Coronaviruses with Special Emphasis on First Insights Concerning SARS
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Virological laboratory diagnosis of SARS

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Introduction

The enormous speed with which, through unprecedented international collaboration coordinated by WHO, the novel coronavirus causing SARS, termed SARS-CoV, was isolated and characterized [1], allowed the development of diagnostic tests when the first SARS epidemic was still in full swing (Severe Acute Respiratory Syndrome (SARS): Laboratory diagnostic tests – 29 April 2003: http://www.who.int/csr/sars/diagnostictests/en/). As early as mid-April 2003, sequences of polymerase chain reaction (PCR) primers were made publicly available on the WHO SARS website (PCR primers for SARS developed by WHO Network Laboratories – 17 April 2003: http://www.who.int/csr/sars/primers/en/) to facilitate the introduction of laboratory testing in affected countries.

Soon afterwards the first commercial assays became available, and numerous laboratories all over the world started preparing for SARS diagnosis using these or various in-house assays. Important research efforts were - and still are, as of April 2004 - directed towards improving SARS laboratory testing, including the development of novel genome targets for nucleic acid detection and of recombinant antigens for serological diagnosis.

Nevertheless, despite these efforts, it has to be recognized that the laboratory diagnosis of SARS so far remains problematic. Problems with test specificity have led to false-positive results for SARS-CoV on occasions, which created enormous public concern and confusion. In addition, some of the results of newly developed antibody tests have yielded results that are difficult to reconcile with epidemiological and other information and may be related to insufficient test sensitivities or specificities.

In this chapter, we will endeavour to briefly list and describe the currently available laboratory test methods for SARS-CoV, including their performance as far as has been published. We will then outline the current WHO-endorsed strategy for the laboratory diagnosis of suspected SARS

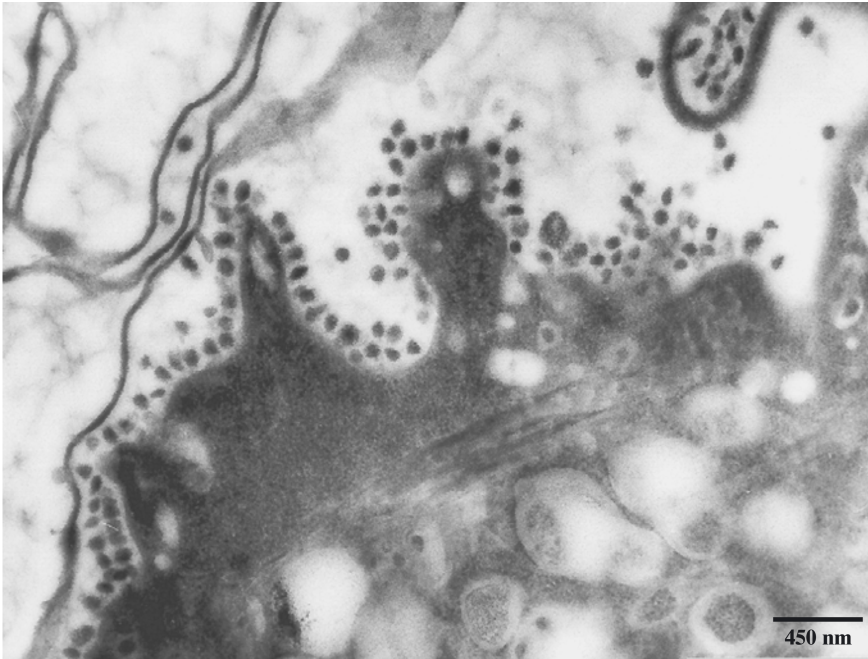

Figure 1. SARS-CoV particles in Caco-2 cell culture.

cases, including recommendations for dealing with such cases, and provide an outlook regarding future developments.

