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Molecular Approaches to the Diagnosis of Infectious Diseases

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Abstract

Infectious diseases are the deadly processes being caused by a number of microbes. However, the timely detection of these agents can save many life's. Although many biochemical techniques are available however, molecular approaches are far better to give authentic and timely results. The molecular diagnostic tools such as polymerase chain reaction (PCR) act as novel armamentarium in fight against existing as well as emerging infectious diseases. Moreover such an approach brings a new dimension in the early detection of infectious agents and would help in the better management thereby providing further strength to our pandemic preparedness. The intent of this review is to summarize the overview of molecular approaches to detect deadly infection agents.

Introduction

Infectious diseases being the leading causes of morbidity and mortality worldwide are a major public health concern (1). Around 50 new infectious pathogenic agents (microorganisms) have been identified during the last 40 years (2). In fact every contagious disease that emerged at some time in the past, remain persistent in the population by a chain of human to human transmission. Rapid and precise diagnosis followed by early therapeutic intervention is thus the cardinal component for arresting the progression of an infectious disease not only at individual level but also within the community. The inherent limitations of conventional microbiological diagnostic modalities to detect and identify the infectious organism in a timely fashion pose inevitable resistance to the meaningful clinical management of the disease (3,4). Additionally, the serological laboratory tests relying on antigen detection by immunofluorescence or enzyme immunoassays may have variable diagnostic sensitivities or specificities (5,6). Thus a rapid, accurate, and reliable diagnostic method is very important, as it allows identification of the disease for suitable therapy, which consequently can reduce the mortality rate by interrupting the human chain of transmission.

The unprecedented advancement in Molecular Biology provides a repertoire of tools for use in the clinical diagnostics and biomedical research. Of these, nucleic acid amplification tools or assays (NAAT or

NAA) have drawn the maximum attention owing to its enormous potential in detecting and identifying infectious agents for which routine growth-based culture and microscopy methods may not be adequate (7–9). Furthermore it is a well-established fact that in the past epidemics or outbreaks, caused by previously unknown infectious agents, the identification and characterization of a new infectious agent usually took years to decades or even centuries. However with the advent of powerful Molecular Biology techniques, such time frames have been decreased to months or weeks, as illustrated by the identification of H1N1/09 pandemic flu virus within weeks of the first case reported. There are several procedures for NAA such as polymerase chain reaction (PCR), strand displacement amplification (SDA), Transcription mediated amplification (TMA), Q-beta replicase amplification, Ligase chain reaction (LCR) etc. (8,10). However, PCR is the simple and most widely used assay in establishing the diagnosis of many infectious diseases (11). In addition to the rapid detection and identification of non-cultivable or very slow growing organisms, it also facilitates strain typing in epidemiological studies, antimicrobial susceptibility determination and monitoring treatment by measuring bacterial or viral loads.

Polymerase chain reaction (PCR)

PCR has emerged as a powerful tool in clinical medicine for the exponential in vitro amplification of specific sequences of interest from the minute quantities of nucleic acid using a thermostable DNA polymerase. Thus instead of biologically increasing the number of microorganisms on artificial media, PCR directly increases the amount of specific nucleic acid target in vitro. The essential materials, reagents and equipment required for performing amplification reaction are summarised in Table 1.

Table 1: Reaction ingredients and equipment for the PCR

Template	Specific sequence of nucleic acid (DNA /RNA) for amplification
Deoxyribonucleotides (dNTPs)	dATP, dGTP, dCTP, dTTP (A-Adenine; G-Guanine; C-Cytosine; T- Thymine)
Primer	Forward and reverse primers complementary to the flanking region of known sequences of the template
DNA polymerase	Thermo stable Taq DNA polymerase for catalysing extension of annealed primers
Themocyler	An equipment which perform the temperature regulated PCR cycles

In order to perform PCR, initially, DNA is extracted from the microorganism present in the clinical specimen under investigation. The known sequence of the target for amplification allows the synthesis of primers. Subsequently a series of cyclic reactions are developed, each cycle consisting of three steps. The first is the denaturation step, which involves the dissociation of double stranded template

DNA into single stranded DNA by heating at 94°C. This is followed by the second step known as annealing of primers to the complementary region of target DNA at an optimized temperature. The third step is the extension step which is developed through the polymerization of the new DNA strand with the help of DNA polymerase at a temperature of 72°C. This results in the accumulation of multiple copies of target segment limited by the primers. Finally amplified products are detected by agarose gel electrophoresis stained with ethidium bromide.

