Measles diagnostics in the elimination setting

L'anàlisi diagnòstica del xarampió en el marc de l'eliminació del virus del xarampió endèmic

Sara Mercader Verdés

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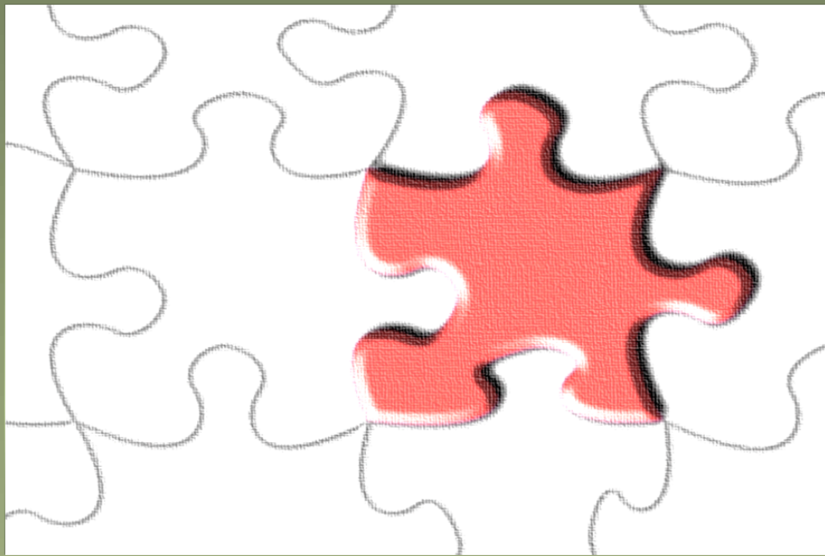
MEASLES DIAGNOSTICS IN THE ELIMINATION SETTING



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Tesi doctoral

2012

Universitat de Barcelona

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Memòria presentada per
Sara Mercader i Verdés
per optar al grau de Doctora en Biologia per la Universitat de Barcelona.

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A vosaltres, família

i especialment

a tu, ALBERT

Patience Clock

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Four. Tw,

Thr,

http://journeytotallulahfalls.wordpress.com/

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TABLE OF CONTENTS

ABBREVIATIONS 1

INTRODUCTION 2

I. THE DIAGNOSIS OF INFECTIOUS DISEASES 2

II. OVERVIEW OF MEASLES 3

A. THE DISEASE 3

B. MEASLES IN THE VACCINE ERA 6

1. MEASLES VACCINE 6

2. MEASLES IN ELIMINATION SETTINGS 8

III. DIAGNOSIS OF MEASLES 10

A. EVOLUTION OF THE DIAGNOSIS OF MEASLES 10

B. LABORATORY DIAGNOSIS OF MEASLES 13

1. MEASLES VIROLOGY 13

a) Measles virus 13

b) Infectious cycle 15

2. DIAGNOSTIC AND SURVEILLANCE METHODS BASED ON MEASLES VIROLOGY 16

a) Cell culture 16

b) Genome amplification by RT-PCR 16

c) Genotyping for virologic surveillance 17

d) Specimens for measles molecular virology 18

3. MEASLES IMMUNITY 18

a) Important antibodies for measles serology 19

i. IgM antibodies 19

ii. IgG antibodies 19

b) Useful antibody properties for measles serology 20

i. Antibody avidity 20

ii. Neutralizing antibodies 20

4. MEASLES SERODIAGNOSTIC TOOLBOX ……………………….......... 21
a) Serum-based enzyme immunoassay ……………………….............. 21
b) Enzyme immunoassays for serum alternative specimens ………........ 24
c) Avidity assay ……………………...……………………….......... 28
d) Assays to measure measles immunity ……………………….............. 30

IV. STATISTICAL TOOLS FOR ASSAY EVALUATION...................................... 31

A. ACCURACY ……………………...………………………......................... 31
B. PRECISION ……………………...………………………........................... 33

OBJECTIVES 35

PUBLICATIONS 36

1. COMPARISON OF AVAILABLE METHODS TO ELUTE SERUM FROM DRIED BLOOD SPOT SAMPLES FOR MEASLES SEROLOGY 41
2. VIRUS INFECTION IN PREVIOUSLY VACCINATED AND UNVACCINATED PATIENTS ………………….................................... 53
3. A MEASLES IgG AVIDITY ASSAY: USE IN CLASSIFICATION OF MEASLES VACCINE FAILURE CASES IN ELIMINATION SETTINGS 65

RESULTS AND DISCUSSION 102

I. IMPLEMENTATION OF DBS USE IN ELIMINATION SETTINGS OF SUSPECTED MEASLES CASES ……………………...….………….… 102
II. COMPLEMENTARY DIAGNOSTIC APPROACHES TO ENHANCE MEASLES CASE CLASSIFICATION ………………….......................... 105
1. DEVELOPMENT OF A MEASLES AVIDITY ASSAY ………..……. 108
2. EVALUATION OF DIAGNOSTIC PERFORMANCE……………...…... 110
III. MEASLES AVIDITY TESTING IN ELIMINATION SETTINGS……...…… 112
1. CLASSIFYING VACCINE FAILURES ………………………............... 113
2. PROVIDING REASSURANCE IN RESULTS OBTAINED BY IgM ASSAYS …………………….....………………………....……...…. 115
3. RESOLVING CASES WHEN AN IGM ASSAY IS NOT AVAILABLE 120

CONCLUSIONS 123

RESUM EN CATALÀ 125

REFERENCES 146

APPENDIX I 160

APPENDIX II 161

APPENDIX III 167

APPENDIX IV 185

ABBREVIATIONS

bp	Base pairs
CDC	Centers for Disease Control and Prevention
DBS	Dried Blood Spots
DEA	Diethylamine
EIA	Enzyme Immunoassay
F	Fusion protein
H	Hemagglutinin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISR	Immune Status Ratio
kDa	Kilo Daltons
L	Large protein
MeV	Measles Virus
mRNA	Messenger Ribonucleic Acid
MMR	Measles, mumps and rubella
N	Nucleoprotein
NPV	Negative Predictive Value
OF	Oral Fluid
ORF	Open Reading Frame
P	Phosphoprotein
pI	Isoelectric Point
PPV	Positive Predictive Value
PRN	Plaque Reduction Neutralization
PVF	Primary Vaccine Failure
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
ROC	Receiver Operating Characteristic
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
Se	Sensitivity
Sp	Specificity
SVF	Secondary Vaccine Failure
WHO	World Health Organization

INTRODUCTION

I. THE DIAGNOSIS OF INFECTIOUS DISEASES

Infectious diseases have existed since the dawn of humanity. However, the ability to identify the specific pathogen responsible for an infectious disease is a relatively recent event. Until the 19th century, the diagnosis of infectious diseases relied on incorrect theories due to lack of sound scientific knowledge[1, 2]. In the mid-19th century, Jacob Henle and Louis Pasteur formulated the germ theory, which explained the transmission of disease by declaring that each infectious disease is caused by a specific microbe. Technical and scientific advances followed, and led to the discovery of numerous pathogenic organisms, including the discovery of pathogens smaller than bacteria: filterable virus[3].

The field of immunology was born hand in hand with microbiology. In fact, the mechanisms behind immunity were not understood until bacteria were recognized as pathogens. By the end of the 19th century, it was demonstrated that blood serum contained substances, known as antibodies, which destroyed bacteria[4]. Serology began in 1896, when Fernand Widal developed the first agglutination test for the diagnosis of typhoid fever[5]. Over time, scientists have invented or modified serological and molecular diagnostic assays to provide the best means to accurately identify disease pathogens and feed information for epidemiological surveillance. Since the late 19th century, these assays have been at the core of the microbiological and public health laboratories, and their impact was well anticipated by Pasteur[6]:

Take interest, I implore you, in those sacred dwellings which are designated by the expressive term: Laboratories. Demand that they be multiplied; that they be adorned. These are the temples of the future . . . Temples of well being and happiness. There it is that humanity grows greater, stronger, better.

This dissertation presents diagnostic tools to help enhance measles control and surveillance in elimination settings, where endemic measles virus circulation has been interrupted.

II. OVERVIEW OF MEASLES

A. THE DISEASE

Measles is a serious and highly communicable acute respiratory disease that typically occurs during childhood, and spreads from human to human. Measles virus (MeV) causes measles. MeV is a *Morbillivirus* in the family *Paramyxoviridae* (Table 1). The regular course of classic uncomplicated measles spans approximately 15 to 21 days and begins with MeV invasion via aerosol droplets. The virus binds to MeV receptors expressed at the surface of cells in the respiratory tract of susceptible individuals[7]. Attachment is followed by transport to local lymph nodes and later into the circulatory system[8-10]. At this point, clinical symptoms appear, such as coryza, cough, conjunctivitis, fever, malaise and measles-specific Koplick's spots, which are observed on the buccal mucosa. Meanwhile, MeV is released from the respiratory tract using nectin-4 on polarized epithelial cells[11-13]. This prodromal phase lasts two to three days and is followed by the eruption of a characteristic maculopapular rash, which starts on the face and centrifugally spreads to the extremities (Figure 1). Measles cutaneous eruption subsides after three to four days, signaling the onset of the specific immunologic response against MeV[14].

Table 1: *Classification of measles virus (in bold font).*

Family	***Paramyxoviridae***	
Subfamily	***Paramyxovirinae***	*Pneumovirinae*
Genera ✓ Species example	*Respirovirus* ✓ Human parainfluenza virus type 1 and 3 *Rubulavirus* ✓ Mumps virus *Avulavirus* ✓ Newcastle disease virus *Henipavirus* ✓ Hendra virus ***Morbillivirus*** ✓ **Measles virus**	*Pneumovirus* ✓ Human respiratory syncytial virus A2, B1, S2 *Metapneumovirus* ✓ Human metapneumovirus

Devastating complications may follow acute infection as a result of MeV-driven immunosupression, complications that can lead to pneumonia, diarrhea, blindness or otitis. Postinfectious encephalomyelitis, measles inclusion body encephalitis, subacute sclerosing panencephalitis and death may follow acute MeV infection. The most destructive effects of measles are observed in children under five years in impoverished countries, as the effects are accentuated by malnutrition and lack of vitamin A[15].

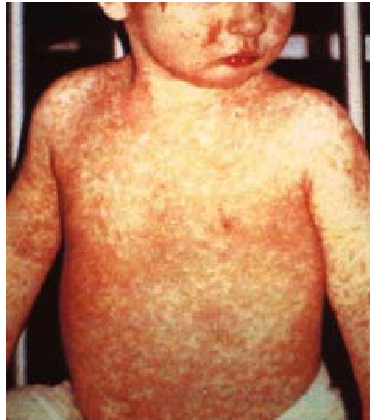


Figure 1 *Child with classic measles rash (4th day). Source: Centers for Disease Control and Prevention, National Immunization Program, Barbara Rice.*

Impact on health in the pre-vaccine era

Measles is an ancient disease that has probably accompanied civilization since its early days, causing major epidemics every two to three years and infecting all susceptible persons. Before a vaccine was available, measles was responsible for an estimated 135 million cases and 8 million deaths worldwide per year, with an estimated mortality rate of 30%. MeV may have evolved from rinderpest virus, a domestic livestock Morbillivirus, in the Middle East and India. Measles spread to Europe (8th century) and the American continent (15th century) through military expansion[16-18]. In medical literature, the first known citation of the disease dates back to the 6th century, but it was Abu Becr, also known as Rhazes of Baghdad, who in the 9th century first distinguished measles from other rash illnesses like smallpox[19, 20]. During an outbreak of measles in the Faroe Islands in 1846, Peter Panum observed the respiratory route of transmission, incubation period and lifelong immunity that resulted, which were key in the understanding of measles epidemiology[21].

Post-measles encephalomyelitis and subacute sclerosing panencephalitis were recognized as neurological complications of measles by James Lucas (18th century) and James Dawson (20th century), respectively[22, 23]. During the 18th century, Francis Home proved the infectious nature of the causative agent of measles; 150 years later, Ludvig Hektoen showed that the infectious agent was a virus[24, 25]. In 1954, Enders and Peebles first isolated MeV from a child with measles, opening the door to the *in vitro* growth in primate and non-primate cells and subsequent generation of attenuated measles vaccines[26-28].

B. MEASLES IN THE VACCINE ERA

1. MEASLES VACCINE

An effective measles vaccine became available in 1963. The live attenuated Edmonston B measles vaccine was developed by adaptation of the Enders' isolate of Edmonston wild-type virus to tissue culture through sequential *in vitro* growth passages in several cell types, and was later further adapted in two vaccines that were even more attenuated, the Schwarz and the Moraten vaccine strains[29, 30]. In the early 1980s, the World Health Organization (WHO) introduced worldwide measles vaccination through the Expanded Programme for Immunisation (Figure 2). In many parts of the world, measles vaccine is administered in a two-dose schedule in combination with the mumps and rubella vaccines (known as MMR)[31]. The availability of a safe and effective vaccine has encouraged the establishment of measles mortality reduction and elimination goals for each of the six WHO Regions. The implementation of the *Measles Mortality Reduction Strategy* led to a 67% decline in the estimated number of annual measles cases from 852,937 in 2000 to 278,358 in 2008, and a 78% decline in the estimated number of deaths from 733,000 in 2000 to 164,000 in 2008[32]. Disease elimination and eradication were defined at the Dahlem

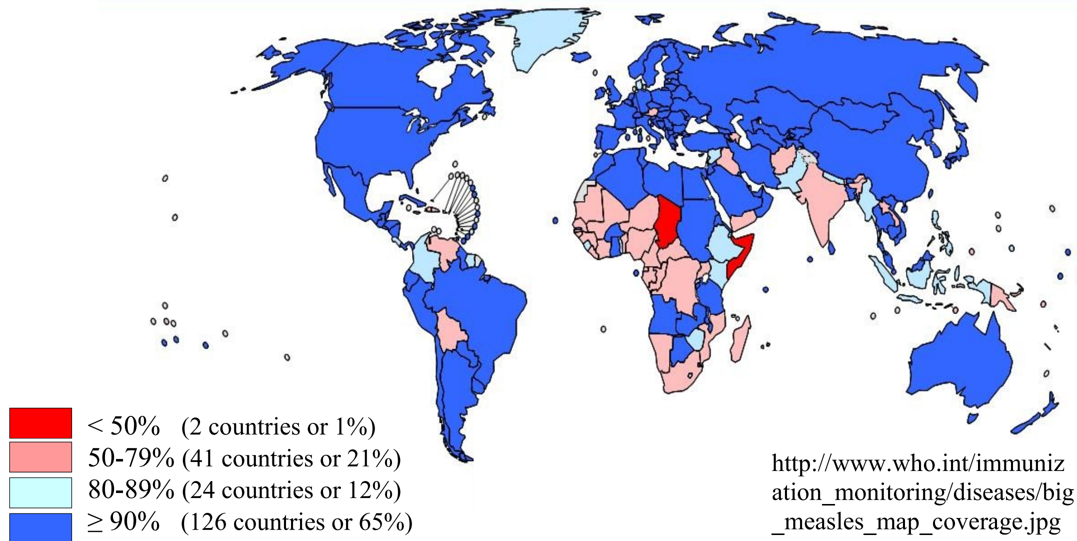


Figure 2: *Worldwide measles vaccine coverage in infants based on 193 WHO member states. Date of slide is 26 July 2011. Source: WHO/UNICEF.*

Workshop[33]. To interrupt MeV circulation in a specific geographic area and thus eliminate measles, a high level of herd immunity (92%-95% vaccination coverage) needs to be achieved and maintained through immunization campaigns[42]. WHO regions have started programs with goals to eliminate measles by 2012 (West Pacific Region), 2015 (European and East Mediterranean regions) and 2020 (African region). As of the year 2012, this international initiative has led to the successful elimination of measles in the American Region and in other geographical areas[34-39]. Although eradication by 2020 is considered feasible, a goal for worldwide measles eradication has not yet been established[40, 41]. Reasons supporting measles eradication and potential impediments to attaining this goal are summarized in Table 2.