Available virological laboratory tests for SARS-CoV

Virus detection

Virus isolation

In contrast to the previously known human-pathogenic coronaviruses, HCoV-229E and HCoV-OC43, SARS-CoV is relatively easily propagated in cell culture. The most commonly used cell lines are Vero (African green monkey kidney) cells, but fetal rhesus kidney (FRhK-4) cells and – interesting in the light of possible gastrointestinal pathology caused by SARS-CoV – the human colonic carcinoma cell line Caco-2 (HTB-37) may also be used (Fig. 1). Cells commonly used for the isolation of human respiratory viruses are however unsuitable. The authors were able to isolate SARS-CoV on Vero cells from lower respiratory tract specimens (sputum, bronchoalveolar lavage) from the two Frankfurt/Main SARS cases; a marked

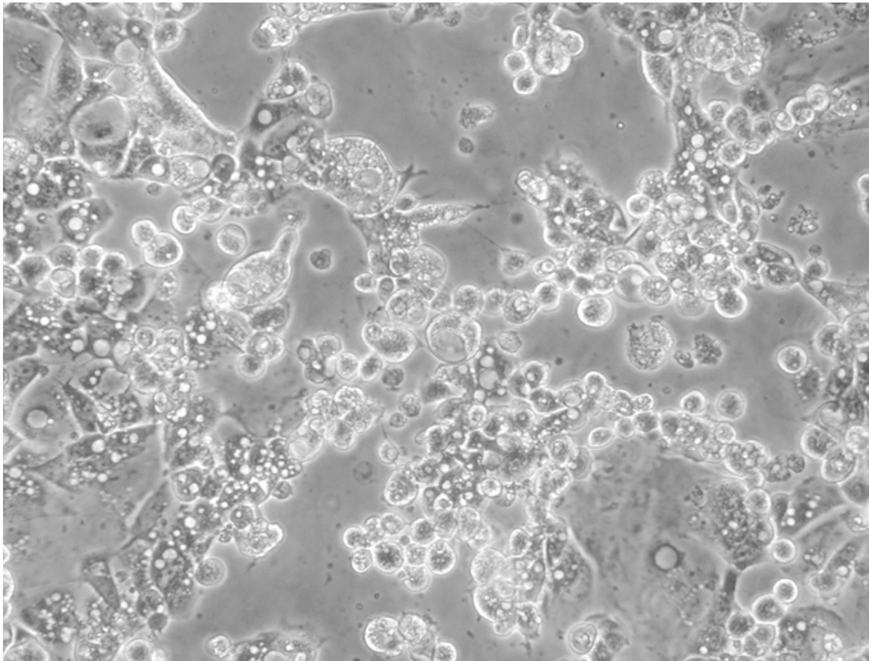

Figure 2. Cytopathic effect (CPE) in Caco-2 cell culture caused by SARS-CoV infection 72 hours after infection.

cytopathic effect (CPE) became visible only a few days after inoculation ([2]; Fig. 2).

To confirm the presence of SARS-CoV, further tests are required. Human SARS-CoV immune (convalescent) serum may be used for immunostaining of infected cells (Fig. 3). Alternatively, viral nucleic acid testing (see below) may be performed on cell culture supernatant. The latter is recommended by WHO (Use of laboratory methods for SARS diagnosis – 1 May 2003: http://www.who.int/csr/sars/labmethods/en/).

Relatively few isolations of SARS-CoV were made during the SARS outbreak which caused more than 8,000 "probable" SARS cases. This may be due to the fact that many laboratories refrained from virus isolation due to biosafety concerns, and if it was attempted, long transport times and the use of patient sample types with a low viral load, such as those from the upper respiratory tract, may have affected its success rate, despite the agent's relatively high degree of resistance to various environmental conditions [3].

The advantage of virus isolation is that it demonstrates the presence of infectious virus and thus proves active – and potentially infectious – SARS-CoV infection in the patient. Of course, negative cell culture results do not exclude a diagnosis of SARS (see below). Virus isolation furthermore allows further studies to characterize the virus strain implicated, which will

