DIAGNOSTIC METHODS OF INFLUENZA A FROM CLINICAL SPECIMENS: A CRITICAL REVIEW OF LITERATURE.

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ABSTRACT
Influenza viruses cause seasonal epidemics with patients displaying respiratory infection leading to high morbidity and mortality in some cases. However, even during periods of epidemic prevalence, clinical diagnoses are problematic. Clinical laboratory tests became more important, especially after the H1N1 pandemics in 2009. To date, several tests are available from classic to molecular approaches, although some of them may present limitations for a promptly diagnostic response, including time consuming, average sensitivity or sensibility. From April 2009 to the present, the influenza A(H1N1)pdm09 virus has been evolving continuously, acquiring new amino acid changes that may alter its antigenic characteristics, virulence, and its antiviral drug susceptibility. This review provides an overview of various techniques, including those recommend by Centers for Disease Control and Prevention, for specific diagnosis of influenza infection.

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INTRODUCTION
Influenza viruses are associated with severe respiratory complications such as bronchiolitis and pneumonia (Rothberg et al. 2008). Two antigenically and genetically distinct lineages of influenza B viruses are also currently circulating among humans and make a considerable contribution to morbidity attributed to seasonal influenza. Such complex co-circulation of multiple types and subtypes of influenza viruses increases the difficulty of diagnosis and virus identification (WHO 2011). Influenza A viruses can be subtyped according to their antigenic properties of the haemagglutinin (HA) and neuraminidase (NA) glycoproteins. There are virus strains from two virus subtypes circulating in humans: H1N1 and H3N2 (H2N2 strains were also circulating in humans from 1957 to 1968). The H5N1 virus is of avian origin, although this subtype was responsible for human deaths in 1997 and in 2004, however none of the strains was easily transmitted from person to person. In addition, none of the H5N1 strains showed evidence of having acquired genes from circulating human influenza viruses. It is likely that such reassortment event between avian and human influenza viruses could have happened many times over, either in humans or in animals (Medina et al. 2011). Recently, a new subtype H3N1 was identified as a result of reassortment in the canine genes of human H1N1pdm09 and canine H3N2 influenza viruses highlighting that companion animals may be a critical determinant to act as intermediate hosts for influenza viruses (Song et al., 2012). The influenza A subtype H3N1 is endemic in pigs and it has been seen that several H3N1 containing A(H1N1)pdm09-like segments could pose a threat to public health (Pascua et al., 2013). In 2009, the subtype H1N1 spotted the headlines due to the quickly spread worldwide through human-to-human transmission leading to high mortality rates. Its genome possesses none of the previously known markers of human adaptation, and its predecessors possibly circulated undetected in pigs for some time, suggesting that reassortment of the precursor swine lineages probably occurred years before the detection of the virus in humans, different of the influenza virus research community (Medina et al. 2011). The 2009 pandemics demanded a global response by World Health Organization (WHO) and Centers of Disease Control and Prevention (CDC) on the real impact on public health, guidelines on prevention and treatment, such access to antivirals and vaccines (World Health Organization, available at: http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html).

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Underestimated by the number of laboratory confirmed cases, the CDC has estimated the global death toll from 2009 H1N1 at more than 280,000 people, especially because many people who died of flu-related causes were not tested for the disease (Dawood et al. 2012).

The optimal utilization of diagnostics must meet the high standards required for public health goals. Influenza viruses are remarkably known for their features of continuous antigenic change through antigenic drift and shift (Webster et al. 1992). This highlights the importance of rapid and reliable diagnostic methods to prevent shortcomings that would underscore the importance and impact on public health policy, decreasing and/or delaying detection of novel strains, prior to its widespread dissemination (Kumar & Henrickson 2012).

In general, influenza A viruses causes high mortality rates, what requires a rapid, sensitive, and specific diagnostic tool in order to facilitate the appropriate management of patients with these infections. However, laboratory techniques used for viral diagnosis are varied and differ in their sensitivity, cost, and time to obtain results (Harper et al. 2009).

Early detection is critical for containment and reduction of morbidity and mortality during a pandemic. Developing countries are more susceptible to a pandemic risk because of the poor hygiene, overpopulation, lack of trained molecular expertise to implement local diagnostic platforms or insufficient modern tools for diagnosis and treatment (Oshitani et al. 2008). Taking these together, it would be expected the worst case-scenarios during an outbreak of respiratory illness caused by a new subtype of Influenza A (World Health Organization, available at: http://www.who.int/bulletin/volumes/89/7/11-086173/en/).