A number of in house and commercial PCR assays have been developed for the diagnosis of a plethora of infectious diseases ranging from bacterial to parasitic infection e.g. Pneumonia, Meningitis Tuberculosis, Hepatitis B and C virus, Herpes simplex virus, HIV, cytomegalovirus, H1N1/09, Malaria etc (12). This review will provide in depth analysis of the molecular diagnosis of Tuberculosis and H1N1/09 pandemic flu or swine flu.

Tuberculosis

Tuberculosis (TB) is one of the key global health concerns that afflicts one third of the world population (13). The causative agent of this disease, *M. tuberculosis* has remarkable power to persist and is a major cause of human mortality and morbidity. TB is an air borne highly contagious disease which primarily involves lungs (Pulmonary TB), but can affect almost every organ of the body (Extra pulmonary TB)(14).

Disease Magnitude:

Based on surveillance and survey data, the World Health Organisation (WHO) in its latest report from the year 2018, estimates that 10.0 million individuals developed active tuberculosis in the year 2017. The most disturbing fact is among the 1.6 million persons who died from tuberculosis in the year 2017, 1.3 million were seronegative and 300 000 seropositive for HIV infection. India has the highest TB burden where more than 3561 people develop disease and nearly 1153 die of TB per day accounting for 0.421 million causalities and 1.3 million new cases annually (13).

Clinical diagnosis:

Prompt diagnosis followed by immediate instigation of anti-tuberculosis therapy (ATT) is the cardinal point in the management of TB. Ironically, the rapid detection of individuals with tuberculosis can be difficult as only 44% of all new cases and only 15–20% of children are identified by acid-fast bacilli (AFB) microscopy on sputum smears (15,16). Undoubtedly, the gold standard for the diagnosis of tuberculosis is the recovering of tubercle bacilli from clinical specimens by microbiological culture (17). However, culture growth of *M. tuberculosis* may take 2 or more weeks on an average for generating a meaningful result. This encourages ad hoc decision to initiate ATT merely on the basis of clinical suspicion which in turn is furthermore misguiding particularly in paucibacillary pulmonary and extra pulmonary cases due to varied clinical manifestations (18). Looking at this perspective, there is a strong need to develop non-conventional rapid diagnostic tools such as PCR that expedite the precise diagnosis of TB thereby achieving the twin goals of the patient care and disease management. There are a substantial number of PCR based molecular diagnostic studies in TB that has enabled: Direct detection of *M. tuberculosis* complex in clinical specimens, differentiation of *M. tuberculosis* from members of MOTT complex, detection of drug resistance and epidemiological studies (19).

Interestingly, PCR results can be available to the clinician within 1 day after obtaining clinical specimen and can have important implication for the management of a patient. Although a number of amplification targets have been used for designing TB-PCR test, yet IS6110 which is a multicopy target remains the target of choice for this purpose (20,21). In microscopically positive disease presentation, PCR reliably confirm the presence of specific mycobacteria whereas PCR has also been employed in smear negative pulmonary and extra pulmonary disease where the diagnosis is conventionally inconclusive. The clinical value of in-house and/or commercial PCR performed on respiratory specimens for diagnosis of pulmonary tuberculosis has been reviewed in meta-analyses (22). In individuals with positive AFB sputum smears, the sensitivity of PCR to detect *M. tuberculosis* nucleic acid on these specimens is greater than 95%. However, a negative amplification result in this situation strongly indicates the presence of a non-tuberculous Mycobacteria (NTM) species in this specimen. In contrast, the estimated sensitivity of PCR test particularly of in house nature for the diagnosis of active tuberculosis in sputum smear negative pulmonary and extra pulmonary individuals is highly heterogeneous and is not consistently accurate enough to be routinely recommended for establishing the disease diagnosis (22).The variations in the clinical performance of PCR in TB diagnosis are attributed to different protocols for the extraction of *M. tuberculosis* DNA, variation in amplification protocols used for diverse PCR targets, presence of PCR inhibitors. So concluding, these aforesaid lacunae need to be addressed seriously in order to smoothly implement this test in the routine clinical diagnosis of TB that in turn positively influence the clinical decision making and thus the disease management.