Table 2: *Measles eradication*

Reasons[33, 36, 43]

- MeV is only transmitted from human to human
- Accurate diagnostic tools are available
- Existing vaccines are effective and inexpensive
- Interruption of endemic MeV circulation in large geographic areas for prolonged periods indicates that eradication is achievable

Potential impediments[36]

- Lack of political will
- Transmission among adults
- Increasing population density
- The human immunodeficiency virus epidemic
- Waning immunity and the possibility of transmission from subclinical cases
- Risk of unsafe injections

2. MEASLES IN ELIMINATION SETTINGS

In settings where endemic MeV has been eliminated, the majority of the population is vaccinated with two doses of measles vaccine and the number of reported measles cases is extremely low. Despite very high vaccine coverage, sporadic measles cases and small-size outbreaks do occur due to travel of infected persons from countries with endemic MeV. This has been observed in the United States and Catalonia, where measles was declared eliminated in the year 2000[38, 44]. In the United States from 2001 to 2008, the size of these outbreaks

ranged from 37 to 140 cases[45]. In the year 2011, a total of 222 confirmed measles cases were reported[46]. In Catalonia, intermittent measles outbreaks have occurred since 2000, with the largest outbreak in 2006-07 involving 381 confirmed measles cases[47, 48]. In elimination settings, outbreaks tend to involve measles cases within the unvaccinated population, including children below the age of vaccination (<15 months of age), vaccine exemptors, and travelers arriving from measles endemic areas. Measles cases, although relatively rare, are also reported within the vaccinated population and are classified into primary vaccine failure (PVF) and secondary vaccine failure (SVF) cases. PVF cases are individuals who never made an immunologic response to the vaccine; about 5% of first time vaccinees do not seroconvert[49]. However, providing a second dose of measles vaccine has made PVFs an extremely rare phenomenon. SVF cases are individuals who did seroconvert, but lost immunity over time or had an incomplete immune response[48, 50, 51].

From a public health perspective, it is paramount that each measles case occurring during the elimination phase is correctly identified to assess the need for outbreak control measures. Equally important, each measles vaccine failure should correctly be classified as a PVF or SVF case, since rates of vaccine failure may be used in the analysis of outbreaks, and the re-evaluation of vaccination programs. Confirmation of SVF cases is also important when studying the role of SVF in measles transmission in the community. However, in elimination settings, measles diagnosis becomes more complex because SVF cases may present modified clinical symptoms and modified immune responses.

III. DIAGNOSIS OF MEASLES

A. EVOLUTION OF THE DIAGNOSIS OF MEASLES

Diagnosis of measles has evolved with vaccination. Prior to the introduction of the measles vaccine, measles was a childhood disease clearly distinct from other rash illnesses and had a defined "periodicity" or cyclical epidemiology. Physicians easily recognized the disease, and clinical diagnosis was highly sensitive[52]. Three phases in the diagnosis of measles unfold as countries heighten measles vaccination campaigns and move from measles control to measles elimination. The first phase can be described as the time during which measles is endemic and a physician's recognition of clinical symptoms of classic measles is sufficient for the diagnosis of acute infections.

The second phase takes place in areas with elimination goals and high vaccination rates. As measles becomes a less common disease, errors in clinical diagnosis may occur. Physicians are less familiar with measles symptomatology, which can easily be mistaken for other febrile rash illnesses like rubella, parvovirus B19, and herpesvirus 6; several different bacterial diseases; or allergic responses to antibiotics and other allergens[53]. Case-based surveillance is then implemented, and physicians refer samples from all patients fitting the clinical case definition to the laboratory for testing and subsequent case classification[54]. Clinically suspected cases are confirmed by detection of measles-specific IgM antibodies or amplification of MeV RNA, and circulating wild-type MeV are identified by genotyping (see *Laboratory diagnosis of measles*). Additionally, immunization levels in the population are evaluated by detection of measles-specific IgG antibodies[55]. Laboratory surveillance coupled with epidemiological surveillance becomes essential to guide measles control efforts and achieve measles elimination goals.

The third phase emerges when MeV has been eliminated. In these regions with no endemic MeV circulation and extremely low measles prevalence, medical personnel may have even less experience with measles, which may result in delays in patient isolation, in outbreak investigation, and in specimen collection[56]. To complicate matters, infections with MeV may result in modified measles symptoms in SVF cases. Modified disease has been described in pre-elimination settings[57]. The difference between pre-elimination settings and elimination settings is that in case-based surveillance, suspected SVF cases also need to be investigated, as they are becoming more relevant. However, current available tools cannot specifically confirm suspected SVF cases. In simple terms, classic measles disease is observed in unvaccinated persons and in PVFs, while modified measles disease is most often observed in SVF cases. The spectrum of classic to modified symptoms observed in SVF cases is summarized in Table 3[58]. Due to the milder and modified presentation of the disease in SVF cases, modified measles cases are not recognized as measles outside of a measles outbreak[51]. Furthermore, an epidemiological link to a measles case is not always easy to establish. International travel from endemic areas may indicate a potential exposure[59]. If appropriate samples are collected, molecular epidemiology can track the origin of transmission[56]. Moreover, MeV is infectious in the environment for at least one hour, and infection with MeV without direct contact is possible[60]. Until measles becomes eradicated, importations from endemic areas may occur with possible spread to unvaccinated persons, or vaccinated persons with insufficient protection against MeV. Therefore, in elimination settings, the clinical diagnosis of measles relies even more on the laboratory confirmation of suspected cases.

***Table 3**: Main characteristics of classic and modified measles*

	Classic measles	**Modified measles**
Case is	Unvaccinated, PVF, or SVF	SVF
Symptoms		
Fever	≥38.3°C	Present or absent
		If yes, could be < 38.3°C
Rash	Generalized rash	Present or absent
	≥ 3 days duration	If yes, rash is milder, distribution may
		be uneven, duration may be shorter
CCC[1]	At least one	None or at least one
Laboratory confirmation		
IgM detection	OK[2]	IgM detected or not
RNA detection	OK[2]	OK[2]

[1] CCC is cough, coryza and conjunctivitis

[2] In timely collected samples. See *Limitations of IgM EIA's in elimination settings.*

B. LABORATORY DIAGNOSIS OF MEASLES

The role of the infectious disease laboratory in public health is to analyze specimens from patients or the environment and deliver timely information to assist in the diagnosis and surveillance of disease. In measles laboratory diagnosis, this is achieved through assays developed based on the virology, immunology, biochemistry and epidemiology of MeV. Once developed, assays are validated using statistics. Public health infrastructure is important for the implementation of assays and the provision of quality specimens and accompanying records. Next is a review of basic measles virology and immunology. Applications to the laboratory diagnosis and surveillance of measles relevant to this dissertation are presented.

1. MEASLES VIROLOGY

a) Measles virus

A cartoon of MeV virion and genome organization is depicted in Figure 3[61-63]. The genome of MeV is described as negative-sense because purified molecules of the MeV RNA are not infectious. MeV genome serves as a template for the synthesis of six monocistronic messenger RNAs (mRNAs) (transcription) and for the synthesis of positive-sense RNA (replication)[64]. Genomic and antigenomic RNAs are closely associated with the nucleoprotein (N; 60kDa), the phosphoprotein (P; 72 kDa), and the large protein (L; 200 kDa) forming a helical complex known as the ribonucleoprotein (RNP). The RNP transcribes and replicates MeV genetic information, and is the template for translation. Within the RNP, L is an RNA-dependent RNA polymerase. The matrix protein (M; 37kDa) coats the outside of the RNP and forms helices around it. The M-coated RNP folds into bundles that are connected by M-M interactions. M plays a role in regulating RNA transcription. The MeV envelope is made of cellular lipids and contains two external surface glycoproteins, the hemagglutinin (H; 78 kDa) and the fusion protein (F; 60kDa)[65].

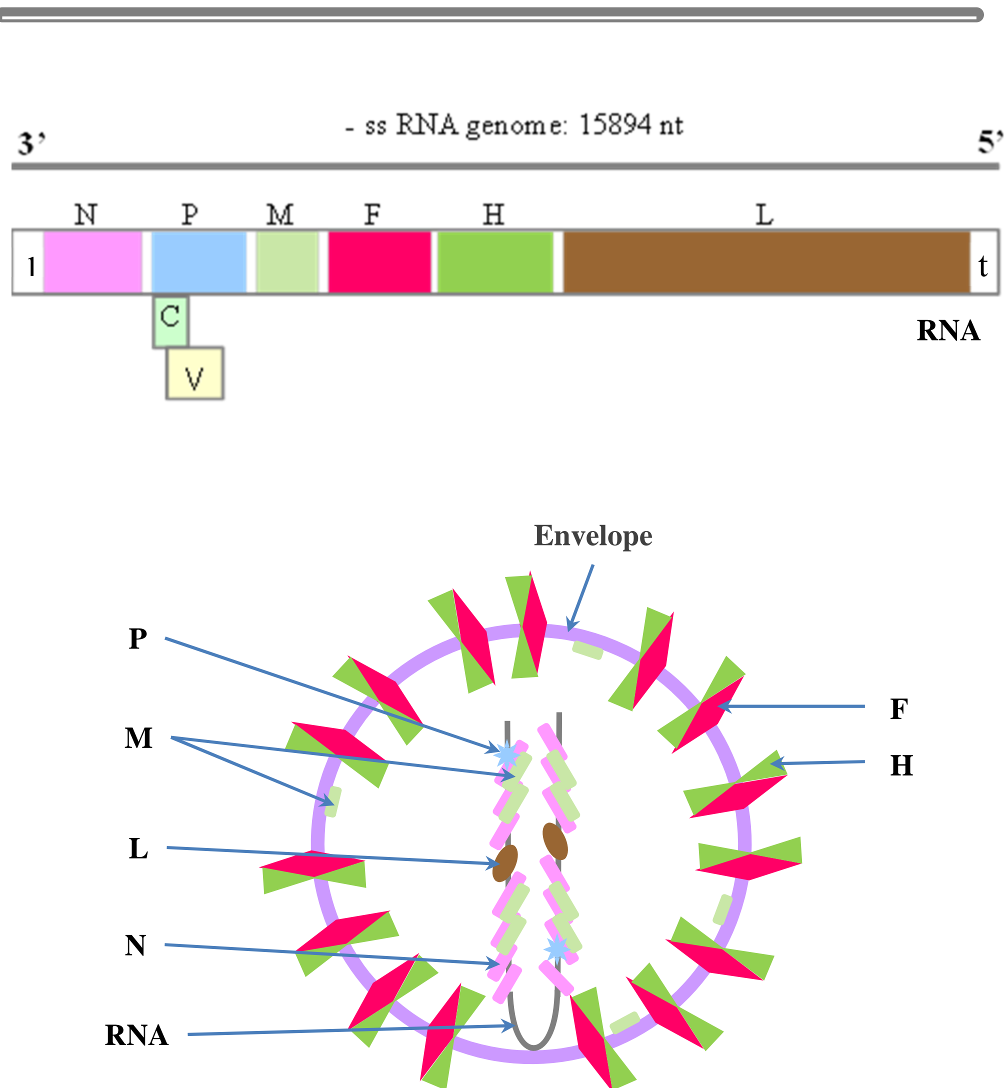


Figure 3: *Schematic of measles virus virion and genome organization. The horizontal solid line and the large box are schematic diagrams of the nonsegmented single-stranded negative-sense MV genome RNA. Each MV gene is represented by a small box. " l " and " t " represent the leader and the trailer sequences at the ends of the genome. N is the nucleoprotein gene; P is the phosphoprotein gene, V and C are genes encoded within the P gene; M is the matrix protein gene; F is the fusion protein gene; H is the hemagglutinin; and L is the large protein gene. White boxes show untranscribed RNA areas. Below the genomic diagram is a representation of measles virion with the location of each viral protein indicated.*

b) Infectious cycle

MeV infectious cycle starts with H and F acting in concert to mediate attachment and penetration into the cell, thus governing MeV tropism and pathogenesis. Specifically, H from wild-type MeV attaches to at least two known receptors: the signaling lymphocyte activation molecules (SLAM, or CD150) located at the surface of dendritic cells, lymphocytes, and macrophages, and the tumor cell marker nectin-4 located at the surface of epithelial cells in the upper respiratory tract and lungs[7, 117, 11]. The binding of H to its receptors provides the F protein with the energy to trigger membrane fusion, which allows infection of targeted cells and fusion of infected and uninfected cell membranes, thus promoting cell to cell spread and the formation of syncytia or multinucleated cells[66]. Once in the cell cytoplasm, RNPs begin transcription at the N protein and, in a sequential fashion, generate capped and polyadenylated mRNAs; mRNAs are not encapsidated[67]. The first and most abundant transcript is the N protein, and the least abundant is the L protein (Figure 3)[68, 69]. Being the most abundant protein, N is also the target of most of the antibodies produced to MeV infection. Hence, N is the most antigenic MeV protein and is used as an antigen in serological assays. During replication, genome RNA is synthesized and immediately encapsidated by the N protein[64, 67, 70]. In the cytoplasm, M coats the newly synthesized RNPs, and the M-RNPs are transported to the plasma membranes for budding. F-, H-, and M-coated RNPs co-localize on membrane domains (rafts) that act as scaffold for assembly into functional and infectious units. After budding, virions contain several RNPs and raft and non-raft membranes[71-73].