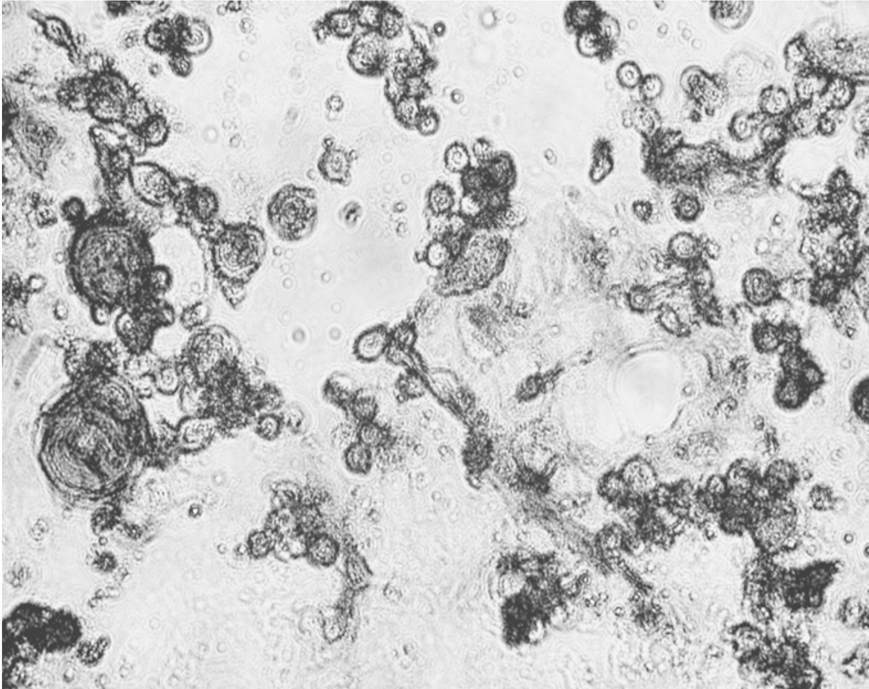

Figure 3. Immunostaining of SARS-CoV infected cells using human immune serum.

be of enormous importance should SARS re-emerge. Such information might not only shed light on the transmissibility and virulence of different strains of SARS-CoV but also on its presumed animal reservoir [4].

While it is internationally agreed that virus isolation from suspect SARS cases has to be performed under at least biosafety safety level (BSL) 3 conditions (WHO post-outbreak biosafety guidelines for handling of SARS-CoV specimens and cultures – 18 December 2003: http://www.who.int/csr/sars/biosafety2003_12_18/en/), it has to be stressed that during the outbreak that ended in July 2003, not a single laboratory infection seems to have occurred. In contrast, there have been several such incidents since; two without further human-to-human spread (Singapore, September 2003, and Taiwan, December 2003), and with spread beyond the laboratory workers themselves (Beijing, April 2004). Therefore, it is obviously not so much the routine diagnostic work-up of suspect SARS specimens that poses a risk to laboratory workers but the neglect of well-known precautions during scientific work which involves much higher volumes and concentrations of infectious materials. If possible, isolation of the causative agent should therefore always be attempted from suspect and probable SARS cases if suitable facilities are available.

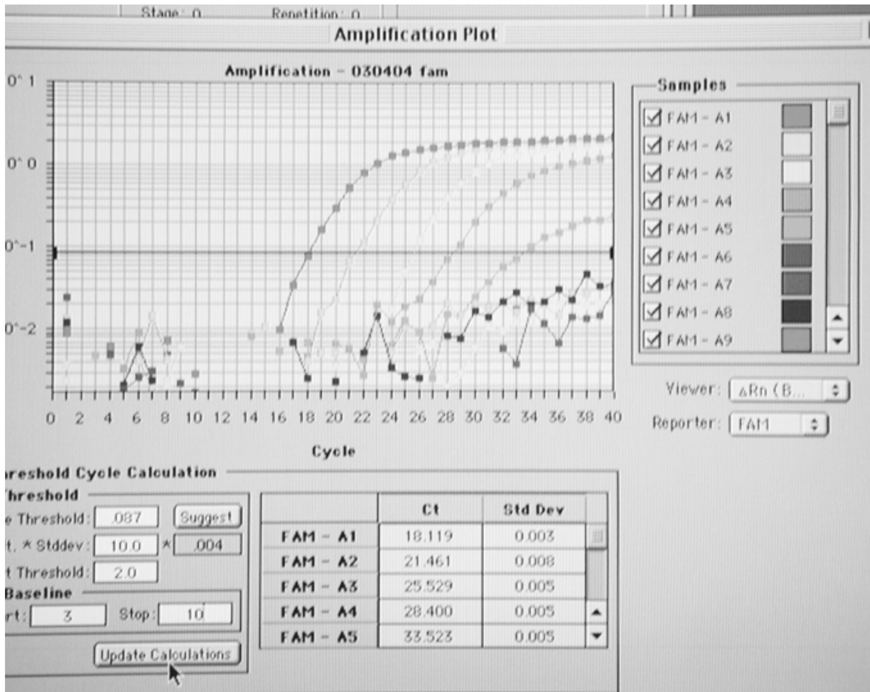

Figure 4. “TaqMan” real-time PCR amplification plot.

