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Serological assays for emerging coronaviruses: Challenges and pitfalls

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ABSTRACT

More than a decade after the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002/2003 the occurrence of a novel CoV termed Middle East respiratory syndrome (MERS) CoV challenges researchers and public health authorities. To control spread and finally contain novel viruses, rapid identification and subsequent isolation of infected individuals and their contacts is of utmost importance. Next to methods for nucleic acid detection, validated serological assays are particularly important as the timeframe for antibody detection is less restricted. During the SARS-CoV epidemic a wide variety of serological diagnostic assays were established using multiple methods as well as different viral antigens. Even though the majority of the developed assays showed high sensitivity and specificity, numerous studies reported on cross-reactive antibodies to antigens from wide-spread common cold associated CoVs. In order to improve preparedness and responsiveness during future outbreaks of novel CoVs, information and problems regarding serological diagnosis that occurred during the SARS-CoV should be acknowledged.

In this review we summarize the performance of different serological assays as well as the applicability of the two main applied antigens (spike and nucleocapsid protein) used during the SARS-CoV outbreak. We highlight challenges and potential pitfalls that occur when dealing with a novel emerging coronavirus like MERS-CoV. In addition we describe problems that might occur when animal sera are tested in serological assays for the identification of putative reservoirs. Finally, we give a recommendation for a serological testing scheme and outline necessary improvements that should be implemented for a better preparedness.

1. Introduction

In 2002/2003, a new disease emerged in Southeast Asia that was subsequently termed severe acute respiratory syndrome (SARS). A previously unknown coronavirus (CoV) was identified as the etiological agent (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). It was only the concerted efforts of public health authorities that made it possible to break the chain of transmission. No new cases have been reported since May 2004. Overall, SARS-CoV infected 8422 people, of whom 916 died, giving a case fatality ratio of about 11% (Chan-Yeung and Xu, 2003). Before the emergence of SARS-CoV, human pathogenic CoVs (HCoVs) such as HCoV-OC43 and HCoV-229E were known to cause mild upper respiratory diseases contributing to 5–30% of the seasonal common cold cases (Isaacs et al., 1983; Larson et al., 1980; Monto, 1974). This explains why, globally, more than 90% of the population has antibodies against the common cold CoV (Gorse et al., 2010).

SARS-CoV belongs to the *Coronavirdae* family within the order of *Nidovirales*. It harbors one of the largest known positive-strand RNA genomes comprising about 29 kb (Rota et al., 2003). The first two-thirds of the genome contain nonstructural proteins (NSP) that are well conserved among different CoV species (Rota et al., 2003). The NSPs include the RNA-dependent RNA polymerase and form the main part of the transcription/replication machinery. The last third of the genome encodes mainly the four structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N) (Rota et al., 2003). Interspersed between the structural proteins are group-specific open reading frames (ORFs) encoding a subset of accessory proteins with mostly unknown function (Narayanan et al., 2008). In the case of SARS-CoV, it was shown that, apart from the four structural proteins, some of the NSPs as well as the accessory proteins p3a and p7a, are incorporated into virions and may elicit an immune response in infected patients as it was shown for NSP13 (Leung et al., 2004; Neuman et al., 2008; Schaecher et al., 2007).

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Diagnostic assays for SARS-CoV detection were rapidly developed after the identification of the virus. Testing of suspected cases helped considerably to contain the outbreak and understand the rapid disease progression that was observed in some of the patients. SARS patients had detectable viral RNA between three and 30 days after the first symptoms appeared, with high viral loads in lower respiratory tract and fecal samples. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays were the first assays that helped identify and subsequently isolate patients who were actively shedding the virus (Drosten et al., 2003). It was reported that viral RNA could be detected by qRT-PCR up to 30 days post onset of illness (dpoi) (Chan et al., 2004a). However, detection rates by these early qRT-PCR assays were often low as they relied on sampling during virus shedding and proper handling of samples (Yam et al., 2003). The specificity of the qRT-PCR assays has also been questioned because of the potential for nucleic acid contamination in the laboratories where many SARS-CoV samples were processed (Patrick et al., 2006).

As a consequence, the development of serological assays became crucial at the time. Many laboratories worldwide generated in-house assays using either virus-derived antigen or recombinant structural CoV proteins. For all laboratories, proper validation of serological assays was highly challenging because of its reliance on numerous well-characterized positive and negative serum samples. Furthermore, the exchange of patient serum samples was a major challenge during the outbreak for logistic and ethical reasons. Assay sensitivity (the number of positive samples that could be determined correctly) and specificity (measured by the number of negative samples that could be identified correctly) were therefore difficult to determine. Consequently, an external quality assurance study revealed that many laboratories had difficulties with SARS-CoV serodiagnostics (Niedrig et al., 2005). In particular, the high seroprevalence in the population of antibodies against the common cold CoV combined with the presence of cross-reactive antibodies against conserved parts of the immunogenic CoV proteins could have contributed to false positive results.

