Role of the Laboratory in the Diagnosis of Viral Exanthems

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Abstract: An important number of viruses (measles, rubella, human parvovirus B19, herpes simplex, varicella zoster, enteroviruses, dengue and others) can cause exanthematic diseases. Laboratory diagnosis of exanthems due to viral infections include the identification of the disease-causing virus (isolation, antigen detection or nucleic acid detection) and the determination of a specific serological response (seroconversion or specific IgM).

The aim of virus isolation is the recovering of infectious viruses, being useful for molecular epidemiological studies. It is of application for measles, rubella, enterovirus, herpes simplex and varicella-zoster. However, due to its methodological complications, it is not of application in most laboratories. For some viruses, isolation is not practical, because there is no cell substrate available (Epstein-Barr, parvovirus B19). An alternative to viral isolation is the detection of antigens on clinical specimens (dengue). Thirdly, nucleic acid detection is a very useful approach for diagnosing viral exanthems, specially when multiplex assays recognising measles, rubella and parvovirus B19, or enterovirus and herpesvirus, are used.

The best tool for the serological diagnosis is the detection of IgM, since it provides a rapid diagnosis during the first days of the disease. However it has some drawbacks when used to diagnose viral rashes (the presence of rheumatoid factor and specific IgG; the multiple reactivity due to polyclonal stimulation of memory lymphocytes; the cross-reactivity between viruses causing a similar clinical picture; the IgM synthesis in herpesvirus reactivations). In such cases, confirmatory serological approaches (establishment of seroprofiles, or avidity assays) are necessary.

Both direct and indirect methods must be considered as complementary and the results obtained for each one should be evaluated in the context of the clinical profile and epidemiological history.

INTRODUCTION

An exanthem (rash) is a skin eruption that may be caused by infections, drug reactions and inflammatory diseases. Various clinical and epidemiological factors should be considered in the evaluation of the possible infectious causes of exanthematic diseases. This includes firstly, the signs and symptoms that accompany the rash, such as fever, respiratory or gastrointestinal symptoms, joint pain, or adenopathy. Secondly, epidemiological aspects, such as disease in habitual contacts, contact with animals, foreign travel, and the time of year, given the seasonality of some exanthematic diseases. Thirdly, the vaccination status against exanthematic diseases included in the current vaccination schedule (rubella, measles, varicella) and possible recent vaccinations.

Infectious rashes may be caused by bacteria, viruses and parasites; many viruses cause exanthematic disease and some may cause different types of rashes (Table **1**) [1].

In addition to the aspects mentioned, the contribution of the microbiology laboratory is fundamental for the etiologic characterization of exanthematic diseases. This work reviews the various laboratory tests used to diagnose viral rashes.

THE LABORATORY IN THE DIAGNOSIS OF VIRAL EXANTHEMS

When a susceptible individual is infected by a virus, the disease appears after a variable incubation period; the virus may be recovered in correct clinical specimens in the days around the onset of symptoms. A few days later, specific antibody responses begin, initially IgM, which appears rapidly and is short-lasting, followed by an IgG response, which remains detectable for life [2]. Laboratory tests for the diagnosis of viral infections include <u>direct diagnosis</u> by identification of the disease-causing virus, and <u>indirect diagnosis</u> by determination of a serologic response of specific antibodies. The two methods may be considered complementary and the results obtained for each should be evaluated in the context of the clinical profile and epidemiological history.

Direct Diagnosis: Virus Isolation

There are three approaches to direct diagnosis: isolation, antigen detection and nucleic acid detection.