Viral nucleic acid detection

All three groups that simultaneously but independently succeeded in isolating and characterizing SARS-CoV published methods for the detection of the agent’s RNA [5–7]. These first-generation reverse transcription (RT)-polymerase chain reactions (PCR) all target the viral replicase gene, and detection of PCR products is *via* agarose gel electrophoresis.

SARS-CoV RNA has since been found in various clinical materials. Published papers describe its detection in respiratory secretions both from the lower and the upper respiratory tract, in stool, in urine, and in the blood, as well as in different tissues and organs. Subsequently developed PCR methods employ real-time formats and allow quantification of SARS-CoV RNA (Fig. 4). Using such a method, Drosten et al. [5] were able to demonstrate high concentrations of viral RNA of up to 10^8 molecules per ml in sputum samples; on the other hand, only low concentrations were detected in specimens from the upper respiratory tract, such as throat swabs, and in plasma during the acute phase of the illness. However, faecal specimens obtained late during convalescence, beyond the time period for which strict isolation of patients is usually recommended, tested positive. In patients from Hong Kong, Peiris et al. [8] found the amount of nasopharyngeal

SARS-CoV to peak around 10 days after onset of symptoms, at which time 19 of 20 patients were PCR positive in nasopharyngeal aspirates, all 20 in stool and 10 in urine samples.

Currently, two real-time RT-PCR test kits, both targeting the viral replicase gene, are commercially available, the RealArt HPA coronavirus LC kit (Artus, Hamburg, Germany) and the LightCycler SARS-CoV quantification kit (Roche, Penzberg, Germany). A recently conducted international external quality assurance study for SARS-CoV nucleic acid testing (NAT) distributed 10 coded, inactivated samples to 58 laboratories in 38 different countries [9]. The seven positive samples contained between 94 and 940,000 RNA copies of SARS-CoV strains Frankfurt 1 or HKU-1 per ml when reconstituted. Almost 90% of laboratories reported correct results for all samples containing more than 9,400 viral RNA copies/ml while not producing false-positive results in the three negative ones. The use of commercial test kits was found to positively influence diagnostic performance. The encouraging outcome of this exercise confirms the success of the considerable efforts undertaken by WHO to support laboratories in establishing SARS-CoV diagnostic tests rapidly as a means to effectively respond to the SARS outbreak. Through the European Network for Imported Viral Infections (ENIVD; http://www.enivd.de), an inactivated standard preparation of SARS-CoV is available for diagnostic purposes.

To improve the insufficient negative predictive value of PCR-based methods to rule out SARS in suspect cases, efforts were made to increase their methodological sensitivity. Besides increasing the volume of patient sample material from which RNA is extracted [10], an attractive approach seemed to target the viral nucleocapsid gene of which, due to the unique transcription strategy of coronaviruses, more than one mRNA copy is present in infected cells [11]. However, in a recent study a technically optimized nucleocapsid in-house assay failed to consistently detect SARS-CoV RNA in 66 clinical samples from confirmed SARS patients with an overall positivity rate around 70% [12]. This is in agreement with results obtained by other groups [13].

The same study, however, found viral RNA detectable in all lower respiratory tract samples by all test methods. It therefore concluded that, rather than further optimizing PCR methods and protocols, efforts should be directed towards developing safe and convenient methods for obtaining specimens from the lower respiratory tract of suspect SARS patients. In the very early phase of clinical illness, prior to the onset of respiratory symptoms, plasma may provide a convenient and safe alternative sample material; Grant et al. [14] were able to detect SARS-CoV RNA in 19 of 24 patients (79%) tested within three days after onset of fever. The same study confirmed the brief duration of viraemia in SARS cases and thus offers reassurance for blood transfusion services. However, should SARS re-emerge on a large scale, methods have already been developed and

evaluated that would allow screening of donated blood for SARS-CoV RNA [15].