Taking into account the existing diagnostic systems, they can present a wide variation in diagnostic capabilities with regard to the cost, sensitivity, specificity, and other important performance characteristics, such as turnaround time, throughput, and complexity of use (Kumar & Henrickson 2012).

The CDC recommends some established laboratory diagnostic procedures and testing methods for influenza, including from the classic virological techniques to the most advanced systems in the field of molecular diagnostics. We aim with this review to highlight the advantages and disadvantages about each guided methodology applied in Influenza A diagnostics.

The importance of the collected samples

The good quality of the sample yields a rapid and precise characterization of virus isolates at specialized reference laboratories. A prompt and reliable result for influenza detection lies on the collection of appropriate specimens from human for viral isolation or viral RNA detection (World Health Organization, available at: http://www.who.int/influenza/resources/documents/RapidTestInfluenza_WebVersion.pdf). The specimen's types of choice are nasopharyngeal swabs and aspirates (Wright et al. 2007). However, for a relative performance of diagnostic tests (RIDTs, for instance) some considerations must be evaluated concerning the type, quality and transport of specimen, as well as the age and immune status of the patient (Gavin & Thomson JR 2003). In some occasions, the doctor may prefer the collection of throat swabs, however the specimen collected will contain less viral particles than aspirates samples, which could not be satisfactory for use in rapid testing (Foo & Dwyer 2009).

Specimen transport has an impact on accuracy of rapid diagnostic influenza tests. After collection, nasopharyngeal aspirates specimens should be transported in sterile vials and nasal or throat swabs in viral transport medium, in order to prevent dry out, contamination or rapid RNA degradation. These procedures will allow viral isolation, rapid testing and molecular methods to be performed on the same specimen (Gavin & Thomson JR 2003). Notwithstanding, only the use of expensive transport kits is not a guarantee of yielding precise diagnostic results. All specimens must be stored refrigerated (recommended between 2º to 8 ºC) for up to 24 hours prior the tests without drastically affecting test performance (Steininger et al. 2002). It is emphasized that samples under long storage and constant freezing and thawing will possibly degrade viral RNA and cDNA. This situation may limit the quantification by molecular techniques on samples with low viral copies (Ward et al. 2004).

Rapid Influenza Diagnostic Test

Rapid influenza diagnostic tests (RIDTs) are immunocromatographic assays that detect influenza viral antigens. Some can identify influenza A and B viruses and distinguish between them, but cannot distinguish among different subtypes of influenza A (Chauhan et al. 2013). It is considered a valuable tool for rapid treatment management during an influenza outbreak event. They are simple to use, and are less expensive than molecular methods, such as RT-PCR (Peci et al. 2014). Results are generated within 15 to 30 minutes, and, in some cases, it can be used at the point of care in a routine clinical setting, such as a physician's office or at an emergency department (Chartrand et al. 2012). Respiratory specimens used are nasopharyngeal swab, nasal wash, and nasal aspirate.

The clinical sensitivity and specificity of rapid influenza diagnostic test vary considerably in the literature. In clinical settings, they are reported to have sensitivities of 45-90% and specificities of 86-100%, compared to viral culture or RT-PCR. Thus, a negative result does not rule out infection. Therefore, the accuracy of the test is usually less than that of viral culture or RT-PCR (Cho et al. 2013).
This high range in sensitivity occurs likely due to the clinical sample, age, and season that could affect the performance of the test. Previous studies reported significantly higher sensitivities when analyzing nasopharyngeal aspirates, likely due to higher viral loads in sputum and nasopharyngeal aspirates than those in throat swabs (Smit et al. 2007), although common practice guidelines have selected nasopharyngeal aspirates as the best specimen type, other studies have not shown a stark difference among them (Charttrand et al. 2012). Influenza rapid detection kits are reported to have higher sensitivity to analyze samples from young children than samples from adults (Alexander et al. 2005). This is presumably a consequence of higher levels and longer duration of viral shedding in the younger age group (Cho et al. 2013). Similarly, recent studies reported that the sensitivity values of rapid detection kits for influenza A are higher in younger patients. However, some studies have also reported lower sensitivities for samples from younger patients (≤10 years) than the ones older (10-18 years) (Sandora et al. 2010). The clinical sensitivity and specificity of a rapid influenza diagnostic test also differ greatly during periods of low prevalence, by region, influenza subtype and risk population (Dale et al. 2008). Furthermore, since higher respiratory viral loads are expected during a pandemic due to the lack of prior immunity to the novel influenza virus subtype, higher levels of sensitivity from rapid tests in pandemic settings might be expected (Cheng et al. 2009).