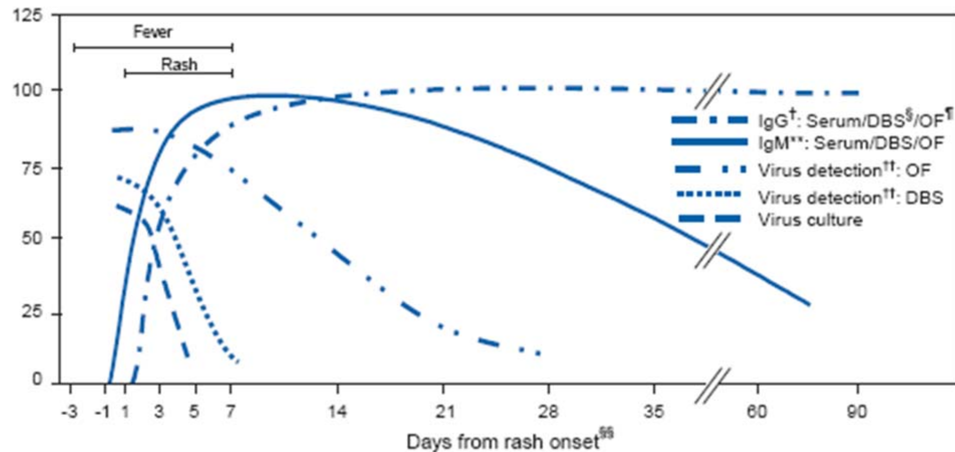
2. DIAGNOSTIC AND SURVEILLANCE METHODS BASED ON MEASLES VIROLOGY

a) Cell culture

Isolation of MeV in cell culture from clinical specimens is necessary for good surveillance of circulating MeV strains and for outbreak investigations[74, 75]. Highly specialized laboratories use cell culture to obtain viable MeV as a source for sequencing, which allows for the collection of reference strains to help identify emergent genotypes. Although cell culture is important for measles molecular epidemiology, this method is rarely used as a diagnostic tool because currently there are faster methods to diagnose measles (Figure 4, see Virus culture).

b) Genome amplification by RT-PCR

The reverse-transcriptase polymerase chain reaction (RT-PCR) method is used to directly detect MeV RNA in clinical samples and to amplify the N and H genes prior to sequencing[76-79]. RT-PCR is especially helpful in the diagnosis of cases whose samples are collected within the first three days after rash onset, when about 20% of samples may still be IgM negative (Figure 4, see Virus detection)[80]. RT-PCR amplification can help classify cases that are inconclusive by serology. For instance, a positive RT-PCR reaction provides reassurance when an IgM positive result is obtained. As newly developed real-time RT-PCR protocols have increased detection sensitivity, it is now possible to detect 10 genome copies[81]. However, RT-PCR may give rise to false negative results due to poor sample quality, inappropriate collection methods, or suboptimal timing of sample collection. Cross-contamination is possible if the laboratory does not have adequate facilities to perform RT-PCR and the persons in the staff are not well trained. Furthermore, samples for virological characterization are not always collected[75].



* Illustrative schematic based on data presented at the Measles and Rubella Alternative Sampling Techniques Review Meeting, convened in Geneva, Switzerland, in June 2007.
† Immunoglobulin G.
§ Dried blood spots.
¶ Oral fluid.
** Immunoglobulin M.
†† Virus RNA detection by conventional, nested, or real-time reverse transcription–polymerase chain reaction.
§§ Incubation period: approximately 14 days.

Figure 4: *Timing of specimen collection for measles confirmation. Percentage of positive results among patients with wild MeV infection, by day from rash onset and type of sampling method used – WHO Measles and Rubella Laboratory Network* - With permission from David Featherstone (WHO)* [82, 83].

c) Genotyping for virologic surveillance

RT-PCR followed by sequencing identifies circulating genotypes, thus providing information on transmission pathways and aiding in distinguishing wild-type strains from vaccine strains when adverse vaccine reactions are suspected or mistaken as measles cases. Specific N primers are used to amplify the most variable region of N. If a new genotype is suspected, then a new viral isolate is obtained and the complete sequence of the H gene is

amplified and sequenced in addition to the N sequence. A review of this topic and a map of the global distribution of the 23 measles genotypes can be found in Rota *et al.*[84, 85].

d) Specimens for measles molecular virology

Specimens for measles molecular virology are optimally collected between zero to three days after rash onset, but this window can be extended to seven days if necessary. The best types of specimens, in descending order, are nasopharyngeal swabs, throat swabs, and urine specimens; they can serve for both virus isolation and genome amplification by RT-PCR[78]. Dried blood spots collected on filter paper and oral fluid samples collected with the Oracol (Malvern Medical Developments, Ltd, Worchester, England) or similar collection device can also be analyzed (Figure 4, see Virus detection)[75, 76, 86, 87].

3. MEASLES IMMUNITY

The immunologic response to MeV infection involves both the innate and the acquired branches of the immune system. Elements of the innate immune system are activated in the initial stages of MeV infection[88]. The specific protective mechanism against MeV is provided by humoral and cellular elements of the acquired immune system, which only develop after contact with the virus by natural infection or immunization. Subsequently, protection from MeV disease is generally believed to be maintained for life through continued maintenance of long-term memory B and T cell populations and production of antibodies and circulation of MeV-specific T cells[21, 89]. Upon challenge by MeV, the humoral response is engaged and specific antibodies are produced. MeV only has one serotype. Immunity generated after MeV invasion neutralizes all MeV strains, wild-type and vaccine. The laboratory diagnosis of measles is based on components of humoral immunity only.

a) Important antibodies for measles serology

(1) IgM antibodies

In individuals inoculated with wild-type MeV or MeV vaccine, IgM antibodies are first produced by B cells and secreted into the circulatory system. IgM is found in serum and saliva. IgM antibodies are pentameric with ten antigen-binding sites. IgM is also produced during the secondary immune response. Figure 4 depicts the kinetics of detection of IgM in classic measles[80]. Detection of specific IgM in serum indicates infection with MeV and is an important criterion in the confirmation of measles in unvaccinated individuals or in PVF. IgM is a less reliable marker in the diagnosis of SVF because the IgM response is short-lived and may not be detected (Table 3)[90-92].

(2) IgG antibodies

IgG antibodies are monomeric with two antigen-binding sites. There are four subclasses of IgG: IgG1, IgG2, IgG3, and IgG4. All, except IgG2, can cross the placenta. For diagnostic purposes, total measles-specific IgG is detected. IgG is found in all body fluids, including serum and saliva. Figure 4 depicts the kinetics of detection of measles IgG after classic measles[93]. Measles IgG isotypes have a marked specificity for the MeV N, F and H proteins. Detection of measles IgG is important in the determination of seroconversion, the identification of acute infection by a four-fold rise in IgG titer between paired acute and convalescent sera or by determination of low avidity antibodies, the evaluation of protection by detection of neutralizing antibodies, and the classification of vaccine failures. In vaccinated individuals, the antibody titer is lower than that induced by natural infection, and serum titer tends to wane with time[94]. Therefore, a second vaccine dose is recommended in

most countries to protect susceptible individuals by providing another chance for PVFs to seroconvert[95-97]. A second dose of the vaccine also helps boost the immune response[98, 99].

b) Useful antibody properties for measles serology

(1) Antibody avidity

Avidity is the term used to describe the net antigen force by which multivalent antibodies bind to multivalent antigens. Antibodies bind to antigens mainly through relatively weak non-covalent interactions. In the naive host, the first antibodies elicited upon challenge with a new antigen are of low avidity and bind less strongly to their targeted epitopes. The force of the antibody-antigen interaction will become stronger as antibody avidity matures over the following weeks to high avidity. Antibody maturation takes place during B cell differentiation. B cells undergo a process known as somatic hypermutation, which is followed by selection of high affinity (avidity) clones[100].

In MeV infections, measles-specific IgG transitions from low to high avidity after infection or vaccination over the first three months; low avidity antibody correlates with recent exposure of naive individuals to MeV or those who are PVF, while high avidity antibody correlates with distant exposure or with instances of SVF[101, 102]. High avidity in measles-specific IgG correlates with protection[103]. The quality of antibodies is believed to play an important role in protection from reinfection, since non-neutralizing low avidity antibodies elicited by a formalin-based vaccine could not protect against atypical measles[104].

(2) Neutralizing antibodies

Neutralizing antibodies bind to F and H proteins at the surface of MeV virions and render the virus non-infectious. It has been shown that a minimum level of neutralizing antibodies (titers ≥ 1:120 mIU/mL) is needed to protect a person against classic measles

disease[57]. Detection of anti-MeV glycoprotein-specific IgG correlates with virus neutralization, and thus with protection[105]. Serum neutralizing antibodies recognize both linear (sequential) and conformational epitopes on the H antigen, and many of these appear to be dependent on the correct glycosylation and processing of H. MeV F antibodies are believed to contribute to the overall neutralizing activity by preventing viral-cellular membrane fusion[106, 107].

4. MEASLES SERODIAGNOSTIC TOOLBOX

a) Serum-based enzyme immunoassays

Contemporary measles laboratories generally use enzyme immunoassays (EIA) to detect IgM and IgG in serum[108]. Briefly, EIAs are based on the formation of specific antigen-antibody complexes bound to a solid phase (usually 96-welled polystyrene plates), which are detected through a specific colorimetric or fluorescent reaction. The amount of visual signal produced is proportional to the bound antigen-antibody complexes. The cartoon in Figure 5

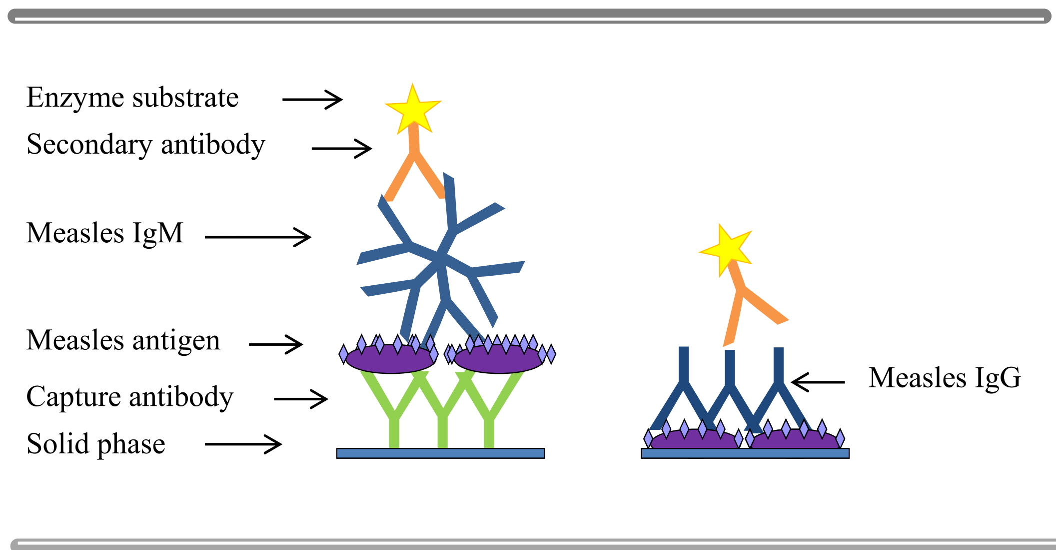


Figure 5: *Schematic of the capture (left) and indirect (right) enzyme immunoassays. Symbols as indicated.*

represents the typical steps involved in the two assay formats used to detect measles-specific antibodies. Antigens may consist of either extracts of MeV-infected cells containing all MeV proteins, or recombinant N protein expressed in cells of the insect *Spodoptera frugiperda* or in *Saccharomyces cerevisiae*[109, 110]. It is recognized that IgG can interfere with IgM detection in indirect EIA formats. Measles-specific IgG present in the serum specimen can compete with IgM, interact with the antigen coated on the plate, and prevent IgM binding, thus resulting in false negative results. Hence, indirect IgM EIAs include a step where IgG is absorbed from serum samples prior to testing. Capture assays do not require serum pre-absorption[55].

EIA has proved to be more sensitive and specific than most previously available assays, and is more easily performed and readily standardized (Table 4). Current IgM EIAs

Table 4: *Measles serodiagnosis before the development of enzyme immunoassays.*

MeV antigen	Assay	Reference
H	Hemagglutination inhibition	111
F	Hemolysis inhibition	112
H and F	Neutralization assays	113
	Mixed agglutination	114
N or whole virion	Complement fixation	115
	Immunodiffusion	116
	Immunofluorescence	117
	Radioimmunoassay	118

provide the advantage of diagnosing measles using a single serum collected at the first visit to the health care center, thus circumventing the need to obtain two serum samples to determine a four-fold increase in IgG antibody. Detection of measles IgM by currently used EIAs is the preferred method of confirming measles. In general, the sensitivity (Se) of measles IgM EIAs ranges from 83% to 89% and the specificity (Sp) from 95% to 100%, in samples collected within four weeks of rash onset. The use of EIAs provides an ample window of opportunity to draw blood and detect measles-specific IgM (Figure 4, see IgM)[55].

Limitations of IgM EIAs in elimination settings

The following limitations are a result of low MeV circulation and low disease prevalence in the population[55, 119]:

1) IgM tests may generate false positive results due to detection of IgM specific to other rash-producing infectious agents, and sometimes due to competition with serum rheumatoid factor[53, 120].

2) IgM tests may also yield false negative results in samples collected in an untimely manner, either too early a collection from an acute measles case, or too late in a SVF case, when the IgM response tends to be more transitory[58].

3) False positive results may be obtained due to the strong influence of the prevalence of disease on the positive predictive value (PPV). In this context, PPV is the probability that a patient infected with MeV will have a positive measles-specific IgM result. This probability is dramatically decreased when the prevalence of the disease in the population is extremely low (<1%). In practicality, large numbers of specimens from individuals with rash and fever disease are typically analyzed by IgM EIA, thus increasing the probability of false reactions (false positives).

4) IgM positive results are often suspected of being false positive in those isolated suspected cases that are assumed to be protected, either by natural infection because they were born before the vaccine era (1957) or by record of vaccination.

5) The important decrease in the number of cases in elimination settings can also affect the availability of IgM assays to confirm cases. For instance, in the United States, there is only one imported capture format IgM EIA and four indirect format IgM EIAs that are commercially available.

6) Detection of IgM cannot distinguish between MeV wild-type and vaccine strains in persons who have been vaccinated days or weeks prior to developing a measles–compatible rash; all strains of MeV elicit IgM antibodies. This limitation is only resolved with genotyping.