While it is clear from the studies cited above – and numerous others – that PCR cannot rule out the presence of SARS-CoV in patients with certainty, there is also a risk of false-positive results being caused by contamination of samples in laboratories performing PCR, or of true-positive results caused by related viruses when methods of low specificity for SARS-CoV are employed. In August 2003, there was considerable confusion when mild respiratory infections in an institution for the elderly in Canada were initially reported to be positive by SARS-CoV tests; these later turned out to be infections with a different human coronavirus, OC43. When a small number of SARS cases occurred in the southern Chinese province of Guangdong in January 2004, there was some uncertainty for several weeks as to the true diagnosis in some of the cases, as independent confirmation could not be obtained for some patients tested positive locally. For these reasons, WHO has issued clear guidance on how such samples should be handled and tested [16].

For the interpretation of laboratory results, WHO recommends that PCR results for SARS-CoV should only be regarded as confirmed positive if (1) at least two different clinical specimens (e.g. nasopharyngeal and stool) or (2) the same type of clinical specimen collected on two or more days during the course of the illness (e.g. two or more nasopharyngeal aspirates) were tested or (3) if two different assays or repeat PCR using the original clinical sample on each occasion of testing yielded positive results on one specimen (Use of laboratory methods for SARS diagnosis – 1 May 2003: http://www.who.int/csr/sars/labmethods/en/). Besides including appropriate negative and positive controls in each PCR run, possible PCR inhibition should be controlled for by spiking the patient sample with a weak positive control and testing it in parallel with the unspiked sample. In the post-outbreak period – when each truly positive SARS-CoV result will have massive implications for public health etc. –, a second aliquot of the original sample should be sent unopened to the national SARS reference laboratory for re-testing, and a third aliquot retained for use by an international SARS reference and verification laboratory nominated by WHO, if necessary.

In summary, PCR is able to detect viral RNA in between 50 and 85% of stool, blood and upper respiratory tract samples from acutely ill SARS patients. While the use of commercial kits may increase the reliability of results, the sensitivity and thus the negative predictive value of PCR require further improvement. While this is unlikely to come from changing the PCR target gene region, increasing the volume of sample from which RNA is extracted [10], testing lower respiratory tract specimens [12], or testing several samples from each patient [17] may be useful approaches. Nevertheless, a diagnosis of SARS cannot currently be ruled out by a negative PCR result.

Antibody detection

Neutralisation test (NT)

This assay became available as soon as SARS-CoV had been isolated and propagated *in vitro*. It is the only antibody test that allows to assess and quantify, by means of serial titrations of patient sera, their ability to neutralize the infectivity of SARS-CoV. Although this has yet to be demonstrated in humans, the NT titer may therefore be correlated to clinical immunity and is used to assess humoral immunity in animal studies [18]. Furthermore, NT is recommended as a means to rule out non-specific antibody reactivity that might occur in other tests, e.g. through cross-reactivity with antibodies directed against other, non-SARS human coronaviruses. One disadvantage of NT is that it is limited to institutions with BSL-3 cell culture facilities as it involves the propagation of SARS-CoV.

Immunofluorescence test (IFT, IFA)

IFT was the first type of solid-phase antibody assay to be used for SARS and remains the widely employed “gold standard”, together with NT (see above). Even before the exact nature of the newly isolated agent was known, specific seroconversion and thus, primary infection, were demonstrated in the Frankfurt SARS patients by means of indirect IFT, using infected cells (and non-infected cells as control), serum samples obtained from the patients at different points of time and anti-human IgG conjugate antibody labelled with a fluorescent dye [5] (Fig. 5). Peiris et al. found seroconversion in 93% of 75 patients by day 30 after onset of symptoms. None had antibodies prior to day 10, and the mean time period to seroconversion was 20 days [18].

An IFT assay is now commercially available (Euroimmun, Lübeck, Germany). As it employs gamma-irradiated infected cells that have been demonstrated to be non-infectious and includes both negative (uninfected) cell controls and appropriate positive and negative control sera, it is suitable for use under BSL-2 conditions; ideally, patient samples should be heat inactivated prior to testing to exclude infectivity. Positive samples should also be tested in serial dilutions to provide a titer which may be helpful to demonstrate acute or very recent infection (see below).