The objective of virus isolation is to recuperate the infectious virus. Collecting the correct sample at the right time is essential. The most useful samples for isolating the viruses causing exanthems are pharyngeal exudates (measles, ru-

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Table 1.   Most-Frequent Exanthematic Disease Causing Viral Infections (1)

	Virus	Disease
Maculopapular Rash	Measles	Measles
	Rubella	Rubella
	Parvovirus B19	Erythema infectiosum
	Human herpes 6	Exanthem subitum
	Enterovirus	Enteroviral disease
	Epstein-Barr	Infectious mononucleosis
	Cytomegalovirus	
	Dengue virus	Dengue fever
Vesicular Rash	Herpes simplex	Herpes simplex
	Varicella-zoster	Varicella
		Herpes zoster
	Coxsackie A	Hand-Foot-Mouth disease
Purpuric Rash	Dengue virus	Hemorrhagic Dengue fever
	Filovirus	Hemorrhagic fevers
	Enterovirus	
	Epstein-Barr	
	Cytomegalovirus	
	Measles	

bella, enterovirus [EV], varicella zoster [VZV], herpes simplex [HSV], cytomegalovirus [CMV]), urine (measles, rubella, CMV), and vesicular fluid (Table **2**). The clinical specimen is inoculated in a living system, embryonated eggs, experimental animals or, most-often, cell cultures. The cultures are incubated, generally to 37ºC, with periodic microscopic observation to observe cytopathologic alterations (cytopathic effect [CPE]) that indicate the presence of the virus in the specimen; the CPE is usually characteristic for each virus and cell culture used. However, once a CPE is observed, the virus should be identified by determination of the biological characteristics (e.g., agglutinating activity in the case of rubella or measles) or, more specifically, using an immunological technique, of which immunofluorescence (IF) using viral antisera or monoclonal antibodies against antigens is the most practical approach.

One modification of conventional culture methods is the shell vial assay, which shortens the time required to isolate the virus [3]. It is based on the use of cultures grown on a cover slip contained in flat-bottomed shell vials. Once the specimen is inoculated the vial is centrifuged. After 24 hours, even when the CPE is not evident, the viral antigens synthesized in the early phases of viral replication may be detected when the cell cultures are stained with virus-specific monoclonal antibodies conjugated with fluorescein. This method may be applied to practically all viruses for which isolation is useful, and especially CMV, HSV and measles.

For some viruses, isolation in cell cultures is not practical, either because there is no cell substrate available in diagnostic laboratories (Epstein-Barr virus [EBV], human herpes-6 virus [HHV6], human parvovirus B19 [PVHB19], and some EV), or, in the case of the Dengue virus, because it is a class 3 pathogen and requires other diagnostic techniques.

The measles virus may be isolated in pharyngeal specimens from 4 days before to 7 days after the onset of rash. Excretion in urine samples may extend to 20 days after the onset of rash. Currently, the best cell lines for the isolation of measles virus are B95a, a lymphoid line persistently infected by EBV, and Vero/hSLAM, derived from the Vero line that has been transfected with a plasmid which codes for the human protein SLAM (signalling lymphocyte-activation molecule), which has been shown to be a receptor both for the wild strains of the virus and for strains adapted to the laboratory [4]. Both lines show an equivalent sensitivity, although Vero/hSLAM is the line of choice, since both the handling and transport of B95a is considered potentially dangerous. An additional advantage of Vero/hSLAM is that it can also be used to isolate rubella. It also requires culture in medium containing the selective antibiotic geneticin (G418 sulfate) to maintain SLAM expression.

The CPE in both Vero/hSLAM and B95a is characterized by the appearance of syncytia with patent inclusion bodies,

Table 2.   Characteristics of the Isolation of Rash-Producing Viruses

Virus	Clinical Specimen (1)	Cell Culture (2)	Cytopathic Effect	Identification (3)	Response Time
Measles	PE, urine	Vero/hSLAM, B95-8	Yes. Syncytia	IF (N)	14-20 days
Rubella	PE, urine	Vero, BHK-21, Vero/hSLAM	Not apparent	IF (E1)	14-20 days
PVHB19	Not applicable				
EV	PE, vesicle	BGM, RD, A549, HEF	Yes. Typical of EV	IF, NT	2-20 days
HHV6	Not applicable				
VZV	PE, vesicular	HEF	Yes. Rounding, cell groups	IF	15 days
HSV	PE, vesicular	HEF, BHK21	Yes. Syncytia, destruction	IF	1-4 days
EBV	Not applicable				
CMV	PE, urine	HEF	Yes. Typical of CMV	IF	2 days*
Dengue	Not applicable				

