycin, atovaquone, or IV pentamidine. In addition, dapsone is an alternative for prophylactic therapy[10].

Viral infection of respiratory tract

The Common Cold

More than 200 different viruses are known to cause the common cold. Viruses coming in Rhinoviruses, coronaviruses, and adenoviruses group are related to common cold. The aerosols produced during the coughing and sneezing persist on environmental surfaces for up to a week[24]. The route of entry of the viruses is nasal mucosa and eyes. The optimum temperature for replication is below normal body temperature (37 °C [98.6 °F]). The localisation of virus is seen in the cooler part of body such as nasal cavities. The attachment of the virus causes irritation of mucosa and causing inflammation. Running nose, congestion in the air passage, sore throat coughing and sneezing are the common signs and symptoms of the disease. A slight increase in body temperature is seen in common cold. The virus after entering the nasal region, it can also enter in ears, pharynx, and larynx causing inflammation. The symptoms subside within a week or two. Once infected by the virus it activates the cell mediated immunity and develops memory cells leading to lifelong immunity.

Influenza

Influenza viruses are enveloped RNA virus and exist as eight segments, each coated with ribonucleoprotein and encoding one or two specific viral proteins. The envelope contains two spike proteins hemagglutinin (H) and neuraminidase (N). The hemagglutinin spike protein binds to sialic acid receptors on the host receptors. Following inhalation, the influenza virus uses the hemagglutinin protein to bind to sialic acid receptors on host respiratory epithelial cells. The virus fuses with the host cell and the RNA enters the host cell where it will be transcribed and translated by using the host mechanism to produce viral proteins. The influenza viruses A (most virulent/pandemic), B(less virulent), and C (mild virulent) make up three of the five major groups of orthomyxoviruses. Influenza A virus can infect a variety of animals, including pigs, horses, pigs, and even whales and dolphins.

Viral Pneumonia

Viral pneumonia is caused by adenoviruses, influenza viruses, parainfluenza viruses, and

respiratory syncytial viruses (RSV). RSV is highly contagious and can be spread through respiratory droplets from coughing and sneezing. The symptoms include mild cold-like and in persons with weak first line defence system virus can enter the lungs and cause Pnuemonia. In persons with co morbid condition the disease will be life threatening.

Coronovirus

Corona viruses are enveloped RNA viruses with high rate of transmission. Pandemic diseases like Severe Acute Syndrome (SARS) and Middle East respiratory syndrome (MERS) are two acute respiratory infections caused by corona viruses. The main reservoirs of these viruses are animals like cats, bats, camel etc. Covid-19 diseases are caused by newly modified varients of coronavirus and have high pandemicity. There are no specific treatments for either MERS or SARS. Several recombinant vaccines, however, are being developed.

Measles, rubella (German measles), and chickenpox

Measles, rubella (German measles), and chickenpox causing skin rashes enters the body through the respiratory tract.

Measles, rubella (German measles), and chickenpox

Measles, rubella (German measles), and chickenpox causing skin rashes enters the body through the respiratory tract and can be included in respiratory infections.

Measles (Rubeola)

The main reason for the death of children is due to infection by rubeola virus[25]. They belong to minus stranded RNA virus possesses an envelope containing hemaglutinin protein. Release of minute particles during coughing, sneezing, breathing are the main agents for the transmission of viruses. Once it enters the body it can cause increase in temperature, inflammation in the conjunctiva, and sore throat. Rashes on the skin surface appear due to viremia and these rashes last for several days. Kopliks spot are the characteristic symptoms of measles disease. The virus can enter the lungs and cause pneumonia; it can cause inflammation of encephalus region of brain and can be a reason for fatality[26].

The appearance of rashes and kopliks spots is used for the diagnosis of disease. Hemagglutination inhibition tests and serological tests may be used to confirm measles infections in low-prevalence settings. MMR (measles, mumps, and rubella) is the vaccine administered for prevention of disease.

Rubella (German measles)

Measles and Germen measles have the common symptoms like fever and rashes on the skin. German measles is caused by Rubella virus. Rubella viruses are belonging to enveloped RNA viruses. Virus enters through respiratory tract and in most of the individuals they are in apparent in nature. Apparent symptoms includes less intense facial rashes which will remain only for two to three days and doesn't have Kopliks spot(2–3 days), not associated with Koplik's spots, and the resulting fever is lower (101 °F [38.3 °C]). Vertical transmission occurs leading to congenital rubella syndrome and can also cause malformation of the foetus or still birth[27]. The disease diagnosis is done by observing the rashes on the faces and can be confirmed by serological methods. Disease subsides within two to three days and can be prevented by MMR.

Chickenpox and Shingles

Varicella zoster belonging to herpes virus family is the causative agent of chicken pox and is also transmitted by direct contact or inhalation of material from the skin lesions. Pregnant ladies will transmitting the virus to the foetus leading to some abnormality or birth defects in the baby called Reye syndrome. The viral infection initially produces pustules on the upper part of the body and the spreads to other part of the body. Varicella zoster, when reaches the lungs can lead to pneumonia in adults. The presence of virus in the blood results in chills and fever. The virus infected individual produces lifelong immunity.

The presence of pustular rashes indicates the presence of Varicella virus and is used for disease diagnosis. Other detection system include antigen antibody testing and isolation viral RNA and its amplification. The disease will subside without any treatment and in severe cases acyclovir is the drug of choice.

Conclusion

The respiratory tract infections are caused by all groups of microbes. In all infections the mode of entry is through respiratory tract and causing cough and later on pneumonia. Even though the organisms mainly cause rashes and pustules but the route of entry of these organisms is also through nasal route causing pneumonia and they are considered as respiratory tract infections. As they are transmitted through air droplets these

infection are rapidly spreading. Diagnosis is by isolation, serological and PCR methods. Most of the respiratory infections are self limiting and others are controlled by antibacterial ,antifungal and antiviral drugs. Prevention of RTI is mainly through vaccines. Vaccines are available for most of the RTI and in the case of viruses the vaccine development is facing problems as variation in structure occurs due to mutation

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Erratum

"A Study of Blood Stream Infections in Critical Care Units"

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The original published version of this Article contained errors in affiliation offirst author mentioned, Now title of article readed as

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