Other serological methods

A number of other serological test methods such as enzyme immunoassay (EIA), Western blot and rapid antibody tests have been developed. They employ infected cell lysates or recombinant proteins as antigens, and some

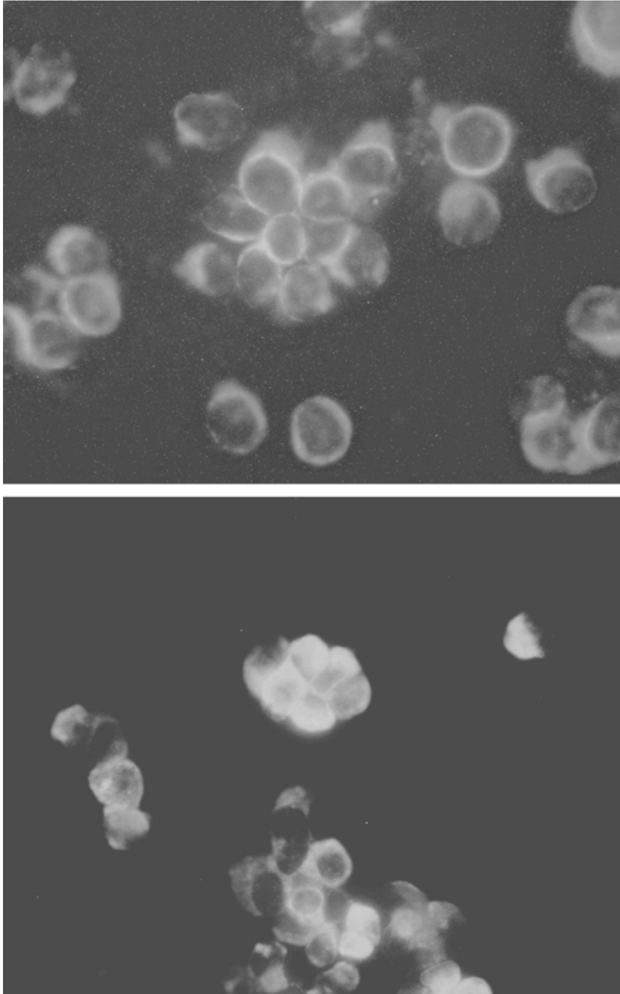

Figure 5. Seroconversion against SARS-CoV demonstrated in Frankfurt SARS patient by IFT. Serum sample obtained 8 days (top) and 14 days (bottom) after onset of illness.

are even commercially available. However, none of these assays has been thoroughly evaluated yet, by means of comparison with either IFT or NT. It is therefore currently impossible to give well-founded recommendations regarding their use. Particularly some well advertised “rapid tests” have caused much excitement in the lay press, as they seemed to offer a quick answer; the speed with which an antibody test result is available, however, does not help at all with the commonest and most urgent practical problem, i.e. to reliably establish a diagnosis in suspect cases, for which antibody testing is unsuitable due to the appearance of antibodies only later in the

course of disease (see above). However, from about three weeks after a possible contact with a SARS patient, e.g. during travel or in a health care setting, antibody testing is a useful tool to exclude that SARS-CoV infection has taken place.

According to WHO recommendations, serodiagnosis may be used for (normally retrospective) confirmation of a diagnosis of SARS if either seroconversion is demonstrated by IFT or EIA (i.e. a negative antibody test on an acute serum followed by a positive antibody test on a convalescent serum) or if an at least four-fold rise in antibody titer is demonstrated between such paired sera tested in parallel. Reassuringly, most studies so far showed seroconversion six and more weeks after the beginning of the illness in almost 100% of SARS patients [19].

Results demonstrating SARS-CoV antibodies in a small proportion of sera obtained from individuals prior to the emergence of SARS remain to be confirmed and may reflect a lack of specificity of the assays used rather than pre-existing circulation of the virus. On the other hand, the finding of SARS-CoV antibodies in animal handlers without a history of clinical disease compatible with SARS and presumably exposed to closely related but possibly non-human-pathogenic coronaviruses isolated from different species of animals in southern China [20, 21] may point to a possible explanation for such phenomena. However, most studies found no background seroprevalence against SARS-CoV in the control populations screened so far.

Therefore, antibody testing is suited to retrospectively confirm the diagnosis of SARS and may help to further elucidate the epidemiology of this novel disease. While of course unsuitable during the acute phase of illness when a reliable diagnosis is needed most urgently, it has the advantage of requiring only a blood specimen and probably being little time-sensitive once patients are beyond the first few weeks of their illness.