To help confirm IgM positive results, measles-specific IgG assays are usually used as a complementary assay. IgG negative tests in sera collected within seven days after rash onset help confirm the diagnostic of a positive IgM result. When there is no history of immunization, IgG positive tests may sometimes help to unveil false positive IgM results. However, in vaccinees, IgG positive tests are not very helpful since the assay is not able to distinguish between primary and secondary immune responses. In some instances, measles cases can also be confirmed by observing a four-fold rise in measles IgG antibodies titers in paired acute and convalescent sera, if a convalescent sample is available.

b) Enzyme immunoassays for serum alternative specimens

Serum is the specimen most frequently used for measles serology, while throat swabs, urine and/or whole blood can be collected for genetic characterization. MeV RNA can be successfully amplified in serum collected during the first three days after rash onset[121, 122].

However, serum is not routinely used for RT-PCR. Thus, several specimens are typically obtained for proper suspected case investigation. Oral fluid (OF) and dried blood spots (DBS) are alternative sampling methods that can overcome limitations posed by serum collection through venepuncture (see comparison in Table 5). One of them is that serology and RNA

Table 5: *Differential characteristics of specimen collection systems alternative to venipuncture*

Serum	Oral Fluid	Dried blood spots
Invasive collection	Noninvasive collection	Minimally invasive
Not used for RT-PCR	Best for RT-PCR (Oracol device)	Good for RT-PCR
Risk of disease transmission	Safer[1]	Safer[1]
Trained personnel	Minimal training	Minimal training
Poor acceptance[2]	Best accepted	Better accepted
Problematic with children	No problem	No problem
Not practical for some settings[3]	No problem	No problem
Electricity required	Electricity required	Electricity not required
Refrigeration required	Refrigeration required	Refrigeration not required
Biohazard	Non-biohazard	Non-biohazard
Shipped as biohazard	Regular postal system	Regular postal system

[1] Oral fluid is safer because there are no needles or glass tubes involved. DBS is safer because there are no glass tubes and retractable single-use lancets are used.

[2] Patient and parents may not accept due to involved pain or bruises, social/cultural barriers, fear to cross infection.

[3] Schools, day-care centers or in epidemiological studies.

amplification can be performed in one single specimen, OF or DBS. In 2008, the WHO published recommendations on the use of DBS and OF: collection of these alternative samples is suitable when there might be resistance to venepuncture or when collection, transport or refrigeration of samples is challenging. Serum continues to be the preferred specimen for measles diagnostics, especially in regions in the elimination phase that already have a surveillance system based on serum82. This dissertation includes a study based on DBS samples.

DBS were invented by Robert Guthrie in 1963[123]. Filter paper cards have made collection of whole blood very easy, and have been used extensively in neonatal screening of metabolic and hereditary disorders[124]. DBS is a simple whole blood collection device in which a few drops of blood are collected and dried on pre-printed cards made of filter paper after the piercing of a finger or foot with a lancet (Figure 6). DBS are then wrapped in glassine paper and stored in low-permeant plastic bags (zip-lock type) with desiccant silica packets at 4°C or at -20°C, depending on how rapidly the samples will be processed. Samples stored in this way are easily transported at room temperature, and no refrigeration is required. The stability of IgM, IgG and RNA in DBS at various temperatures has been studied[86, 125-129]. DBS samples do not break or spill, are light, and are easily handled and stored. Blood collection by DBS is very appealing no t only for the collection of samples in areas where

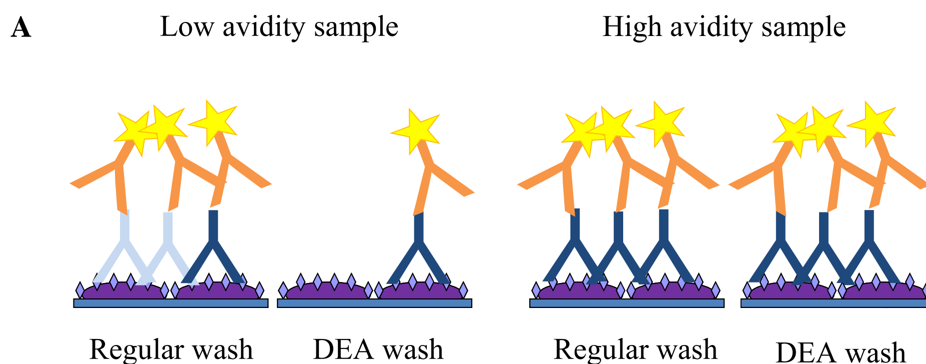
Figure 6: *Photography of a good quality dried blood spot specimen*

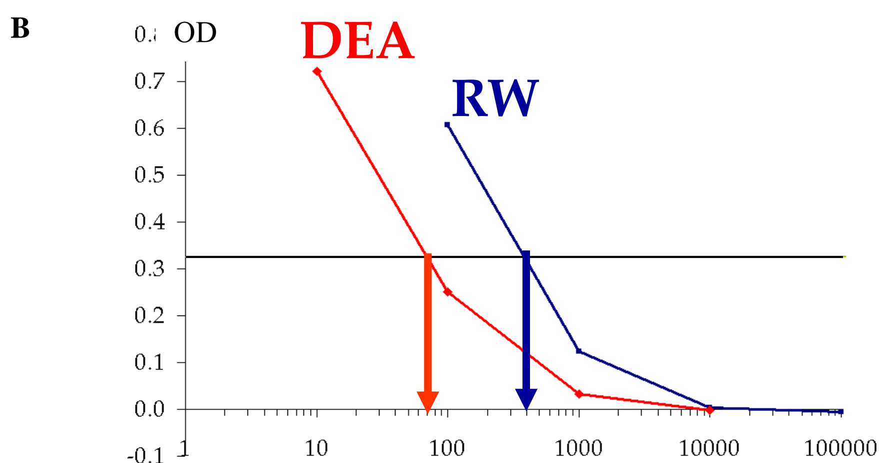
there is little infrastructure for phlebotomy, but also in the industrialized world, since the less invasive character of the sample collection is more accepted by the pediatric patient and by their parents. DBS specimen collection is easy, but some precautions need to be taken during collection to ensure that an acceptable sample is obtained and that tests results are not jeopardized[124, 130]. As in venepuncture, serum can be readily recovered from DBS and can be used for antibody detection. Over the years, several approaches have been used to extract serum antibodies from DBS. These approaches are either based on passive elution or on active elution. In passive elution protocols, eluates are obtained by soaking punch-out disks in elution buffer, without any further extraction steps. In active elution protocols, further extraction is achieved by manual extrusion, microplate or vial centrifugation, or extrusion through a column. A comparison of available protocols would facilitate the implementation of DBS in measles serology laboratories in elimination settings, as well as other laboratories interested in the use of DBS.

For detection of measles antibodies in DBS eluates, WHO recommends the use of the Enzygnost® Anti-MeV IgM and Enzygnost® Anti-MeV IgG indirect EIAs (Siemens, Germany), which have been adapted for the detection of measles antibodies in DBS eluates[75]. For instance, wash buffer is enriched with 5% milk to reduce background possibly caused by hemoglobin released from red blood cells[125, 128]. Evaluation studies have shown that detection of MeV antibodies in eluted DBS correlates extremely well with detection in venepuncture-derived serum by both indirect and capture EIA formats[125, 128, 129, 131-133]. For these studies, DBS were processed by passive elution, and by active elution via microplate centrifugation (Appendix I).

c) Avidity assay

Avidity assays answer the relative temporal diagnostic question of when the encounter with the antigen occurred. The basic principle of avidity assays plays with the quality of interactions established over time between antibody and antigen. Avidity assays differentiate between early and late antibody responses by using chaotropes (substances that disrupt non-covalent bonds) to either prevent antibody binding to antigen, or to elute antigen-bound antibody (Figure 7)[134-138]. Some chaotropes that have been used include diethylamine (DEA), urea, guanidine hydrochloride and ammonium thiocyanate[137, 139]. EIA is the preferred assay, although the avidity principle has been applied to radioimmunoprecipitation, Western blot and immunofluorescence assays[140-142]. Generally, an EIA that is already available is modified to include a wash with the selected chaotrope. Two parallel treatments are performed onto the antigen-antibody complex: one treatment consists of a wash with the EIA wash buffer, while the other consists of a wash with EIA wash buffer containing the selected chaotrope. A ratio between the optical density values of the chaotrope wash over the regular wash is calculated to determine an avidity index. The gold-standard procedure is based on the serial dilution of serum, followed by the calculation of an end-titer avidity index result as described in Figure 7. A logistically easier approach is to perform single dilutions. In this case, the ratio of optical density values with and without the chaotrope is calculated to obtain the avidity index result. In pre-natal screening, avidity assays are widely used to diagnose recent infections with rubella virus, *Toxoplasma gondii* or human cytomegalovirus[144-146]. Hepatitis C primary and chronic infections can also be distinguished using avidity assays [147]. Avidity testing has also been helpful in the serodiagnosis of other diseases[141, 148, 149]. Testing for measles antibody avidity has been used primarily to estimate the role of vaccine failure in outbreaks and as a research tool to study antibody maturation after infection, administration of experimental vaccines, or in persons with immunodeficiencies[50, 101, 150-156].





C

$$etAI\% = \frac{\text{end-titer DEA curve}}{\text{end-titer RW curve}} \times 100$$

Figure 7: *Schematic of an avidity assay enzyme immunoassay using the dilution approach with serially diluted samples. Symbols as in Figure 5. A: Serum IgG in light blue represent low avidity antibodies, in deep blue represent high avidity antibodies. B: Serially diluted curves. Blue is a serum curve washed with regular wash. Red is a serum curve washed with a chaotrope wash; here, deathylamine or DEA. C: General formula to calculate the end-titer avidity index (etAI%)*[143] .

Most of the measles avidity assays described in the literature use urea at 6M to 8M, and a few use DEA at 20mM or 70mM, or ammonium thiocyanate. Although several measles avidity

assays have been described, there is no standardized protocol to test for measles avidity and no independent evaluation of these assays has been reported. Furthermore, the availability of commercial avidity assays is limited in some areas. There is currently a need for a well validated measles avidity assay to be used as a complement to IgM testing in the seroconfirmation of suspected measles cases and as a tool to classify vaccine failures. Ideally, such an assay should be based on a commercially available platform to widely facilitate its use.

d) Assays to measure measles immunity

The measles-specific *in vitro* plaque reduction neutralization test (PRN) was developed in the early 1980s. This assay offered higher Se and Sp than previously available assays (Table 4)[105, 157]. PRN offers the best correlation with protection and is the adopted gold standard for measuring natural and vaccine-induced MeV immunity. PRN uses whole infectious MeV particles as the assay antigen which is added to sequential dilutions of serum. Protective antibodies will bind to H and F and neutralize MeV virus particles, impeding cell infection and plaque formation[158]. Criteria for immunity based on pre-exposure serum PRN titers have been established (Table 6)[57].

Table 6: *Level of plaque reduction neutralization titers in relation to protection*

If PRN titer in mIU/mL is	then individuals are
PRN titer ≥ 1052	protected from MeV infection and from disease
120 ≤ PRN titer <1052	may become infected by MeV but will not develop classic measles
PRN titer < 120	susceptible to MeV infection and to disease

IV. STATISTICAL TOOLS FOR ASSAY EVALUATION

Diagnosis determines the current state of health of an individual and, if the person is found to be diseased, accurately identifies the illness afflicting the person[159]. Statistics can help in the validation of diagnostic assays by presenting tools to design and interpret the studies that ultimately will deliver a working description of the performance of the assay. A useful diagnostic assay is accurate and can thus discriminate between true disease and true non-disease; it is precise, and can thus be repeated over time with the same level of performance. Accuracy can be estimated using Receiver Operating Characteristic (ROC) analysis.

A. ACCURACY

Receiver Operating Characteristic analysis

Assays to diagnose infectious diseases are often based on a continuous scale, such as the optical density resulting from EIAs. In the development of EIAs, at least a threshold needs to be established to have a binary system: positive or negative, infected or not, measles or not. Two intrinsic characteristics are typically used to describe the performance of an assay: the Se and Sp, both of which are contingent to the established threshold [160]. Additionally, two values are used to describe assays based on Se and Sp that take into account the prevalence of disease in the population to which the assay will be applied to: the positive (PPV) and the negative predictive values (NPV). It is important to note that each threshold is associated with Se and Sp values that do not describe the assay at any other threshold. Limitations of Se, Sp, PPV, and NPV include dependence on threshold level and dependence on disease prevalence. To overcome these limitations, one can use ROC analysis. ROC analysis is a widely used statistical tool for describing and comparing the accuracy of

medical diagnostic tests[161-166]. The beauty of ROC analysis lies in the advantages that the method offers: (1) independence from prevalence of disease; (2) two or more assays can easily be compared; and (3) the overall accuracy of the assay is captured in a single index by estimating the full area under the ROC curve.

ROC is a representation of the relationship between Se and Sp at all possible thresholds (Figure 8); each curve point of the ROC is associated with each threshold. The performance of a new assay is then compared to the gold standard. The gold standard is the procedure that makes possible the identification of true disease status. For many diseases, a gold standard is not available due to cost or practical or ethical reasons. There is no unifying guideline on how to proceed in situations where the gold standard is missing (i.e., the gold standard cannot be performed in all subjects), imperfect or absent. Even when the perfect

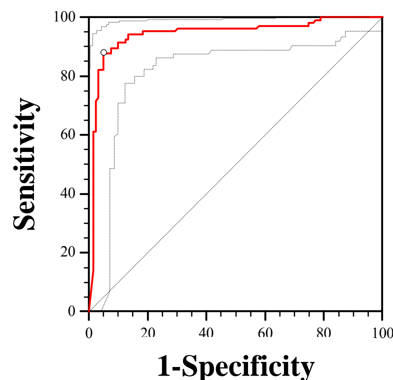


Figure 8: *Receiver Operating Characteristic (ROC) curve. An ROC curve that passes through the top left corner of plotted area provides an area under the ROC (AUC) curve of 1, interpreted as perfect accuracy. Diagonal line is AUC=0.5, interpreted as random guess. 95% confidence intervals are in plotted as dashed lines. Circle indicates point with maximal accuracy, or highest sensitivity and specificity.*

gold standard is available (for instance, viral isolation in a viric disease), there may be laboratory or other errors. One solution consists of relating the results of the assay being evaluated to relevant clinical characteristics, test results or future events[169]. This approach was applied in this dissertation. Using the selected gold standard, it is possible to create a diseased and a disease-free group,against which the assay is compared. Formulae are available to calculate the sample size of the gold standard groups for different applications[170]. Parametric and nonparametric approaches can be used to create an ROC[164, 171, 172]. Software packages are available to assist in ROC analysis[173]. The area under the ROC curve (AUC) describes the overall accuracy of the assay. The values of AUC range from 0.5 to 1 (Figure 8). AUC values have been described as follows: $0.5 \leq AUC \leq 0.7$ is low accuracy, $0.7 \leq AUC \leq 0.9$ is intermediate accuracy, and $0.9 \leq AUC < 1$ is high accuracy[167]. The AUC can be used to easily compare the accuracy of assays[174]. ROC analysis is also used to establish the optimal threshold for an assay. The most upper left point in the ROC curve is the point with the highest Se and Sp. The threshold that is associated with this coordinate is the optimal threshold. Selection of the most appropriate threshold often requires taking into account other important associated aspects such as disease, epidemiology and costs[167].