Serological diagnosis

Antibodies are produced after the onset of influenza virus illness and can be detected using serological diagnostic techniques like hemagglutination inhibition assay (HIA), en-zyme immunoassay (EIA), complement fixation, and neutralization tests (Kim and Poudel 2013). Serological diagnosis of influenza is based on the detection of a four-fold or greater rise in specific antibody titre in paired serum samples, measured by haemagglutination inhibition, EIA, complement fixation, or neutralisation tests. The need for paired serum samples, the first collected as soon as possible after onset of illness and the second collected 10–14 days later, limits the usefulness of serology in diagnosis and treatment of acute illnesses (Cox et al. 1999). The haemagglutination inhibition assays have been widely used to diagnose seasonal influenza and assess response to seasonal influenza vaccines (Chen et al. 2010).

Chen and colleagues observed during 2009 pandemic that antibody titers increased rapidly in the first 2 weeks, and collection of acute samples less than 5 days from illness onset, and convalescent samples more than 2 weeks after illness onset, maximized the proportion of RT-PCR confirmed infections which seroconverted on hemagglutination inhibition assays. In addition, showed that more than 80% of RT-PCR confirmed H1N1-2009 cases seroconvert using hemagglutination inhibition and virus microneutralization assays, and demonstrated the development of cross-reactive antibodies to other influenza A strains following H1N1-2009 infection (Chen et al. 2010).

Direct fluorescence antibody assay (DFA)

This is a diagnostic approach for detection of viral antigens by immunospecific assays (immunofluorescence microscopy) which confers cheap and results within 1–4 hours (Bakermann et al. 2011). The technique is based on the staining of cells from the sample, followed by bioconjugation of antibodies to the fluorescent dye. DFAs are widely available, have variable sensitivity (range 47–93%) for 2009 H1N1 influenza virus, and have a high specificity (≥96%) (Pollock et al. 2009). They enable technicians to distinguish between influenza A and B viruses but do not distinguish among different influenza A subtypes (Dawood et al. 2012).

DFA test requires specialized and experienced staff, specialized equipment. The sensitivity is comparatively higher than that of rapid antigen detection kits and lower than the PCR tests. Thus, the negative results need to be confirmed by the PCR-based assays (Lee et al. 2008; Ganzenmueller et al. 2010; Gordon et al. 2010). Some studies recommend the use of DFA, as well as shell vial cultures (SVC) together, in order to increase the sensitivity of the test by 5.0-15.0% than the DFA alone (Mahony 2008). Generally, the sensitivity of DFA is higher than that of the rapid antigen test. Studies have shown that DFA sensitivity is higher among pediatric patients, especially displaying signs of high fever and spreading high amounts of viruses and decreasing with patients above 30 years of age (Heinonen et al. 2011).

A study comparing DFA, rapid antigen test and RT-PCR highlights low sensitivity rates (38.7% for DFA and 18.2% for rapid antigen test) in comparison to RT-PCR, advising the use of RT-PCR as confirmatory test for H1N1 (Ganzenmueller et al. 2010).

Virus isolation

Laboratory isolation of influenza is essential for confirmation of the arrival of influenza in a community, as well as allowing characterization of novel influenza viruses and vaccine production (Gavin & Thomson Jr 2003). In culture, the viruses cause different cytopathic effects, according to the main cell lines used for influenza isolation, such as: monkey kidney cells, Madin Darby canine kidney (MDCK) cells and A549 cells (Li et al. 2009). This classic laboratory diagnostic method is recognized as an important gold-standard technique to be available in reference laboratories worldwide (Kumar & Henrickson 2012).

However, modern molecular assays have been replacing viral culture to detect influenza (Rodinka & Kaiser 2013). In addition to present lower sensitivity for
specimens with low viral loads; the technique is time consuming, as it takes up to 14 days for a confirmatory result, thus delaying the initiation of antiviral therapy (Landry 2011).