It needs to be borne in mind that the international case definition for SARS was based on a combination of clinical and epidemiological factors and did not require specific virological or serological testing (although evidence of SARS-CoV infection was added from May 1st, 2003, as a facultative criterion to upgrade a suspect to a probable case). Since the clinical manifestation rate of SARS-CoV infection appears to be high (>80%), the epidemiology of SARS does not need to be revised. However, much remains to be done to define the optimal antigens for use in EIA and other assays, including large-scale comparative evaluations on panels of well-defined sera. WHO has called for countries in which large numbers of SARS cases occurred to make such panels available to the scientific community.

Current status of virological laboratory diagnosis of SARS

In the light of currently available information, it is regarded as unlikely that SARS-CoV continues to circulate in the human population of previously

affected areas. Nevertheless, the cases in Guangdong at the beginning of 2004 have demonstrated that a re-introduction from the still unknown animal reservoir is possible, and repeated laboratory accidents have underlined the need for stringent infection control procedures and their strict application in laboratories working with the agent, as these are the only places of which it is known for sure that infectious SARS-CoV still exists [22].

It is therefore imperative that vigilance for SARS be maintained. WHO has defined three geographical zones according to their presumed risk for potential SARS recurrence and issued recommendations for SARS surveillance for each of them [23].

In the “potential zone of re-emergence”, comprising Guangdong and other, adjacent areas where animal-to-human transmission of SARS-CoV might again occur, “SARS alert” plus enhanced SARS surveillance should be in place and special studies for SARS-CoV infections in animal and human populations conducted. In “nodal areas”, comprising Hong Kong, Vietnam, Singapore, Canada, and Taiwan, that experienced sustained local transmission in spring 2003 or the entry of numerous persons from the potential zone of re-emergence, only “SARS alert” plus enhanced SARS surveillance are deemed necessary. In “low risk areas”, i.e. the rest of the world, surveillance should be directed to clusters of “alert” cases among health care workers, other hospital staff, patients and visitors in the same health care unit.

A “SARS alert” is defined as at least two health care workers developing clinical illness, or at least three individuals – health care workers and/or other hospital staff and/or patients and/or visitors – in the same health care unit developing hospital-acquired illness, within the same 10-day period, that fulfills the clinical case definition of SARS. Fulfilling the clinical case definition also means that “no alternative diagnosis can fully explain the illness”, i.e. it requires certain other laboratory tests for infectious agents – such as influenza and other viruses – to be conducted if appropriate.

For such cases, and in those recognized through “enhanced SARS surveillance”, such as otherwise causally undiagnosed cases of atypical pneumonia, a laboratory case definition was developed based on PCR, antibody tests or virus isolation to be undertaken in a national or regional reference laboratory. Only clinically compatible and laboratory-confirmed cases should be reported to WHO.

At the SARS Laboratory Workshop held in October 2003 in Geneva, experts from the enlarged international laboratory network made several recommendations [24]. These included

(1) the establishment of a quality assurance programme and the standardisation of laboratory tests and protocols;
(2) the establishment of a WHO SARS reference and verification laboratory network to undertake verification of suspected cases of SARS-CoV

infection, with terms of reference requiring such laboratories to be active members of the quality assurance programme;

(3) to require all sporadic (non-epidemic) cases of suspected SARS-CoV infection to be verified by a WHO reference and verification laboratory external to the country in which the case occurred;
(4) to acquire and assist in the development of a panel of positive SARS-CoV control sera;
(5) to develop algorithms for assessing the need to test patients for SARS-CoV infection based on an epidemiologic and geographic risk assessment;
(6) to endorse the guidelines for biosafety in laboratories handling diagnostic specimens potentially containing SARS-CoV or culturing SARS-CoV, which should be done in biocontainment level 3 facilities where tests involve virus propagation, or in biocontainment level 2 facilities with level 3 work practices where tests involve manipulations of live virus without propagation;
(7) to strongly recommend that countries maintain an inventory of laboratories working with SARS-CoV, and an inventory of cultures of SARS-CoV in those laboratories.

Because of the risks of inappropriate use of scarce resources and of overwhelming the health system unnecessarily by false alarms, the low pre-test probability for SARS has to be taken into account in low risk settings during the post-epidemic period.