Nevertheless, as antibodies can be detected over a long period, developing reliable post-infection serological assays for SARS-CoV became a high priority. Serodiagnostic assays were applied to address epidemiological questions about transmission patterns, to observe silent infections, to analyze disease progression and to identify the origin of SARS-CoV. Patients usually developed IgM and IgG antibodies within 17–21 dpoi (Woo et al., 2004b). To identify infected contacts, including those that are asymptomatic, it is recommendable to analyze paired serum samples. Ideally, samples should be taken on day 0 and day 42 post exposure since it was shown that seroconversion of IgG or IgM occurred during that period (Chen et al., 2004a). According to the WHO criteria for serological diagnosis, a patient was considered to have seroconverted if one of the following statements were true: "*Negative antibody test on acute-phase serum followed by positive antibody test on convalescent-phase serum tested in parallel*" or a "*Fourfold or greater rise in antibody titer between acute- and convalescent-phase sera tested in parallel*" (WHO, 2004). Of note, WHO further recommended using a virus neutralization test (VNT) to exclude serological cross-reactions with other human or animal CoVs (WHO, 2004).

In this review, we summarize the various types of serological assays that were developed during the SARS-CoV outbreak and analyze the challenges and potential pitfalls of serodiagnostics. It should be acknowledged that the multitude of developed assays and the lack of standardized procedures and assay validation make it difficult to directly compare all studies. Finally, we discuss the implications and challenges facing serodiagnosis of the novel Middle East respiratory syndrome (MERS-CoV).

2. Testing parameters: IgM vs. IgG subclasses

Testing for different immunoglobulin (Ig) subclasses is common in serodiagnostics. Being pentameric with ten antigen-binding sites, IgM is characterized by a higher antigen avidity but lower antigen affinity than IgG (Murphy et al., 2008). As antibodies of IgM subclass are usually the first to develop following a primary challenge (Murphy et al., 2008), IgM is considered a parameter of the early phase of infection. However, Woo et al. (2004b) found that IgM could not be detected earlier than IgA and IgG subclass antibodies in SARS-CoV serodiagnostic assays. The low affinity of IgM antibodies also carries the increased risk of cross-reactivity with antigenically related epitopes, which are common in CoVs. IgM detection has an added value for CoV serodiagnostics as it is only present in very recently infected patients, but does not allow differential diagnosis. In contrast IgG antibodies comprise a higher specificity than IgM and can be detected even years after the infection. In this review, we therefore focus on studies describing assays for the detection of IgG antibodies.

3. Virus-based serological assays

During the outbreak of SARS-CoV, a wide variety of serological assays were established, including immunofluorescence assays (IFAs), enzyme-linked immunosorbent assays (ELISAs) and Western blot (WB) analysis.

Conventional IFAs (cIFAs) using virus-infected African green monkey kidney cells (VERO E6) spotted on glass slides and ELISAs using extracts or supernatant of infected cells were among the first assays used in serological diagnosis of SARS-CoV (Chan et al., 2004b; Chen et al., 2004a; Hsueh et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003; Wu et al., 2004). Both cIFAs and ELISAs for SARS-CoV were relatively easy to set up for experienced laboratories, as they primarily only required susceptible cell cultures and the virus. An obvious disadvantage of virus-based serological assays is the need for a biosafety level (BSL) 3 facility. In addition, IFAs can neither be properly standardized because the interpretation of fluorescence staining patterns is subjective, nor are they appropriate for high-throughput screening. In the case of ELISAs, antigen production can be an obstacle if it necessitates ultracentrifugation under BSL3 conditions. Ensuring that the antigen is properly inactivated can also be problematic. Ultimately, validation of the ELISA is dependent on access to a well-characterized serum collection for the determination of the assay-specific cut-off value.

A detailed summary of studies applying SARS virus-based cIFAs and ELISAs is given in Table 1. Since SARS-CoV seroconversion generally occurred during the second week of illness (Hsueh et al., 2003; Ksiazek et al., 2003), only those studies in which sera was taken at least 14 dpoi are included in this overview. In the majority of cases, patient serum was tested in assays using virus-derived antigens. Most of the studies found that serum from between 85% and 100% of previously diagnosed SARS patients tested positive (Table 1, column 3), suggesting that the cIFA and ELISA used were highly sensitive. Of note, the studies with the most reliable results were those that used a large number of patient serum samples ($n = 90$; Chan et al. (2004b) and $n = 224$; Wu et al. (2004)), which found that 98.2% and 99.1%, respectively, were positive.

Specificity of serological assays is vital to avoid false positive diagnoses. Critically, some of the HCoVs are antigenically closely related (Bradburne, 1970). This means, in combination with the observed high prevalence of HCoVs, such as HCoV-OC43, HCoV-229E and HCoV-NL63, in the population (Che et al., 2005; Dijkman et al., 2012), that the specificity of the SARS-CoV assay is of particular concern. We therefore analyzed and summarized the rate of false positive results found in serological studies and differentiated

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