B. PRECISION

Precision is a measure of *closeness of agreement between independent test/measurement results obtained under stipulated conditions*[175]. Precision is important in diagnostic assays to ensure the robustness of the procedure in replicating results. It is measured by repeatedly testing a set of samples. Repeatability and reproducibility are different levels of precision. Repeatability is the lowest level and is the measurement of precision using the same operator, method, equipment, and laboratory during a short period of time. Reproducibility is the highest level and is measured using different operators,

equipment, and laboratories over long periods of time. When precision is measured using conditions that satisfy repeatability but not all those of reproducibility, then the measurement is considered an intermediate measure and needs to be defined. In this dissertation, an intermediate measure of precision was applied using two operators, with different sets of pipettes, measuring at different times, within the same laboratory. Precision is expressed in terms of standard deviation; low standard deviation indicates high precision[176].

OBJECTIVES

The goal of this dissertation is to evaluate diagnostic laboratory tools for use in settings where measles has been eliminated. These diagnostic tools included alternative specimen collection systems and complementary confirmatory assays, which were studied with the following objectives:

- To compare protocols for the elution of serum from dried blood spots for use in the laboratory diagnosis of measles.

- To analyze and describe laboratory and clinical data from samples collected from secondary vaccine failures with waning immunity in elimination settings.

- To find biomarkers for the confirmation of measles infections in secondary vaccine failures.

- To develop and validate an avidity assay for measles IgG antibodies to:
    1. Classify vaccine failures
    2. Further confirm measles cases in unvaccinated persons

PUBLICATIONS

1. **Sara Mercader, David Featherstone, William J. Bellini.** Comparison of available methods to elute serum from dried blood spot samples for measles serology**.** *Journal of Virological Methods (2006), 137, 140–149.*

2. **Hickman, C., T. B. Hyde, S. B. Sowers, S. Mercader, M. McGrew, N. Williams, J. Beeler, S. Audet, B. Kiehl, R. Nandy, A. Tamin, and W. J. Bellini.** Characterization of the Antibody Response to Measles Virus Infection in Previously Vaccinated and Unvaccinated Patients. *Journal of Infectious Diseases (2011), 204 (suppl 1): S549-S558.*

3. **Sara Mercader, Philip Garcia, William J. Bellini.** A Measles IgG Avidity Assay: Use in Classification of Measles Vaccine Failure Cases in Elimination Settings. *Clinical and Vaccine Immunology (2012), manuscript published ahead of print at the web site http://cvi.asm.org/content/early/2012/09/07/CVI.00406-12.full.pdf+html (Last accessed: September 17th, 2012)*

The first publication compares methods for eluting DBS for measles diagnostics. A protocol that extracts an ample volume of serum to perform successive analysis needed in elimination settings is identified. The paper provides data for elution method selection.

The second publication describes the symptomatolgy and serological responses of laboratory-confirmed SVFs. A biomarker for SVF in elimination settings is suggested based on parameters of high measles IgG avidity and very high neutralization titers.

The third publication describes the development, evaluation, and applicability of a measles avidity assay for use in vaccine failure classification. In elimination settings, the assay is particularly useful in providing reassurance for case confirmation in situations where routine IgM testing may be ambiguous.

The impact factor of all publications and the contributions of Sara Mercader in the co-authored publication are attached and signed by the director of this dissertation.

Abstracts in Catalan can be found in Appendix IV.

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Centers for Disease Control
and Prevention (CDC)
Atlanta, GA 30329

Date: July 11, 2012

Ph.D. Comissió
Facultat de Biologia
Universitat de Barcelona
Avinguda Diagonal, 645
08028, Barcelona, Spain

Re: Impact factor and participation of Sara Mercader in authored and co-authored publications.

Dear Commission Members:

The following are my assessments of the relative impact of each of three publications that Sara Mercader has chosen as part of her dissertation. These represent only a small portion of the contributions that Sara Mercader has made to my laboratory over the course of the last ten or so years that she has been with us.

1. **Comparison of available methods to elute serum from dried blood spot samples for measles serology**. Mercader, Featherstone, Bellini. (2006). J. Virol. Meth. 117:140-49. Impact factor: **2.258**. This paper was timely and served to clearly demonstrate that dried blood spots could easily be handled efficiently and safely and at the same time yielded excellent results when used in conjunction with the commercially available IgM and IgG assays supported by the World Health Organization (WHO) for measles testing. At the time this paper was published, the WHO had little information to offer resource poor regions regarding inexpensive ways to elute serum from dried blood spots (DBS). Sara Mercader's paper was the first to provide such information. This publication led to her teaching a WHO-sponsored course on the subject of DBS elution methods in Africa. The impact was even broader than anticipated, since subsequent to this publication, we have received numerous inquiries (7-10) from groups working in HIV, malaria, hepatitis and yellow fever wishing to know what method Sara would recommend for the best recovery of serum from DBS. This alternative collection method and companion recovery method has even more relevance now in regions such as Africa and South Asia as we approach global elimination.

2. **Laboratory Characterization of Measles Virus Infection in Previously Vaccinated and Unvaccinated Individuals.** Hickman, Hyde, Sowers, Mercader, et al. (2011). J. Infect. Dis. 204:S549–S558. Impact factor: **6.410**. This paper was the first to describe a possible biomarker for secondary vaccine failure – an extremely high neutralizing antibody titer in conjunction with high avidity IgG in response to exposure to measles subsequent to

measles vaccination in the past. What was actually being described was a secondary immune response to measles infection, because the vaccinated individual had waning immunity but could respond rapidly to infection once antigen presentation occurred post-infection. Sara Mercader accepted the challenge of developing, evaluating and implementing an in-house measles IgG avidity assay. Initially the assay was to be developed using the nucleoprotein antigen. After discussions with Sara, it was decided that we would use a commercial kit platform that would be more widely available to other clinical and research workers. Ms. Mercader was able to show that the avidity assay based on the whole antigen produced results that were very similar to those obtained using the nucleoprotein. Sara Mercader made available a standardized, validated in-house measles IgG avidity assay that aided in the determination that many of the cases that we observed among the Pacific island and United States populations described in this paper were actually secondary vaccine failure cases. In this study, Sara Mercader worked as a team member reviewing epidemiological information, laboratory results and clinical information. She learned how the information from each of the disciplines impacted the decision making and how her results fit into the overall picture of secondary vaccine failure.

3. **A Measles IgG Avidity Assay: Use in Classification of Measles Vaccine Failure Cases in Elimination Settings**. Mercader, Garcia, Bellini. (2012). Clin. Vaccine Immunol. Submitted. Impact factor: **2.471.** This paper represents Sara Mercader's best individual effort as a diagnostic virologist, assay developer and statistician. She had very little help in performing, evaluating and validating this measles avidity assay. Sara understood the importance of developing this assay for our laboratory, as a confirmatory assay for primary measles cases in low disease prevalence settings (such as the US or Spain for that matter) when there are ambiguities in patient records or in routine confirmatory testing (IgM serology). It became apparent, however, that we could not use the avidity assay to rule out a case of measles. In other words, while high measles IgG avidity responses were almost always indicative of past infection or immunization with measles, there were a few individuals who made high avidity measles IgG during the early stages of measles infection. These are, of course, the secondary vaccine failure cases. As mentioned above, the avidity assay when used in conjunction with the plaque-reduction neutralization assay has now provided us with a bioassay (biomarker) to detect and classify such cases.

The measles avidity assay has become part of the catalogue of measles diagnostic assays run a the Centers for Disease Control and Prevention in Atlanta, GA. State and local public health laboratories have begun to appreciate the value of the test when, as I mentioned, clinical ambiguities arise, or when their IgM testing is suspicious. As a result, requests for avidity testing from these customers have increased steadily over the last 3 years. We at CDC often refer specimens with unusually high measles IgG EIA values for testing by avidity and PRN as possible secondary vaccine failure cases, particularly if there are known exposures. Sometimes such individuals are asymptomatic or have only cold-like symptoms.

As I mentioned earlier, Sara wished to make this assay broadly accessible to others and chose a commercial IgG platform as the basis for the assay. We hope that once the details of the assay are published more laboratories will incorporate the method into their routine testing.

4. **Two Case Studies of Modified Measles in Vaccinated Physicians Exposed to Primary Measles Cases: High Risk of Infection but Low Risk of Transmission**. Rota, Hickman, Sowers, Rota, Mercader, Bellini. (2011). J Infect Dis. 204:S559-63. Impact factor: **6.410**. This paper is actually a counterpart of paper number 2 above and can be found in the appendices of this dissertation. Ms. Mercader performed the avidity assays for this manuscript. Even more importantly, she participated as a team member in discussions regarding the relevance of the findings that professions, such as emergency room physicians, associated with high risk exposure to measles when examined for the biomarkers of secondary vaccine failure demonstrated very low risk of transmission. This is a major finding; one that Sara shares in with the remainder of the group and one that she has worked very hard in helping to characterize as part of our collective contributions to measles elimination. Our team, which Sara Mercader serves as an integral part, continues to collect information on secondary vaccine failure cases and attempts to characterize their role in transmitting measles. This far, we have been able to confirm infections by RT-PCR and occasional cell culture isolation, but spread cases generated from these individuals have been exceedingly rare. Clearly others have been observing these modified infections that surround classical outbreaks, and have noted the two publications above a total of six (6) times in the literature thus far.

In summary, Sara Mercader has made considerable impact on measles diagnostics and in particular the diagnosis of measles in elimination settings. I have no doubt that she will continue to contribute to our efforts to eradicate measles and rubella as well as other infectious agents. Sara Mercader has become an invaluable resource and provides critical diagnostic and analytical leadership and guidance to the programs within MMRHLB and we look forward to her continuing contributions to the mission of the MMRHLB.

Sincerely,

William J. Bellini, Ph.D.
Director of Ph.D. Thesis
Chief, Measles, Mumps, Rubella and
Herpesviruses Laboratory Branch
Division of Viral Diseases
National Center for Immunization and Respiratory Diseases
Centers for Disease Control and Prevention

1. Comparison of available methods to elute serum from dried blood spot samples for measles serology

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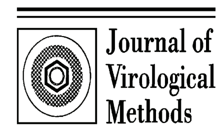

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Comparison of available methods to elute serum from dried blood spot samples for measles serology☆

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Abstract

Six existing protocols for the extraction of serum from blood spots dried onto filter paper were compared. Assessment criteria included: detection of measles IgM and IgG by the Dade Behring Enzygnost® immunoassays, volumes of recovered eluates, reproducibility, processing time and throughput, difficulty of protocol, equipment required, safety and estimated costs. Detection of measles IgM in eluates obtained by four of these protocols was as in serum, and significant differences were only observed in eluates from the two remaining protocols ($p < 0.05$). Significant differences were found between extraction protocols regarding measles-specific IgG detection when an IgG indeterminate DBS was analyzed ($p < 0.05$), but not when an IgG positive and negative DBS were studied. Sufficient eluate volumes were recovered for testing in the IgM Behring assay following all protocols but two. Sufficient eluate was recovered for testing in the IgG Behring assay following all six protocols. While all protocols were relatively easy to perform, only two protocols required less than 2 h for completion. In general, compared protocols performed well on the extraction of antibodies from DBS for serology with differences being observed with eluate volume recovery, turn around time, required equipment and cost. An easy-to-implement protocol is proposed for the rapid extraction of serum for measles/rubella serology in outbreak situations for use in the World Health Organization Global Measles and Rubella Laboratory Network.

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Keywords: Filter paper blood spot; Enzyme immunoassay; IgM detection; IgG detection; Protocol; Elution

1. Introduction

Measles remains a serious vaccine-preventable disease, and is estimated to be responsible for more than half a million childhood deaths worldwide (World Health Organization, 2005). Laboratory surveillance coupled with epidemiological surveillance is essential to guide measles control efforts in the quest for reducing global measles mortality and achieving regional measles elimination goals. Three key laboratory activities are (1) confirmation of measles suspected cases, (2) description of population seroprevalence profiles through serology, and (3) verification of measles virus circulation through genetic characterization (Featherstone et al., 2003). Serum is the specimen most frequently used for measles serology, while throat swabs, urine and/or whole blood are collected for genetic characterization. Thus, proper suspect case investigation requires that several specimens be obtained. However, these specimens can pose problems in: (1) their collection, i.e., the invasive nature of venipuncture and its poor acceptance by the patient and parents, and (2) their storage and transportation, i.e., maintenance of the specimens at the site of collection and reverse cold chain from hard-to-reach areas to testing locations (Bellini and Helfand, 2003).

The World Health Organization (WHO) supports the use of oral fluid and filter paper dried whole blood spots (DBS) as specimen collection alternatives to overcome the abovementioned limitations. Inclusion of oral fluid and DBS in the list of specimens collected for measles will facilitate collection and transport of specimens, as well as offer the potential for performing serology and genetic characterization using a single sample (Helfand

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services. Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services.

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et al., 2001; Nigatu et al., 2001; Nokes et al., 2001). The advantages of collecting DBS have been well-documented elsewhere (Mei et al., 2001; Parker and Cubitt, 1999). Recent performance evaluation studies using commercially available and in-house enzyme immunoassays, demonstrated that collection of DBS is a feasible and reproducible alternative to phlebotomy for measles diagnostics (Chakravarti et al., 2003; De Swart et al., 2001; el Mubarak et al., 2004; Helfand et al., 2001; Riddell et al., 2003, 2002). Similar studies have been performed and are currently underway for rubella serology (Condorelli et al., 1994; Helfand et al., 2001; Karapanagiotidis et al., 2005). Amplification of measles virus RNA from DBS by reverse transcriptase PCR and subsequent sequence analysis has also shown promising results (De Swart et al., 2001; el Mubarak et al., 2004; Katz et al., 2002; Mosquera et al., 2004). This paper is a collaborative pursuit with the World Health Organization to establish a standardized method to recover serum from DBS for measles serology.