(1) PE: pharyngeal exudate.
(2) BGM: African green monkey kidney; RD: human rhabdomyosarcoma; A549: human lung carcinoma; HEF: human embryonic fibroblasts; VERO: African green monkey kidney; BHK21: baby-hamster kidney; B95-8: EBV transformed lymphoid cells in monkeys; Vero/hSLAM: Vero with CD150 receptor or SLAM.
(3) IF: immunofluorescence; NT: neutralization.
* When shell vial assay is used.

generally two days after inoculation. Identification is made by IF, mainly using monoclonal antibodies against viral phosphoprotein (P). Cultures are only determined as negative after carrying out two blind passages, which may take 20 days. However, a negative result does not mean viral infection can be excluded, and other diagnostic approaches should be applied.

The rubella virus is excreted in the pharynx from one week before to four days after the onset of rash and can be isolated in urine for longer. There is no cell line in which an apparent CPE is produced, although it grows in some cell lines (Vero, RK13, SIRC, BHK-21, Vero/hSLAM). Vero/hSLAM is very useful, since the diferential diagnosis of rubella and measles is often required from the same sample, in the context of attempts to eliminate these diseases. Identification is made by IF using monoclonal antibodies against glycoprotein E1 of the viral capsid.

There are a number of susceptible cell lines for EV (Table **2**) that produce a typical CPE, characterized by nuclear pyknosis, rounding, refractility, degeneration and detachment from the solid phase. The virus can be isolated from faeces or pharyngeal specimens. Cultivation of the maximum number of serotypes requires a combination of cell lines. Identification is made in two steps: firstly, IF allows differentiation into polio virus or non polio virus using antisera that recognize a highly conserved epitope within the group of EV, located in VP1; secondly, neutralization, using antiserum mixtures in different combinations, identifies most EV [5]. However, one group of EV does not grow optimally in cell cultures (most Coxsackie A viruses) and their diagnosis requires molecular techniques.

HSV and VZV may be isolated from vesicular fluid or pharyngeal specimens. HSV grows rapidly, yielding a CPE in 2-4 days, whereas isolation of VZV may require up to 15-20 days. Identification is made by IF. CMV grows in human fibroblasts, where it produces a characteristic CPE with cell enlargement and intranuclear inclusions.

Currently, isolation is still considered a reference technique, because infectious virus is obtained; therefore it is very useful for molecular epidemiology studies and assays of susceptibility to antiviral drugs. It also permits the identification of serotypes in EV outbreaks and the diagnosis of atypical infections [3]. However, it is not usually applied in most laboratory diagnoses, due to its methodological complications, high cost, the need for staff trained in biosafety, and the time needed to obtain results. Viable viruses are recovered by isolation, which means that the specimen should reach the laboratory within a few hours in order to achieve the correct sensitivity. In addition, isolation may be tried from different types of specimen: in the case of rashes these include pharyngeal exudate, urine, faeces and vesicular fluid, each with different handling requirements before inoculation.

Even so, isolation continues to be highly useful as it provides the most complete characterization of viruses, although it is restricted to highly specialized laboratories.

Direct Diagnosis: Antigen Detection

Antigen detection directly on clinical specimens is useful in the diagnosis of Dengue virus infection. It is based on immunological techniques, such as enzyme linked immunoassay (ELISA) or immune chromatography (IC), which uses specific antisera for identification.

The assays detect the viral antigen NS1 in sera and are available commercially. The sensitivity is 60-70% and the specificity 86-94%. Not all types of Dengue virus are recognized equally, as the test is less useful for types 2 and 4 [6].

Antigen detection tests are especially useful between days 2 and 4 after disease onset in primary infection [7].

Direct Diagnosis: Nucleic Acid Detection

Genome amplification carried out by polymerase chain reaction (PCR) is currently used to detect nucleic acid. It involves three different steps: extraction and purification of nucleic acid, amplification of a selected fragment of the genome by PCR, and detection of the amplified product.

PCR methods used in the detection of viral exanthems have a better sensitivity than isolation or viral antigen detection. The samples are the same as those used for isolation, although saliva dried on filter paper [8], and blood dried on filter paper [9] have also been used.