If a case is to be investigated, respiratory samples – ideally including nasopharyngeal aspirates, provided full infection control procedures are in place to protect staff and other patients – and stool specimens should be routinely collected for virus isolation and/or detection by RT-PCR during the first and second weeks of illness, as well as plasma or serum specimens early on and in the second or third week to demonstrate seroconversion or a significant rise in antibody titer by testing acute and convalescent sera in parallel.

Laboratory testing is to be conducted through a three-tier system. Therefore, all clinical samples have to be separated into three aliquots at the time of collection or in a secure laboratory in which there is no ongoing work on SARS-CoV. One aliquot is for use by the local diagnostic laboratory, and the second has to remain unopened for use by the national reference laboratory. The third aliquot should be retained in case verification by a WHO SARS reference and verification laboratory becomes necessary. This happens if a positive clinical specimens is reported by either the local diagnostic laboratory or the national reference laboratory.

Regarding the interpretation of laboratory results, specimens found positive by PCR or virus isolation require confirmation on the second, previously unopened aliquot of the same specimen in a second laboratory, and on a second, independently collected specimen, too. The genome region

amplified by PCR should be specific for SARS-CoV and not conserved amongst other known coronaviruses, and PCR products should be sequenced to verify the presence of SARS-CoV.

A four-fold or greater rise in antibody titer on parallel testing by IFT, EIA – using a well-characterised antigen – or another serological test between acute and convalescent phase sera are collected at least 8-10 days apart indicates a suspect case but confirmation by NT is mandatory, because of the possibility of antibodies cross-reacting with other human coronaviruses.

The purpose of these guidelines is clearly to ensure a maximum specificity of laboratory results in order to avoid false alarms which would put unnecessary stress on health systems and might – in the long run – lead to lowered vigilance. Should SARS re-emerge on a larger scale, some of the confirmatory requirements might have to be modified again.

Outlook

Despite the rapid discovery of the causative agent and the early development of diagnostic tests, further progress on the laboratory diagnosis of SARS has been somewhat slower than might have been expected. Although numerous PCR-based assays have been developed, some of which are technically superb, there is still no test that could be used to rule out the diagnosis of SARS in a suspect case, due to the comparatively low virus excretion during the early course of SARS.

In the current post-outbreak phase, thorough evaluation of suspect cases for other agents known to cause atypical pneumonia, such as influenza and parainfluenza viruses, *Legionella pneumophila*, *Mycoplasma pneumoniae* etc. is even more important. Furthermore, if a decision is made to also include SARS in the differential diagnosis – which necessitates thorough and up-to-date information about the patient's personal, work and travel history as well as about the current epidemiological situation worldwide – the recommendations made by WHO (see [23] above; but check for updates!) should be followed meticulously.

The two laboratory-acquired SARS cases in Singapore and in Taiwan in the second half of 2003, and the sporadic SARS cases reported from Guangdong at the beginning of 2004, demonstrate that through a functioning surveillance and alert system, as recommended by WHO, newly emerging cases may be detected in a timely fashion, confirmed by laboratory testing including independent confirmation, and further spread thus prevented. The most recent laboratory-associated cases in Beijing (April 2004), however, cast some doubt on the quality of the local surveillance system.

In little more than one year, a lot has been learned about this novel virus and the disease it causes, but undoubtedly much remains to be understood. The availability and prudent application of SARS-CoV laboratory tests has

allowed to address several important questions, but it has also become clear that much more remains to be done. Given how little attention the previously known human coronaviruses had received in human virological laboratory diagnosis, SARS even offers a chance to improve our understanding of their epidemiology and clinical relevance by developing and applying suitable tests.

For the time being, however, great care has to be exercised when using SARS-CoV tests in clinical settings. It is a prerequisite to stay up-to-date on current recommendations issued by WHO, relevant national organizations, and scientific bodies. A recent meeting of the WHO Scientific Research Advisory Committee on SARS [25] has identified several priority issues for laboratory research, including the evaluation and standardization of current and future diagnostic tests for SARS, the improvement of serological tests with respect to specificity and sensitivity, studying the nature of cross-reactions between different coronaviruses, determining the importance of virus strain variation for diagnostic tests, and finding new technologies that can be applied to develop inexpensive, sensitive and specific laboratory tests that are reliable in the first few days of illness.

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