Over the last 20 years the shell vial culture (SVC) techniques were developed to provide results quickly, and it is based on inoculation of clinical specimens over cell lines on a coverslip for 24 hours (Mathey et al. 1992; Operario et al. 2010). The detection of Influenza A or B antigen is performed by staining with the cell monolayer with fluorescent monoclonal antibodies (Mills et al. 1989). Due to the need to produce more rapid results, some sensitivity may be lost using SVC (Sandora et al. 2010). Additionally, the commercially available R-Mix cells and R-Mix Too (Diagnostic Hybrids, Athens, OH, USA), have shown the advantage of influenza virus identification within 1-2 days of inoculation. The first one is a mixture of A549 and Mink lung cells and the latter is a mixture of A549 and MDCK cell lines. During the 2009 H1N1 epidemics, the kit named D3 Ultra 2009 H1N1 Influenza A ID kit (2009 H1N1 ID kit; Diagnostic Hybrids, Inc., Athens, OH, USA) was developed using the R-mix Too cells allied to immunofluorescence method for the specific diagnosis of H1N1 (Higgins et al. 2010).

Rapid tests are best recognized for their detection ability, more sensitivity and specificity in comparison to viral isolation or PCR-based methods (Ruuskanen et al. 2011). However, the rapid tests results depend on the viral titer present in the sample and those with low viral titer should be analyzed by PCR assays, given that the molecular technique yields higher sensitivity and specificity (Chu et al. 2012).

The main concern of extensive use of rapid test lies on the possibility of false-negative results, which could lead to serious impacts on an effective anti-influenza therapy from individual patients or their contacts (Centers for Disease Control, available at: http://www.cdc.gov/flu/professionals/diagnosis/rapidlab.htm). Many test manufacturers highlights the use of cell culture to confirm the cases in which patients displaying clinical illness that were negative for influenza in rapid diagnostic tests (Kim & Poudel 2013).

**Conventional and Real Time Reverse-Transcriptase Polymerase Chain Reaction**

The first PCR based assay in detecting influenza virus was described in 1991 by Zhang and Evans (Kim & Poudel 2013). Since then, many modifications and highly sensitive methods based on PCR have been developed. The design of PCR primers and probes will differentially detect only one influenza type or subtype. Genes that encode the internal virus proteins such as the M1 matrix protein or the non-structural NS1 protein are highly conserved among influenza viruses and are thus useful targets for the universal detection and differentiation of type A and type B influenza viruses. Because influenza A subtypes are defined by their surface HA and NA proteins, primers that specifically amplify the corresponding genes are effective for determining the subtype of influenza A viruses (WHO, 2011). The conventional RT-PCR has the advantage of being relatively sensitive and specific and allows further sequence analysis, but it is not easily quantitative, and not ideal for high-throughput (Wang & Taubenberger 2010). As long as the time to get test results can be as little as 3 hours, routine surveillance samples are most often processed within 24 hours (Chauhan et al. 2013). Probe-based real time PCR (TaqMan) shows high sensitivity and specificity, what is ideal for quantitative and multiplex detection, as well as it can be high-throughput. Notwithstanding, the high-cost probe, special equipment required and further sequence analysis is not generally possible (Wang & Taubenberger 2010).

When the H1N1 outbreak occurred in 2009, CDC recommended the use of one step RT-PCR technique targeting the matrix gene of H1N1 09, this technique involves the use of oligo-dT or random primers for reverse transcription and using the HA gene as target in a single tube, which minimized the cost and time required to analyze the sample for each type and subtype of the influenza virus (Kim & Poudel 2013).

Real Time PCR assays are far more rapid and sensitive than traditional techniques including virus isolation by cell culture (Whiley et al. 2009). Most of the real-time PCRs used for influenza A virus surveillance and diagnosis are based on the TaqMan approach, in which a probe is designed to hybridize to an internal region of the PCR product, so that the highest sensitivity and specificity can be achieved during the PCR amplification. Because of the conserved nature of the matrix gene segment among different type A Influenza virus, specific matrix gene primers and probes have often been designed for influenza virus typing in human sample. This assay is especially important when negative results are found for HA or neuroaminidase (NA) detection. Generally of high sensitivity and specificity, TaqMan probes required for each PCR target are relatively expensive for large-scale screening and surveillance in many applications (Wang & Taubenberger 2010). During the procedures of the Real Time PCR protocols for Influenza detection, the CDC highlights that for each RT-PCR run the RNA extract’s sample must be tested by separate primer/probe sets: InfA, Universal swine(swFluA), Swine H1 (swH1) and RNaseP. The RNaseP primer and probe set targets the human RNase P gene and thus serves as an internal positive control for human nucleic acid.