Taking into account the large number of viral agents able to cause exanthematic disease, the need for assays that allow the differential diagnosis of these viruses is obvious. Assays that permit the detection of more than one virus simultaneously are especially useful for instance assays that allow the simultaneous detection of rubella and measles [10], and rubella, measles and PVHB19 [11]. The latter assay which is based on amplification of fragments of 289 base pairs (pb) of the rubella virus glycoprotein E1 gene, of 229 pb of the measles virus nucleoprotein gene and of 94 pb of the PVHB19 protein VP1 gene, has shown excellent sensitivity for the detection of measles and PVHB19, with a lower sensitivity for rubella. It has recently been confirmed that the addition of betaine, a reducing agent, to the amplification reaction, significantly improves the sensitivity of assays used for detecting the rubella virus genome [12].

Another important group of exanthematic viruses are EVs and the herpes viruses for which assays allowing their simultaneous detection and identification exist [13]. They are based on amplification of the conserved non-coding extreme 5´region for EV, and the polymerase gene, for herpes [including HSV, VZV, EBV, CMV). Characterization of EV, which is important epidemiologically, is carried out by sequencing the protein VP1 gene.

The combined use of multiplex assays [11, 13] allows the recognition of almost all exanthem producing viruses.

An important advance in the application of molecular methods has been to combine amplification with detection of the amplified product by fluorescence in the same reaction using real-time PCR. The great advantage of this approach is that it allows monitoring the progress of amplification as it occurs by the use of fluorescent probes; the rapidity with which the fluorescence reaches a specific level correlates with the amount of nucleic acid present in the specimen. Methods of real-time PCR have been developed for measles [14-16], rubella [17], PVHB19 [18], EV [19], herpes viruses [20, 21] and Dengue virus [22].

Indirect Diagnosis: Serology

Indirect diagnosis by identification of a specific serologic response to the virus is an alternative and complementary method to direct diagnosis.

There are two different approaches to serologic diagnosis: on the one hand, detection of seroconversion of total antibodies or specific IgG, which requires two serum samples, one taken at the onset of symptoms and the second 1-2 weeks later, and, on the other hand, detection of specific IgM in samples taken in the acute phase.

Seroconversion evidently does not provide a rapid diagnosis, and therefore the best approach is the detection of IgM, although sometimes, if the specimen is taken near to the onset of symptoms , IgM cannot be detected. In this case, confirmation of the diagnosis by analysis of a new specimen is necessary.

Generally, solid phase methods (ELISA, IF, chemiluminescence immunoassay [CLIA], immunochromatography [IC], immunofiltration and immunoblot [IB]) allow the detection of specific antibodies to the different isotypes, and are especially useful for the determination of IgG and IgM in the serologic diagnosis of viral infections; in contrast, agglutination, complement fixation and neutralization assays detect total antibodies and are useful to detect seroconversion.

Table **3** summarizes the serologic methods applicable to exanthematic viruses.

IgM detection is the best approach, since it provides a rapid diagnosis from the first days of the disease, although it has some drawbacks when used to diagnose viral rashes. Firstly, the simultaneous presence of rheumatoid factor and specific IgG may result in false positives, which are avoided by the elimination of IgG from the specimen before analysis by treatment with anti-human IgG antisera. Secondly, in some infections, simultaneous IgM reactivity to more than one pathogen occurs due to polyclonal stimulation of memory lymphocytes, a frequent phenomenon in cases of infectious mononucleosis; it is therefore not unusual to observe cases in which IgM specific for pathogens that infected the patient in the past are present. Simultaneous IgM reactivity to other exanthematic viruses has also been described in PVHB19 infections [23, 24].

In addition, some viruses have a high degree of cross-reactivity, which may be a problem when the viruses that cause this reaction have a similar clinical profile, such as EBV and CMV in mononucleosis syndromes, or HSV and VZV in vesicular exanthems, and EV. Another potential problem is latent infections of the herpesvirus family after primary infection; the latent virus may reactivate under specific conditions and cause disease. Virus-specific IgM can be detected in both primary infections and reactivation; therefore correct serologic differentiation of these infections into primary or secondary types is essential. Finally, virus-specific IgM may persist during prolonged periods in the absence of clinical symptoms, as a result of previous infection [25].