Several methods have been used to extract blood products including serum from DBS for serology of infectious diseases, among them measles and rubella. Some of the protocols are ideal for field situations, while others require well-equipped laboratories. In all protocols, discs are excised from DBS and soaked in elution buffer, which helps to diffuse serum components from the rehydrated filter paper matrix (Chanbancherd et al., 1999; Condorelli et al., 1994; el Mubarak et al., 2004; Farzadegan et al., 1978; Fortes et al., 1989; Hanna et al., 1989; Helfand et al., 2001; Hogrefe et al., 2002; Lindhardt et al., 1987; Pannuti et al., 1991; Parker et al., 1995; Steger et al., 1990; Tappin et al., 1998; Varnier et al., 1988; Vejtorp and Leerhoy, 1981). In some instances, serum diffusion is integrated in the immunoassay procedure (Condorelli and Ziegler, 1993; Eaton et al., 1996; Kalter et al., 1992; Neto et al., 2004). In other protocols, DBS serum elution is taken one step further by manually extruding or by centrifuging the soaked discs to actively recover DBS components (Brody et al., 1964; Chishty, 1971; De Swart et al., 2001; Khare et al., 1985; Monto et al., 1969; Nakano et al., 1983; Riddell et al., 2002; Saha and Saxena, 1983). Additionally, protocols may differ in the number and diameter of discs for elution, depending on the subsequent serological immunoassay (Chanbancherd et al., 1999; Chishty, 1971; Condorelli et al., 1994; De Swart et al., 2001; Draper and Kelly, 1969; el Mubarak et al., 2004; Fortes et al., 1989; Hanna et al., 1989; Helfand et al., 2001; Hogrefe et al., 2002; Khare et al., 1985; Mosquera et al., 2004; Pannuti et al., 1991; Parker et al., 1995; Riddell et al., 2003; Saha and Saxena, 1983; Tappin et al., 1998).

The goal of this paper is to compare available methods for DBS serum extraction for measles serodiagnosis and propose one that will permit the recovery of the maximum volume of eluted sample in the minimum time, effort and cost. We compared six protocols using the measles Dade Behring Enzygnost® immunoassays which are widely used in the WHO Global Measles and Rubella Laboratory Network. The paper attempts to provide a general overview of available methods to elute serum from blood samples dried on filter paper and proposes an extraction method suitable for general use in the Global Measles and Rubella Laboratory Network.

2. Materials and methods

2.1. Literature review

PubMed from the United States National Center for Biotechnology Information was utilized to identify publications which described methods to elute DBS for serodiagnosis.

2.2. Samples

A panel of five simulated DBS samples was prepared and consisted of an IgM positive, an IgM negative, an IgG positive, an IgG equivocal and an IgG negative DBS. These DBS samples were prepared at the Centers for Disease Control and Prevention (CDC, Atlanta, USA) using serum of known IgM and IgG antibody optical density values (as tested by the measles Dade Behring Enzygnost® immunoassays) and washed red blood cells as described elsewhere (Merideth and Hannon, 1993). The sample hematocrit was adjusted to 50%. Negative controls consisted of (1) red blood cells and saline instead of serum, (2) red blood cells and antibody negative serum and (3) a control made of plain filter paper. Before testing, the quality of the DBS was determined by visual inspection (NSPWC, 2003).

2.3. Elution of serum from DBS

A step-by-step description of each of the six compared protocols is provided in Table 1. Protocols 1, 2, and 5 were adapted to the Dade Behring Enzygnost® Anti-Measles Virus IgM and IgG enzyme immunoassays (Marburg, Germany) as follows. First, the required number of dried blood filter paper discs to satisfy the manufacturer's specifications regarding serum dilution (1:21) was calculated by making the assumption that a 6.35 mm (1/4 in.) filter paper disc contained about 6 μL of dried serum (Mei et al., 2001). Second, the volumes of elution buffer used per extraction were 250 and 125 μL per sample for IgM and IgG antibody detection, respectively. Hence, two 6.35 mm discs were removed from each DBS sample using a hole perforator to prepare the extracts for IgM antibody detection, while a single disc of the same size was sufficient for IgG antibody detection. Third, the DBS elution buffer composition was phosphate buffered saline containing Tween 20 and 5% non-fat dry milk (Condorelli et al., 1994; Helfand et al., 2001; Riddell et al., 2003, 2002). This elution buffer has been reported to reduce background interference in the detection of measles antibodies in DBS sample eluates by enzyme linked immunosorbent assay (ELISA) (Condorelli et al., 1994). Protocol 4 is a modification of protocol 3, where discs were soaked in elution buffer in microcentrifuge tubes instead of 96-welled plates (Table 1) (Akoua-Koffi, 2004; Korukluoglu, 2004; Riddell et al., 2002, 2003). In protocols 3 and 4, elution buffer volumes of 220 and 250 μL per sample were used for IgM and IgG antibody detection as described elsewhere (Riddell et al., 2002, 2003). Finally, protocol 6 was developed at CDC and was performed as follows. Two 6.35 mm diameter discs were excised from DBS, and were placed in labeled 24-welled plates. Discs were soaked in 250 μL of elution buffer, covered with a lid and incubated for 30–60 min

Table 1
Step-by-step protocol description

	Protocols					
	1	2	3	4	5	6
Reference	Helfand et al. (2001), Fortes et al. (1989), Hogrefe et al. (2002)	Condorelli et al. (1994), Hanna et al. (1989), Tappin et al. (1998)	Riddell et al. (2002), Riddell et al. (2003)	Korukluoglu (2004), Akoua-Koffi (2004)	Draper and Kelly (1969), Chishty (1971), Monto et al. (1969)	
Elution container	96-Well plate	96-Well plate	96-Well plate	Microcentrifuge tube	Syringe barrel	48-well plate
1. Punch DBS[a] disc 2. Add elution buffer	R[b,c]	R[c]	R[d]	R[d]	R[c]	R[c]
3. Agitate on an automatic shaker	–[e]	30 min	15 min	15 min	–	–
4. Incubate	o/n[f] 4 °C	o/n 4 °C	o/n 4 °C	o/n 4 °C	1 h RT[g]	1 h RT
5. Agitate	–	–	15 min	15 min	–	–
6. Transfer disc/buffer into column	–	–	–	–	–	R
7. Apply force	–	–	10 min 2200 × g[h]	10 min 2200 × g	Manual	30 min 1800 × g
8. Remove eluate from container with a pipette	R	R	R	R	–	–
9. Test/store sample	R	R	R	R	R	R

[a] Dried blood spot.
[b] R: step required in protocol.
[c] Two discs and 250 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgM testing; 1 disc and 125 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgG testing.
[d] Two discs and 220 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgM testing; 1 disc and 250 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgG testing testing.
[e] Step not required in protocol.
[f] Overnight as 16 h.
[g] Room temperature.
[h] Relative centrifugal force.

at room temperature. Following incubation, a pipette tip was used per sample to transfer soaked discs and elution buffer into the corresponding centrifuging system. The centrifuging system for simultaneous elution for IgM and IgG testing consisted of a labeled 10 mL centrifuge tube that held a microtube (Bio-Rad Titertube®), and supported an uncapped 3 mL syringe barrel at the open end (Fig. 1). Samples were centrifuged at room temperature for 30 min at 1800 × g relative centrifugal force in a Thermo IEC Centra CL3R centrifuge. Eluate-containing microtubes were removed from inside the centrifuge tube using a pre-cut 1250 μL pipette tip that fit tightly inside the microtube. Microtubes were then placed in testing racks, and were stored at −20 °C until used. The microtube was used in the simultaneous elution for IgM and IgG testing to minimize loss of eluate volume through pipetting. However, the microtube was not strictly required for elution for IgM testing alone, since ample eluate volume was recovered. When the microtube was not required, the eluate was collected in the labeled 10 mL centrifuge tube.

2.4. Commercial IgM indirect enzyme immunoassay

DBS eluted by all six protocols were tested on the Dade Behring Enzygnost® Anti-Measles Virus IgM enzyme immunoassay (Marburg, Germany) per the manufacturer's instructions, with the following modifications indicated per protocol. The reactivity of IgG and rheumatoid factor (RF) was blocked by the addition of RF absorbent as per manufacturer's instructions. In protocols 1, 2, 5, and 6, 160 μL of the absorbent was added to each eluted sample, whereas in protocols 3 and 4, 170 μL of the absorbent was added to each eluted sample, as described elsewhere (Riddell et al., 2002). When less than 170 μL of sample was recovered, i.e., following protocols 3 and 4 (see Table 2 for mean recovered volume ± standard deviation and median), sufficient RF absorbent volume was added to bring the final volume to 340 μL. As per kit instructions, a volume of 150 μL of this mixture was loaded onto the antigen-coated plate. The final sample dilution in each well was approximately 1:44. For sample eluates of all six protocols, the rest of the testing procedure was as described in the insert supplied with each assay kit. Protocols 3 and 4 were also assayed by the modified conditions described elsewhere, with extended incubation times (1.5 h instead of 1 h) and number of washes (5 instead of 4) (Riddell et al., 2002). Serum samples that were used to prepare the mock DBS samples were tested in parallel with the DBS following the kit insert instructions. Assay validation, calculation of final optical density values and result classification were performed as described in the insert. No measurement correction was performed on delta values from DBS eluates and sera, since it remains unknown how the serum-based EIA values used

Fig. 1. Column centrifugation systems before and after centrifugation of dried blood spot sample. Arrows point at filter paper discs, showing appearance before and after centrifugation.

for the calculation of the correction factor relate to DBS eluate samples.

2.5. Commercial IgG indirect enzyme immunoassay

DBS eluates obtained by protocols 1 through 6 were tested using the Dade Behring Enzygnost® Anti-Measles Virus IgG enzyme immunoassay (Marburg, Germany) per the manufacturer's instructions, including assay validation, calculation of final optical density values and result classification. The only modification was that 50 μL of eluates from protocols 3 and 4 were added to wells containing 170 μL of sample buffer (Riddell et al., 2003). Serum samples that were used to prepare the mock DBS samples were also assayed per Dade Behring's instructions, but not in parallel with the DBS. No measurement correction was performed on delta values from DBS eluates and sera.

2.6. Data analysis

Protocols 1 through 6 were compared using criteria that included antibody detection, eluted DBS sample recovery, protocol difficulty, processing capabilities, required time and equipment, safety, and cost. Each protocol was repeated up to six times and eluates from all protocols were assayed in parallel by the measles IgM and IgG Dade Behring assays for up to five different runs. For IgM detection, cognate serum specimens were run each time in parallel with the eluates. Nonparametric statistics were used for analysis, because the data may not be normally distributed (Lehmann, 1975). Elution protocols for IgM detection on positive and negative DBS eluates were compared to the cognate serum by using the Wilcoxon rank-sum test. Elution protocols for IgG detection on positive, intermediate, and negative DBS eluates were each compared by using the Kruskal–Wallis test. The significance level was <0.05. Statistical analysis was performed using SAS 9.1.

3. Results

3.1. Selection and comparison of DBS elution protocols

A PubMed literature search led to the identification of 74 articles where DBS were collected for serodiagnosis of infectious diseases. Although the methods of DBS serum extraction varied from paper to paper, four core protocols were recognized. For reference purposes, these protocols were labeled as protocol 1, 2, 3, and 5 (Table 1). For laboratories without an ELISA plate centrifuge, protocol 3 was modified and is referred to as protocol 4 (Table 1). Protocol 6 was developed at CDC (Table 1). In protocols 1 and 2, blood components were passively eluted from DBS samples without any applied force, while in protocols 3 through 6 a force (centrifugal or manual) was applied to actively elute the samples.

Protocols 1 through 6 were compared to provide an overview of available methods by both passive (protocols 1 and 2) and active (protocols 3 through 6) extraction. Protocols 1–4 were similar in that 96-welled plates or microcentrifuge tubes were used to soak the DBS discs and the eluted sample was removed from the vessel with a pipette. The difference among these four protocols was the use of instruments to facilitate specimen transfer. In contrast, protocols 5 and 6 differed in that both used a syringe barrel as a means of sample recovery. In protocol 5, following incubation with elution buffer, the syringe plunger was pressed down the column and the resultant pressure expressed the hydrated specimen from the filter paper and into a labeled collecting tube. In protocol 6, DBS discs were soaked in a 24- or 48-welled plate, syringe columns containing soaked discs and partially eluted samples were centrifuged and the generated centrifugal force efficiently removed the hydrated specimens from the discs into the labeled tubes (Fig. 1). The six protocols were compared using the criteria presented in Tables 2–7. Tables 2–4, 6 and 7 summarize the findings related to protocol performance (antibody detection and final eluted sample volume), as well as time needed to perform the protocol and cost. Table 5 identifies within each protocol different practical features and presents required equipment. The studied protocols were modified so that the recovered samples from DBS could subsequently be tested in the aforementioned commercial immunoassays.

3.2. Antibody detection in DBS eluates

The ability to detect measles IgM antibodies in samples eluted from DBS under the six protocols was determined using DBS

Table 2
Volume of recovered dried blood spot eluate

Protocol	Elution for IgM detection[a]		Elution for IgG detection[a]	
	Mean volume (S.D.)[b]	Median	Mean volume (S.D.)	Median
1	200 (5.50)[c,d]	200.0	98 (7.12)[c,e]	100.0
2	195 (9.43)[c,d]	192.5	93 (6.61)[c,e]	95.0
3	158 (8.90)[f,g]	162.5	206 (7.41)[c,h]	210.0
4	168 (5.87)[f,g]	167.5	215 (6.55)[c,h]	215.0
5	205 (13.12)[c,d]	210.0	67 (10.75)[c,e]	60.0
6	215 (7.82)[c,d]	215.0	101 (5.27)[c,e]	100.0

[a] Number of samples measured per protocol is 10.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Enough volume was recovered for testing.
[d] Assay requires 160 μL of eluate sample.
[e] Assay requires 40 μL of eluate sample.
[f] Not enough volume was recovered for testing.
[g] Assay requires 170 μL of eluate sample.
[h] Assay requires 100 μL of eluate sample.

constructed with IgM positive and IgM negative serum specimens. No significant differences were observed when detection of measles virus IgM in DBS eluates extracted by protocols 3 through 6 (active elution methods) was compared to detection in the corresponding serum used to prepare the DBS (Fig. 2 and Table 3). However, IgM antibody detection was found to be significantly different from serum ($P \leq 0.05$) in (a) eluates from protocols 1 and 2 obtained by passive elution, where optical density values were lower than in serum (Fig. 2, panel A) and (b) in eluates from protocols 3 and 4 assayed by the modified version of the Dade Behring enzyme immunoassay (Fig. 2, panel C) (Riddell et al., 2002). The latter resulted in higher optical density

Table 3
Detection of IgM anti-measles virus antibodies in dried blood spot samples and cognate serum by the Dade Behring Enzygnost® assay expressed as optical density values and percentage detection rates

Protocol	IgM detection on IgM positive dried blood spot eluate[a]		
	Optical density (S.D.)[b]	Median	Detection rate (%)[c]
1[d]	0.455 (0.034)[e]	0.467	100 (5/5)
2[d]	0.456 (0.020)[e]	0.453	100 (5/5)
3[d]	0.478 (0.026)[f]	0.477	100 (5/5)
4[d]	0.484 (0.024)[f]	0.484	100 (5/5)
3[g]	0.648 (0.041)[e]	0.654	100 (5/5)
4[g]	0.649 (0.030)[e]	0.666	100 (5/5)
5[d]	0.464 (0.018)[f]	0.454	100 (5/5)
6[d]	0.477 (0.018)[f]	0.479	100 (5/5)
Serum	0.482 (0.008)	0.482	n/a[h]

[a] Number of elution and antibody detection repeats is 5.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Number of eluate samples with *P* result/ number of repeats.
[d] IgM detection by Dade Behring Enzygnost® assay per manufacturer's instructions.
[e] Optical density significantly different from serum ($p \leq 0.05$).
[f] Optical density not significantly different from serum.
[g] IgM detection by Dade Behring Enzygnost® assay per modified instructions (Riddell et al., 2002).
[h] Not applicable.