H1N1/09 flu

Origin:

Influenza commonly referred to as flu is an acute respiratory contagious disease caused by RNA viruses belonging to the family orthomyxoviridae. These influenza viruses can afflict birds as well as mammals. Influenza viruses are categorized into three classes namely influenza A, B and C. It is pertinent to mention that most of the global pandemics are known to occur due to influenza type A viruses which are having three hosts as human, avians and swine. The genome of type A influenza virus codes for two important surface glycoproteins namely Haemagglutinin (H or HA) and Neuraminidase (N or NA). In avian and animal influenza viruses, 16 distinct HA (H1-H16) and nine distinct NA (N1-N9) have been recognized on the basis of sequence and antigenic analysis (23). Importantly only three HA subtypes (H1, H2, H3) and two NA subtypes (N1, N2) have caused extensive outbreaks in human population (Fig 1).

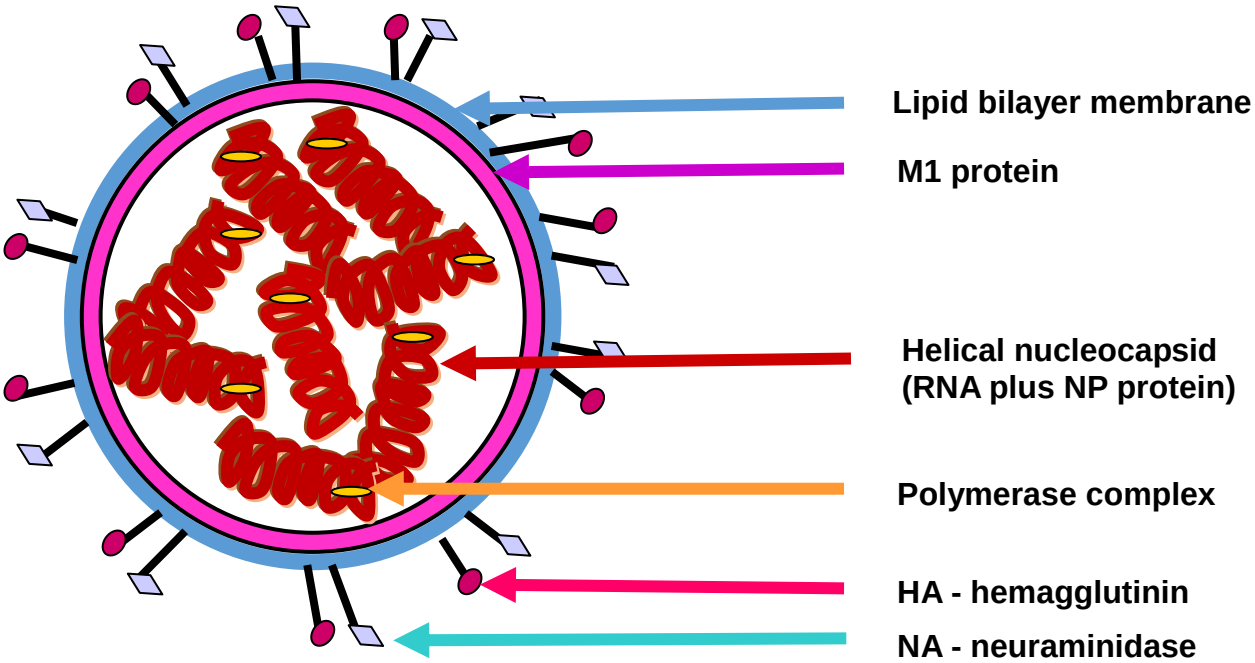

Figure 1: Structural diagram of H1N1 Virus.

There are ample evidences that significantly revealed that new strains of influenza viruses are continuously evolving either by antigenic drift or antigenic shift (24). Antigenic drift is the gradual but significant change in the antigenicity of type A and B viral subtypes due to the frequent mutations that occurred in the surface HA and NA glycoproteins during error prone replication of the virus e.g in 2003-04, the circulating H3N2 virus developed over 80% drift from the virus that was used to make one of the three major vaccine component in that year.