In order to resolve these problems, diverse confirmatory methods are used for each virus. Firstly, antibody profiles may be considered. The clearest example of their use in exanthematic viruses is EBV infections. The serologic diagnosis of EBV infections is based on the study of IgM and IgG

Table 3.   Serologic Methods Used for the Serologic Diagnosis of Viral Exanthems*

Virus	Elisa	IF	CLIA	IC	WB/IB	AGL/HI	CF	Neut
Measles	X	X	X			X	X	X
Rubella	X	X	X	X		X		X
PVHB19	X	X						
EV							X	X
HHV6		X						
VZV	X	X	X			X		
HSV	X	X	X		X			
EBV	X	X	X	X	X	X		
CMV	X	X	X		X	X		
Dengue	X	X		X				X

*ELISA: Enzyme linked immunosorbent assay; IF: Immunofluorescence; CLIA: Chemiluminescence immune assay; IC: Immunochromatography; WB: Western blot; IB: Immunoblot; AGL: Agglutination; HI: Hemagglutination inhibition; CFT: Complement fixation; NEUT: Neutralization.

antibodies to the viral capsid antigen (VCA) and EBV nuclear antigen (EBNA). If tests show the simultaneous presence of IgM antibodies to EBV and CMV, which occurs frequently due to the previously-mentioned cross-reactions, primary EBV infection is confirmed by the detection of IgG and anti-VCA IgM in the absence of anti-EBNA, which appears only 2 or 3 months after disease onset.

Another approach is characterization of the response to viral proteins by Western blot, a technique based on the use of proteins separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. This support is used as the solid phase for antibody identification. This can establish which viral proteins are detected by antibody, enabling antibody profiling.

IgG avidity assays are a third confirmatory method. Avidity is the strength of binding between an antibody and the antigen, and depends on the affinity between the corresponding epitopes and paratopes. The specific IgG response that occurs during the first days after primary infection is of low avidity. Since IgG avidity increases in subsequent weeks or months [26] low avidity IgG is a marker of acute primary infection. Assays exist for measuring the avidity of specific IgG, which use indirect methods, either ELISA or IF. Two simultaneous determinations are required, one using the normal procedure and another that includes a washing step after incubation of the antigen with antiserum in partially denaturing conditions, which permits the IgG to separate from the antigen if it is low avidity. The assay is then completed in the normal manner. IgG is identified as being of low avidity if there is a reduction in the titre in the specimen analyzed in the assay carried out in denaturing conditions compared with the normal procedure, which indicating primary infection. Methods have been developed to characterize the avidity of IgG against measles [27], rubella [28, 29], PVHB19 [30], HHV-6 [31]; EBV [32]; CMV [33], Dengue virus [34, 35] and VZV and HSV [29].

IgG avidity assays are applicable to the diagnosis of primary infection, and permit differentiation between primary

and secondary infection in herpes infections, especially those produced by CMV and VZV infections [29], since both diseases may produce specific IgM. In addition, they permit the confirmation of EBV infection [32] by detection of IgM anti-CMV, and are also useful to confirm or exclude primary rubella virus infection in pregnancy by detection of specific IgM [28, 36].

Evidently, serum is the best type of specimen for serologic studies. However, alternative types of specimen may have some advantages in the diagnosis of viral rashes. Saliva is easier to collect, especially in children. The yield of rubella-specific IgM in serum and saliva is equivalent [37], as is the detection of EBV-specific IgG [38] and measurement of the avidity of rubella-specific IgG [39]. Dry blood on filter paper is also useful, due to its easy preservation and transfer, and also has a good correlation with serum in the determination of measles-specific IgM [9].

Both direct and indirect diagnostic approaches should always be contemplated in the clinical and epidemiological context of the cases whose etiologic characterization is sought. In addition, the two methods are highly complementary, since the laboratory yield is optimized when both types of results are combined, as has been shown for exanthematic viruses including rubella, [40], measles [41], PVHB19 [42] and Dengue virus [35, 43].

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