Table 4
Detection of IgG anti-measles virus antibodies in dried blood spot samples by the Dade Behring Enzygnost® assay expressed as optical density values and percentage detection rates

Protocol	IgG positive dried blood spot eluate[a]		
	Optical density (S.D.)[b,c]	Median	Detection rate[d], %
1	1.478 (0.137)	1.469	100 (5/5)
2	1.496 (0.148)	1.497	100 (4/4)
3	1.468 (0.169)	1.504	100 (5/5)
4	1.526 (0.154)	1.554	100 (6/6)
5	1.517 (0.010)	1.517	100 (2/2)
6	1.431 (0.161)	1.428	100 (6/6)
Protocol	IgG equivocal dried blood spot eluate[a]		
	Optical density (S.D.)[e]	Median	Detection rate, %
1	0.153 (0.053)	0.137	80 (4/5)
2	0.134 (0.018)	0.126	100 (5/5)
3	0.175 (0.024)	0.174	80 (4/5)
4	0.178 (0.020)	0.167	80 (4/5)
5	0.130 (0.008)	0.130	100 (2/2)
6	0.136 (0.011)	0.138	100 (5/5)

[a] Number of elution and antibody detection repeats is ≤5.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Optical density values not significantly different between protocols.
[d] Number of eluate samples with *P* (or *E*) result/number of repeats.
[e] Optical density values significantly different between protocols ($p \leq 0.05$).

values, which were likely due to the extended incubation periods (1.5 h instead of 1 h). Similarly, detection of IgG antibodies was studied using DBS prepared with sera classified as IgG positive, equivocal and negative. No significant differences in IgG detection were found between protocols in the positive and negative DBS samples (Fig. 3 and Table 4). In contrast, significant differences were found in the IgG equivocal DBS sample ($P \leq 0.05$). In the latter, all measurements performed on eluates from protocols 2, 5 and 6 fell within the defined 0.100–0.200 interval for an equivocal sample. However, 1 in 5 EIA measurements on eluates from protocols 3 and 4 where higher than the 0.200 upper cut-off value of the aforementioned interval, while one outlier was detected in eluates from protocol 1 (Fig. 3 and Table 4).

IgM detection reproducibility and detection rates were excellent for all eluate samples recovered using the six protocols. A low standard deviation and 100% concordance was observed when eluted DBS specimens were compared with the cognate serum specimens (Table 3). IgG detection rates on the positive DBS eluted samples were excellent regardless of elution protocol, however equivocal DBS samples eluted with protocols 1, 3 and 4, had detection rates of only 80% (Table 4). All eluated specimens obtained using all protocols yielded an EIA result that was classified as positive, as expected for a DBS prepared with an IgG positive control serum. All specimens obtained from the IgG equivocal DBS were classified as equivocal, except for three that were positive. These three were eluted following protocols 1, 3 and 4. Eluted samples from DBS prepared with an IgG negative serum yielded the expected negative result. In general, IgG detection reproducibility was very good, except for protocol 1, where the coefficient of variation was 35%.

Table 5
Comparison of practical and logistic aspects of evaluated protocols

	Protocols					
	1	2	3	4	5	6
Easy to perform	x[a]	x	x	x		x
Fast (less than 3 h)					x	x
Minimal material preparation	x	x	x	x		
No electricity required	x				x	
Required equipment/materials easily found in serology labs	x				x	x
Low cost and easy-to-implement in serology laboratories	x				x	x
High sample throughput	x	x	x			
Eluate sample volume is completely extracted from filter paper					x	x
Eluate sample volume is adequate for the Dade Behring IgM assay	x	x			x	x
Eluate sample volume is adequate for the Dade Behring IgG assay	x	x	x	x	x	x
Good reproducibility in eluate volume recovery	x	x	x	x		x
Labeling is easy				x	x	x
Minimal risk of sample misplacement/cross contamination				x	x	x
Minimal risk for sample aerosolization	x	x	x		x	x
Required equipment						
Microplate shaker		x	x			
2 mL tube shaker				x		
Microplate centrifuge			x			
2 mL tube centrifuge				x		
15 mL tube centrifuge						x
Required disposables						
Hole perforator	x	x	x	x	x	x
96-Welled plate	x	x	x			
Microcentrifuge tube				x		
1 mL syringe					x	
24-Welled plate						x
3 mL syringe						x
10 mL centrifuge tube						x
Microtiter tube						x[b]
1 mL pipette tip						x[b]
0.3 mL pipette tips	x	x	x	x	x	x

[a] x: applies to protocol.
[b] Only required to simultaneously elute samples for IgM and IgG antibody detection. Not required to elute sample for IgM antibody detection alone.

3.3. Volume of recovered DBS eluate

Protocol performance based on volume of recovered specimen was found to be highly reproducible in all procedures, except for protocol 5 which, due to handling during manual extraction, had the highest standard deviation in sample recovery (Table 2). For testing on the anti-measles Dade Behring IgM assay, addition of 250 μL of elution buffer per extraction was found to yield ample specimen for testing. Elution by protocols 3 and 4 with 220 μL of elution buffer resulted in suboptimal volumes of recovered specimen. It was difficult to recover the required 170 μL for testing. Therefore, an eluate volume smaller than recommended was used only for the purpose of this comparison. Such modification in the protocols should not be applied for diagnostic purposes. In contrast, specimen recovery using protocol 6 resulted in sufficient sample volume to allow testing for both IgM and IgG Dade Behring assays. For IgG detection, elution of specimens from DBS in either 125 μL (protocols 1, 2, 5 and 6) or 250 μL (protocol 3 and 4) also resulted in adequate volumes of recovered specimen. Of all methods, protocols 5 and 6 were those that resulted in what appeared to be complete removal of blood products from the DBS since the resultant filter paper discs were practically white and dry after the procedure (Fig. 1). In contrast, filter paper discs following protocols 1–4 were seemingly saturated with hydrated blood, indicating that a portion of the eluted sample was trapped in the filter paper fibers, and between the filter paper discs and could not be recovered.

Table 6
Turn around time per protocol

Protocol	Processing time
1	o/n[a] + 45 min
2	o/n + 1.5 h
3	o/n + 2.5 h
4	o/n + 1.5 h
5	1.5 h
6	2 h

[a] Overnight as 16 h.

Table 7
Estimated cost per protocol per DBS[a] and per 96 DBS samples

Protocol	Per DBS[b] ($)	Per 96 DBS[c] ($)
1	1.69	0.05
2	1.69[d]	0.05[d]
3	1.69[e]	0.05[e]
4	0.06[f]	0.06[f]
5	0.14	0.14
6	1.02[g]	0.40[g]

[a] Dried blood spot.
[b] Cost to process 1 DBS sample.
[c] Cost to process 96 DBS samples.
[d] Add cost of an orbital shaker.
[e] Add costs of an orbital shaker and a centrifuge with 96-well plate adapters.
[f] Add costs of an orbital shaker and a centrifuge for microcentriguge tubes.
[g] Add cost of a centrifuge for serology tubes.

3.4. Logistic and practical aspects

In general, all six protocols were found to be relatively easy and safe to perform (Table 5). Protocol 5 was less straightforward due to manual handling during elution. Protocols 4–6 were found to be somewhat more laborious due to material preparation (protocols 5 and 6), and due to handling of microcentrifuge tubes (protocol 4). Protocol 4 was found to be relatively less safe due to the potential of sample aerosolization when opening and closing the microcentrifuge tubes. Protocols that do not require any electric powered equipment are obviously more suitable in field situations, where infrastructure may be limited (protocols 1 and 5). Protocols that do not require overnight DBS elution were found to be suitable in outbreak situations, when rapid processing of small numbers of samples is needed (protocol 5 and 6) (Table 6). Protocols using 96-welled plates and that require overnight incubation were found suitable for large stud-

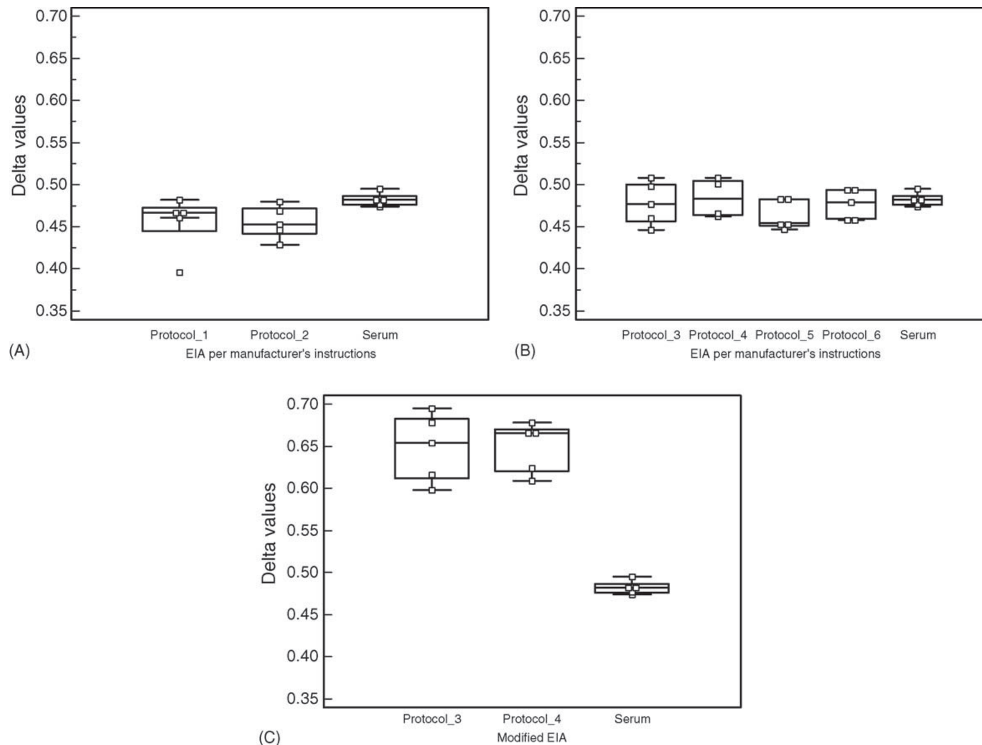

Fig. 2. Detection of antibody by the anti-measles virus IgM Dade Behring EIA after elution of an IgM positive DBS sample by six different extraction protocols and compared to detection in serum. An IgM positive DBS was eluted up to five times per each extraction protocol. Eluates resulting from the six extraction protocols were tested in parallel in five separate runs. Panel A: delta values from eluates from passive elution protocols 1 and 2 tested per manufacturer's instructions were significantly different from serum ($p \leq 0.05$). Panel B: delta values from eluates from active elution protocols 3–6 tested per manufacturer's instructions were not significantly different from serum. Panel C: delta values from eluates from active elution protocols 3 and 4 per modified assay conditions were significantly different from serum ($p \leq 0.05$). Delta value is the optical density value of the positive well minus the optical density value of the negative well. Assay cut-off value for a positive sample is a delta value of 0.200. *Y*-axis minimum value is set at 0.350.

ies when high throughput of samples would be required, but use of 48-welled plates would probably work better for more easy handling of DBS discs, labeling and minimizing sample cross-contamination (protocols 1–3) (Table 5).

3.5. Estimated cost per protocol

Protocols that do not require any electrical equipment (protocols 1 and 5) were the most economical ones (Table 7). Among protocols that would need one or more pieces of sophisticated equipment for sample processing, protocol 3 was the most expensive one due to the cost of the centrifuge plate carrier. For laboratories with readily available equipment infrastructure, protocol 4 would be the least expensive procedure regardless of the number of samples to be processed, followed by protocol 5. The cost of protocols 1–3 and 6, which use multi-well plates for sample elution, varied depending on the number of samples being eluted. Overall, protocols 1, 5 and 6 would be easy and less costly to implement in any laboratory currently performing serology, with protocol 6 being the most advantageous in terms of sample volume recovery and effective liquid extraction.

4. Discussion

In the near future, DBS may become, together with oral fluid specimens, a practical specimen for the serology of rash illnesses, and it would be reasonable to think that several tests would be performed on a patient's DBS for differential diagnosis (Cubel et al., 1996; de Oliveira et al., 1999, 2000; Helfand et al., 2001). The goal of this paper was to provide an overview and a comparison of methods that have been used over the years to extract serum from DBS for the serodiagnosis of infectious diseases, and in particular for measles. Although our comparison was limited by the type and number of DBS samples used and by the use of a specific measles commercial enzyme immunoassay, our findings may serve as a guide on currently available protocols for DBS elution.