On the other hand antigenic shift is the marked changes in HA, with or without similar changes in NA gene segments acquired by re-assortment of genetic segments during dual infection of cells by a human and an animal virus (Fig 2).

Antigenic shifts are associated with Influenza A epidemics and pandemics. As there exists no immunity against these new viruses, so they may cause devastating pandemic whenever they get a chance to make an entry into human population (24). The pandemic influenza virus A/H1N1 2009 that emerged in March 2009, and a pandemic was declared the following June. Genetic analysis revealed that H1N1 2009 influenza virus resulted from the quadruple reassortment of North American H3N2 and H1N2 swine viruses (triple reassortment viruses: avian/swine/human with Eurasian swine viruses) (25).

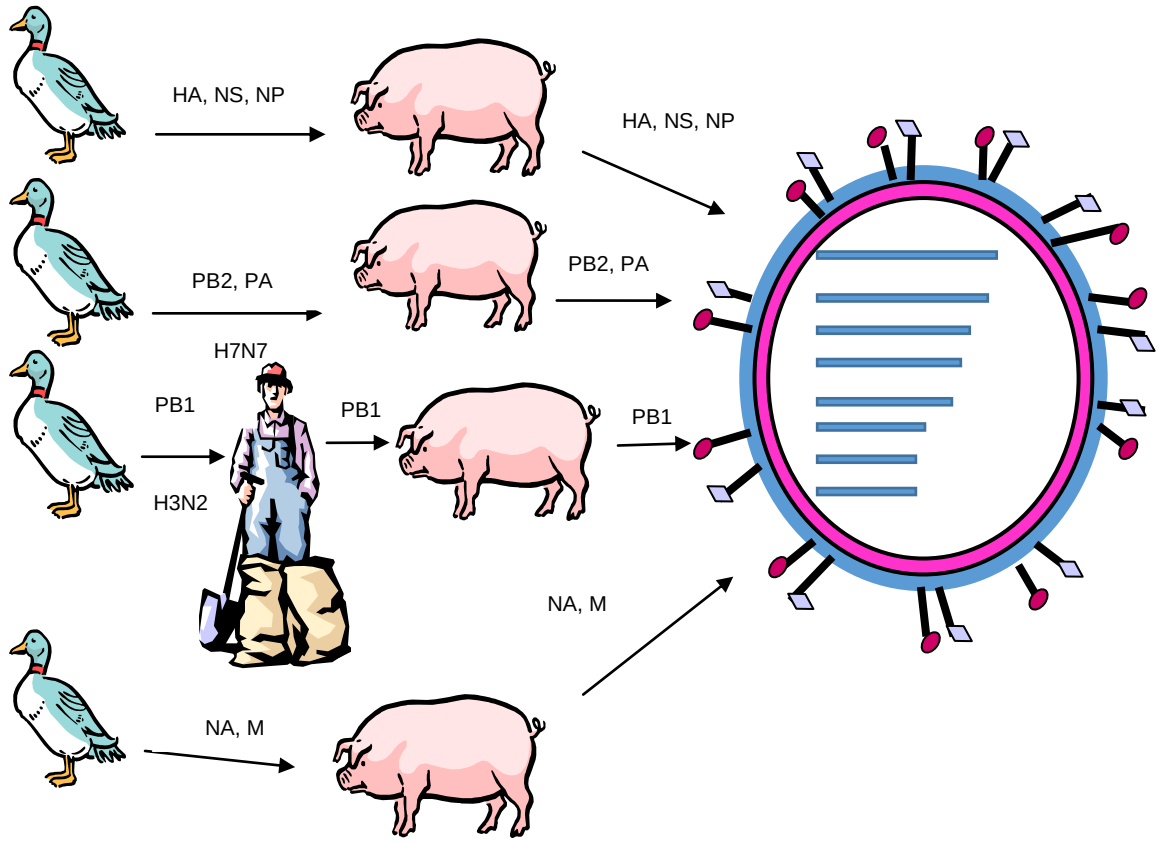

Figure 2: Genetic re-assortment in Influenza virus (Origin of 2009 H1N1) Where HA-Hemagglutinin, NS-Nuclear export protein, NP- Nucleoprotein, (PB2, PA, PB1)-Polymerases, NA- Neuraminidase & M-Matrix Proteins.