A limitation of PCR methods is that false-negative results may occur due to sequence variation in primer and probe targets and this is particularly relevant for the detection of emerging viruses. However, the use of multiple targets can reduce such limitations, and may serve as means to confirm positive results (Whiley et al 2009).
Drug Resistance for Influenza Infection

Monitoring influenza antiviral susceptibility has become a vital part of virological surveillance within the WHO Global Influenza Surveillance and Response System (WHO-GISRS) (Okomo-Adhiambo et al. 2013). Two currently available NA inhibitors – zanamivir and oseltamivir – therefore became the only drugs recommended for the treatment and prophylaxis of both influenza A and B infections. Both drugs (as well as the candidate drug peramivir) were developed to act specifically against viral NA which removes the terminal sialic acid present on cellular receptors (to which haemagglutinin binds) and on virus glycoproteins to promote the release of progeny virions from infected cells thus facilitating their dispersal in the respiratory tract. Early treatment with either drug reduces both the severity and duration of influenza symptoms and associated complications (WHO, 2011).

The oseltamivir is one of the most widely used drugs against influenza infection and the resistance arises due to histidine to tyrosine substitution in the neuraminidase active site (H275Y) (Kim and Poudel, 2013). It was noted that the H275Y mutation caused impaired growth of the viral strains leading to compromised viral fitness and hence, was of little clinical significance. Subsequently, from the period 2007–2008 till the emergence of the 2009 H1N1 pandemic, it was observed that seasonal H1N1 strains predominantly showed the H274Y mutation with significantly improved viral fitness causing oseltamivir resistance worldwide (Behera et al. 2015).

A chemiluminescent neuraminidase inhibition (NAI) is recommended by WHO for the detection of resistance. This assay is used as a primary tool to assess the susceptibility of current circulating seasonal influenza isolates to the NA inhibitors zanamivir and oseltamivir. The assay was chosen because it requires a small volume of virus, even for a low-titre virus preparation. The other advantage is that the reagents are provided in the form of virus, even for a low-titre virus preparation. The other advantage is that the reagents are provided in the form of a kit (NA-Star from Applied Biosystems). Although the kit includes a protocol supplied by the manufacturer, there are several differences between this protocol and the actual protocol used at the CDC as outlined below. NA activity and drug susceptibility are measured using the fluorescent substrate 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA). This assay when performed in the presence of inhibitors allows the concentration of drug required to inhibit enzyme activity by 50% (IC50) to be determined. The assay has been used to assess the susceptibility of circulating seasonal influenza; of sporadic zoonoses (such as H5N1 and H7N7); and of pandemic (H1N1) 2009 isolates (WHO 2011).

CONCLUSION

Influenza virus is a significant global health issue. The interpretation by a clinician based on the patient’s symptoms is not enough to discern between influenza and other known respiratory viruses. The use of suitable tools for influenza tests is essential for early detection of cases, proper management of patients and understanding the epidemiology of the disease in order to help in outbreak control and development of effective vaccines in a timely manner.

During the outbreak of pandemic H1N1 in 2009, molecular detection methods rapidly became the most powerful tools for detection and identification and provided accurate genetic data for policymakers on drug treatment, vaccine development, hospital management and surveillance. Therewith many new studies on influenza virus molecular diagnosis and subtyping continue to be published. Among the methods herein reviewed, RT-PCR is considered a gold standard method for detection of H1N1 by reason of rapid and accurate diagnosis of novel H1N1 infections, what is critical for minimizing further spread through timely implementation of antiviral treatment and public health based measures.

Virus isolation culture has been considered the gold standard for respiratory virus diagnosis so far, nonetheless this classic technique takes days to weeks to obtain results. For this reason, there are some rapid techniques for diagnosis of influenza A virus, RIDTs and DFA, although these techniques vary widely in sensitivity and specificity, they are useful to reduce the spread of infection.

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