Disease Magnitude:

Epidemiological data significantly suggested that H1N1 influenza 2009 flu pandemic or Swine flu started as an outbreak in the Mexican town of La Gloria, Veracruz, in mid Feb 2009. Subsequently the international spread and human to human transmission of this virus prompted World Health Organization (WHO) to declare it as a global pandemic on 11th June 2009 i.e. the first influenza pandemic of 21st century (26). According to grim statistics revealed by WHO, around 1.56 million people got infected and 17,000 die globally due to this alarming pandemic by Jan 2010. Currently this novel influenza virus has made its entry in 213 countries worldwide. Most patients in the world with swine flu have been teenagers and young adults. With respect to the Indian scenario approximately 28,000 people developed infection while 1100 succumbed to death.

Clinical manifestations:

As far as clinical presentation is concerned, the symptoms of H1N1 infection resemble seasonal influenza infection. The frequently observed symptoms in patients with H1N1 influenza A 2009 infection includes sudden onset of fever, cough, sore throat, runny and stuffy nose, lack of appetite, lethargy, nausea, vomiting and diarrhoea. Complications of H1N1 appear similar to seasonal influenza and include myocarditis, bacterial co-infections, and neurological complications such as encephalitis (27).

Transmission:

Recent evidences indicate that the new H1N1 influenza virus A is primarily transmitted via large particles droplet released during coughing and sneezing. The incubation period for the 2009 H1N1 virus has been estimated to be betwe9+en 1 and 7 days (27). It is assumed that infected persons start shedding virus 1 day before the onset of symptoms and shed at least until symptoms resolve, however, Children and immunosuppressed persons may shed virus for weeks. Contact with the contaminated surfaces is another potential source of viral infection as it has been documented that these viral particles after depositing over an environmental surface remain contagious for about 2-8 hours (28).

Laboratory diagnosis:

There are two essential conditions which a diagnostic test must have in order to establish the diagnosis of H1N1 influenza A/2009 infection:

1. Given the H1N1 09 influenza virus is highly contagious so much so that the infected person start spreading the virus even when he himself does not know about his clinical status. Thus there is an inevitable need to have a rapid and sensitive diagnostic approach to prevent the further transmission of this virus.

2. There is an absolute need to categorically differentiate the H1N1/09 influenza virus from the seasonal influenza. So the diagnostic test should be specific enough.

The clinical diagnostic approaches for establishing the diagnosis of H1N1/09 influenza virus includes conventional viral culture, serological methods and molecular methods (rRT-PCR) (5,26). The serological tests e.g. RIDT, DFA etc. are easy to perform and results can be obtained swiftly. However, these tests have lower sensitivities than rRT–PCR tests or viral culture and cannot distinguish between pandemic (H1N1) 2009 and seasonal H1N1 or H3N2 influenza A viruses. Isolation of pandemic (H1N1) 2009 virus by a positive viral culture is diagnostic of infection; however, the results may be too slow to guide clinical management. Furthermore a negative viral culture does not exclude pandemic (H1N1) 2009. Therefore, the molecular tool i.e. real time reverse transcriptase PCR (rRT-PCR) which can detect the concerned virus rapidly with an unprecedented sensitivity and specificity is the only recommended method for the confirmation of H1N1 09 virus in the suspected patients (26). This rRt-PCR facilitates the detection of virus using primer and detector sequences tailored to the specific detection of this virus. However the facility of this test is limited particularly in the resource constrained settings and the test generates

heterogeneous findings with respect to the different clinical specimen. So in order to contain this pandemic and to prevent its sporadic outbreak, it is essential to standardize further and make cost effective this rRT-PCR in such a way that rapid and precise diagnosis ensures timely commencement of antiviral therapy and arrest the further advancement of this disease.

Conclusion

The molecular diagnostic tools such as PCR act as novel armamentarium in fight against existing as well as emerging infectious diseases. Moreover such an approach brings a new dimension in the early detection of infectious agents and would help in the better management thereby providing further strength to our pandemic preparedness.

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Conflict of interest

Authors declares no conflict of interest

Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors

Author contributions

The authors have equivocal contributions in the present study.

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