Our data suggest that any protocol based on active elution, i.e., protocols 3–6, would work well for the extraction of antibody for IgM detection in an indirect format EIA. In contrast, statistical differences were observed in eluates obtained by passive elution, i.e. protocols 1 and 2. Although, the optical density values obtained in the latter eluates were lower than in serum in the tested IgM positive sample, this did not lead to a result

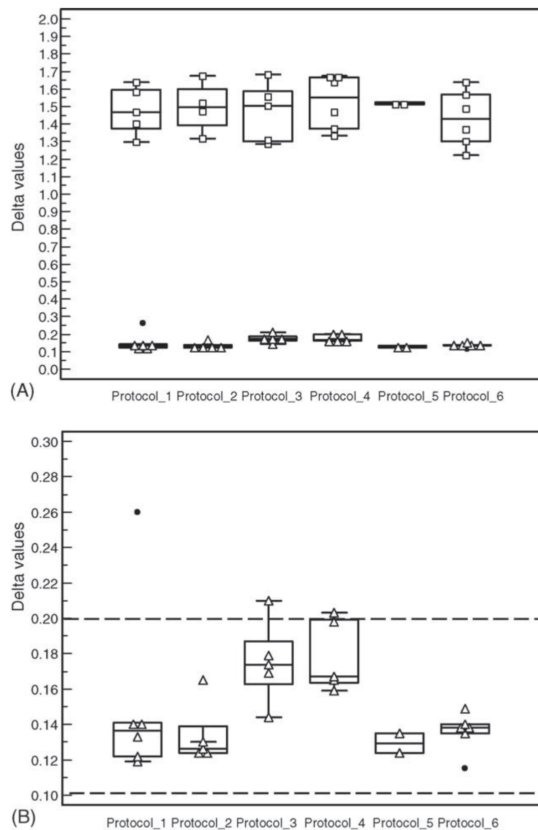

Fig. 3. Detection of antibody by the anti-measles virus IgG Dade Behring EIA after elution of an IgG positive and an IgG equivocal DBS samples by six different extraction protocols. Delta value is the optical density value of the positive well minus the optical density value of the negative well. Assay cut-off value for a positive sample is a delta value of 0.200. Assay cut-off value for a negative sample is a delta value of 0.100. A sample with a Delta value that falls in the interval 0.100–0.200 is considered equivocal. *Y*-axis minimum values in panel B is 0.100. Square markers: IgG positive DBS sample. Triangle markers: IgG equivocal DBS sample. Round black marker is a far out value.

misclassification. Unfortunately, an IgM positive sample with antibody reactivity close to the cut-off values was not available to study the effect of the observed statistical difference on a putative change of result classification, i.e., from positive to equivocal or negative. Interestingly, a passive elution protocol has been used in at least one evaluation study of DBS as alternative sample for serology that demonstrated an excellent correlation with serum by using a capture format EIA (Helfand et al., 2001). This could be explained by the fact that capture EIAs are designed to concentrate the target antibody resulting in higher detection sensitivity compared to indirect EIA. As for IgG detection, any of the protocols are likely to be useful for antibody elution. Unfortunately, differences among protocols detected with the IgG equivocal sample could not be further studied with a parallel comparison with serum. Hence, taking into account the EIA formats used for antibody detection, it is not surprising the good concordance observed between DBS and serum in performance evaluation studies despite using different DBS elution methods (Chakravarti et al., 2003; De Swart et al., 2001; el Mubarak et al., 2004; Helfand et al., 2001; Riddell et al., 2002, 2003).

On the other hand, differences among protocols were mainly found at the logistic and costing level. Protocol 6 was found to be the most advantageous procedure for outbreak situations, because it features a rapid removal method with recovery of ample specimen. In less than 8 h, ten DBS specimens received in the laboratory from remote outbreak areas can be eluted by protocol 6 and assayed by the Dade Behring IgM EIA. Furthermore, implementation of protocol 6 in serological laboratories should be seamless. Alternatively, protocols that employ 96-welled plates would work best for elution of large number of samples.

Limitations of this evaluation include the use of laboratory prepared DBS instead of clinical samples, which were not available at the time of this research. Also, testing of a low positive IgM or an indeterminate antibody sample - not available at the time – would have been beneficial to show differences in antibody detection at IgM levels close to the cut-off. Therefore, further evaluation would be needed using field specimens to verify that the presented observations hold true for clinical samples.

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2. Laboratory Characterization of Measles Virus Infection in Previously Vaccinated and Unvaccinated Individuals

SUPPLEMENT ARTICLE

Laboratory Characterization of Measles Virus Infection in Previously Vaccinated and Unvaccinated Individuals

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Waning immunity or secondary vaccine failure (SVF) has been anticipated by some as a challenge to global measles elimination efforts. Although such cases are infrequent, measles virus (MeV) infection can occur in vaccinated individuals following intense and/or prolonged exposure to an infected individual and may present as a modified illness that is unrecognizable as measles outside of the context of a measles outbreak. The immunoglobulin M response in previously vaccinated individuals may be nominal or fleeting, and viral replication may be limited. As global elimination proceeds, additional methods for confirming modified measles cases may be needed to understand whether SVF cases contribute to continued measles virus (MeV) transmission. In this report, we describe clinical symptoms and laboratory results for unvaccinated individuals with acute measles and individuals with SVF identified during MeV outbreaks. SVF cases were characterized by the serological parameters of high-avidity antibodies and distinctively high levels of neutralizing antibody. These parameters may represent useful biomarkers for classification of SVF cases that previously could not be confirmed as such using routine laboratory diagnostic techniques.

The incidence of measles has been dramatically reduced because of the availability of live attenuated vaccines, either as single-antigen vaccines or as combined vaccines, such as measles-mumps-rubella (MMR) vaccine. Measles is no longer endemic in the Americas because of high 2-dose vaccination coverage rates with MMR vaccine [1]. Consequently, vaccination strategies that include a 2-dose schedule for measles vaccination have been adopted by many countries, which has facilitated tremendous advancements towards the goal of reducing global measles-related morbidity and mortality [2].

Measles outbreaks continue to occur, however, even in highly vaccinated populations, largely as a result of the exposure of vaccine-exempt populations (eg, those with religious and philosophical objections to vaccination) to imported cases and, much less frequently, as a result of exposure of those with primary or secondary vaccine failure (SVF) [3–12]. Following the adoption of a 2-dose MMR schedule in the United States in 1989 [13], measles cases decreased to an average of 63 cases per year for the period 2000–2007 [14]. In 2008, however, US measles cases were at the highest level seen in more than a decade, with nearly half of those cases involving children whose parents had rejected vaccination [14]. Since 2008, Israel, Ireland, Switzerland, Austria, Italy, Australia, Germany, France, Britain, and Canada

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have reported substantial outbreaks of measles among populations that have refused vaccination, and importations from these and other countries have fueled US outbreaks [15–25]. It is worth noting that occasional spread from unvaccinated individuals with measles to 2-dose vaccine recipients has recently been observed [5, 10, 14, 26].

Immunity to wild-type measles is generally thought to be lifelong [27]; however, life spans have increased, and the length of protection afforded by natural infection or vaccination in the absence of circulating wild-type MeV and subclinical boosting is unclear. It has long been recognized that an intense force of infection and/or an extended duration of exposure can produce a range of symptoms from classic to mild/modified or asymptomatic in previously immune individuals [28]. Waning immunity would be most likely to occur in vaccine recipients, because vaccinated persons have lower levels of measles-specific antibody than do those with immunity derived from exposure to wild-type MeV [29–32]. In addition, the decrease in measles antibodies is more rapid in vaccinees than in those who have recovered from measles disease [4]. A prospective cohort study in the United States demonstrated that, although measles plaque reduction neutralization (PRN) antibody persisted in all vaccinees 10 years after a second dose, there was a progressive decrease in levels of measles antibody as time since vaccination increased [33].

Laboratory confirmation of acute measles infection in previously immune individuals presents a greater challenge to the diagnostic laboratory than does detection of acute disease in unvaccinated persons, because immunoglobulin (Ig) M responses may be absent or short-lived. Moreover, because of restricted viral replication, molecular detection using RT-PCR is also limited. The lack of IgM antibodies and an inability to detect MeV in conjunction with the presence of modulated symptoms could lead to an underestimation of measles disease among the previously vaccinated population and suggests that more-sensitive assays or alternative approaches to detect MeV infection may be needed. In this study, we evaluated paired serum samples and clinical information obtained from measles cases among vaccinated and unvaccinated individuals in the Republic of the Marshall Islands (RMI) and presumptive SVF measles cases identified in the United States. RMI is an isolated Pacific island nation with high 1-dose vaccine coverage that implemented a 2-dose requirement in 1998 and was free of reported measles cases for 14 years. In 2003, the RMI experienced a large measles outbreak [6, 7, 34]. During the subsequent investigation, it was noted that some individuals exhibited a milder disease course. Serum specimens from these individuals, as well as from individuals with more-classical presentations, were collected. Subsequently, paired serum samples from US measles outbreaks were also examined. Clinical symptoms and measles IgM, IgG, IgG avidity, and serum neutralizing antibody titers were compared to characterize responses to measles infection in previously vaccinated and unvaccinated individuals.

MATERIALS AND METHODS

RMI Clinical Case Definition

A suspected measles case patient in RMI was defined as a patient with fever, rash, and either cough, coryza, or conjunctivitis who resided in the RMI during the period 13 July–7 November 2003. A laboratory-confirmed case patient was defined as a patient with serological (defined as positive measles IgM enzyme immunoassay [EIA] results or a 4-fold increase in measles PRN antibody titer) or virological (defined as measles virus RNA detected in blood or secretions by reverse-transcription polymerase chain reaction [RT-PCR]) evidence of acute measles infection. For the purposes of this study, acute primary measles infection was defined as a laboratory-confirmed case in an unimmunized individual who had not received a dose of measles vaccine during the outbreak and whose initial serum sample had low-avidity measles antibodies. Individuals meeting the clinical case definition (CCD) who had documented previous measles vaccination and high-avidity antibodies were considered potential SVF cases. Because such individuals could not be distinguished from measles-vaccinated individuals with a rash illness other than measles, only those with laboratory-confirmed measles disease were used for comparison of clinical symptoms among those with acute measles and SVF.

RMI Case Investigation Form

A standardized case investigation form, developed and distributed to all RMI health care providers, collected detailed demographic information, self-reported clinical features, vaccination history, and illness outcome for all suspected measles cases. Vaccination history was obtained from parental/patient recall, personal and medical records, and immunization logs maintained by the local health department. Patients were classified as vaccinated if recall or documentation provided the number and/or dates of vaccinations; as having no history of vaccination if they reported no receipt of previous measles vaccine and had no documentation of vaccination; as having unknown vaccination status if their vaccination status was uncertain or lacked documentation.

Collection of Clinical Samples

Serum specimens were collected for serological testing and nasopharyngeal swab samples were collected for virus isolation and genetic characterization from a subset of RMI case patients with suspected measles. Commonly, specimen 1 was collected during the first medical contact and specimen 2 was collected approximately 1 month later. Patients were selected by convenience, and those patients who were examined do not represent a systematically selected, representative sample of the outbreak

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population. Anecdotal reports from co-investigators suggested that patients with milder symptoms may have been over-sampled. RT-PCR assays to detect MeV RNA were performed on all nasopharyngeal samples that were collected, as previously described [34]. Specimens from patients with suspected measles disease who were involved in US outbreaks were referred to the Centers for Disease Control and Prevention (CDC) for initial or confirmatory testing.

Measles IgM Testing

Serum specimens were tested for measles-specific IgM antibodies using an IgM EIA with a capture format, as previously described [35]. ImmunoWELL Measles Recombinant IgM Test (GenBio) was used for the quantitative detection of IgM to MeV according to the manufacturer's directions.

Measles IgG Testing

The ImmunoWELL Measles Recombinant IgG Test was used for the qualitative detection of measles IgG. This test utilizes an EIA microtiter plate technique in an indirect format for the detection of measles antibodies. Serum is added to microtiter plates coated with baculovirus expressed recombinant measles nucleoprotein (N) and allowed to react. After removal of unbound antibodies, goat anti-human IgG antibody conjugated with horseradish peroxidase is allowed to react with bound antibodies. After a series of washes, colorless chromogenic substrate (3, 3', 5, 5'-Tetramethylbenzidine-H2O2) is added, and bound peroxidase reacts developing a color change. The substrate-peroxidase reaction is stopped by adding 2M phosphoric acid, and the resulting OD is read with a spectrophotometer.

Avidity Testing

Avidity testing of RMI specimens was accomplished using an ImmunoWELL rubeola assay (GenBio) using purified recombinant N protein. Specimens were tested at 4 dilutions (1:100, 1:1000, 1:5000, and 1:25,000). After incubation with the antigen, samples were washed with either the manufacturer's wash buffer or with avidity reagent. An avidity index was calculated by dividing the optical density value of the well washed with the avidity reagent by the optical density value of the well washed with the manufacturer's wash buffer. The linear range was below 1.5 absorbance units. Only the first ratio within the linear range was interpreted. Receiver operating characteristic (ROC) analysis was performed on blood donors with known measles exposure histories and on unvaccinated laboratory-confirmed cases to determine an avidity cutoff value. Avidity ratios $<45\%$ were considered to be low, whereas ratios that $\geqslant 50\%$ were considered to be high. An avidity index between 45%–49% was considered to be an equivocal response.

Avidity testing for measles-specific IgG in US specimens was performed as described (S. Mercader, personal communication). Briefly, a commercially available EIA platform for measles IgG detection was modified to include 3 protein-denaturing washes to elute low-avidity antibodies. Serum was diluted in 2 dilution series: one series was washed with the manufacturer's wash buffer (WB), whereas the other was washed with diethylamine in WB (DEA). The titer value at optical density signal extinction for each dilution series was calculated, and the ratio of the 2 titers was obtained and expressed as a percentage; end-titer avidity index (etAI%) = (end-titer DEA curve/end-titer WB curve) $\times$ 100.

PRN Test

PRN tests were performed as described previously using low-passage Edmonston MeV on Vero cell monolayers [36], and end point titers were calculated using the Kärber method [37]. Serum specimens were run in parallel with the Second World Health Organization (WHO) International Standard Reference Serum (66/202), and samples with reciprocal titers of <8 were assigned a value of 4 for calculating conversion rates. In this assay, a titer of 1:8 corresponded to 8 mIU/mL.

Radioimmunoprecipitation

Immunoprecipitation using ^{35}S-methionine-labeled lysates of MeV-infected cells was performed as previously described, except that Vero/hSLAM cells were used instead of Vero cells [38]. H-protein specific monoclonal antibodies (MAbs) CV-2, CV-4, CV-5 and CV-11 have been described previously [38]. Mabs79VV17D (V17), 80IIIB2 (B2), and 81-I-366 (366) were obtained from the laboratory of Dr D. McFarlin (National Institutes of Health).

RESULTS

Laboratory Testing of Paired Serum Samples From RMI

Paired serum samples (63 pairs) were collected from case patients with suspected measles who met the CCD but did not receive a dose of vaccine during the outbreak response immunization campaign during 2003. Twenty five (40%) of the 63 specimens were IgM positive by the quantitative IgM EIA and were therefore designated as laboratory-confirmed measles cases. In contrast, 38 (60%) of the cases in this cohort were measles IgM negative. Within the IgM-negative group, 2 additional cases (for a total of 27 cases) could be identified because they were either RT-PCR positive for wild-type MeV or had a 4-fold increase in neutralization titer. Although the remaining 36 individuals met the CCD, all had negative measles IgM results and thus were not considered to have laboratory-confirmed measles; therefore, they were excluded from the analyses described below.

Avidity. Antibody avidity measurements were performed on serum specimens from the 27 laboratory-confirmed cases of measles (Figure 1). Of the 8 cases in patients with recorded documentation of vaccination, 2 (ages 1.4 years and 18 years) had low-avidity IgG antibody detected, which suggested that they had experienced primary vaccine failure, whereas 6 cases

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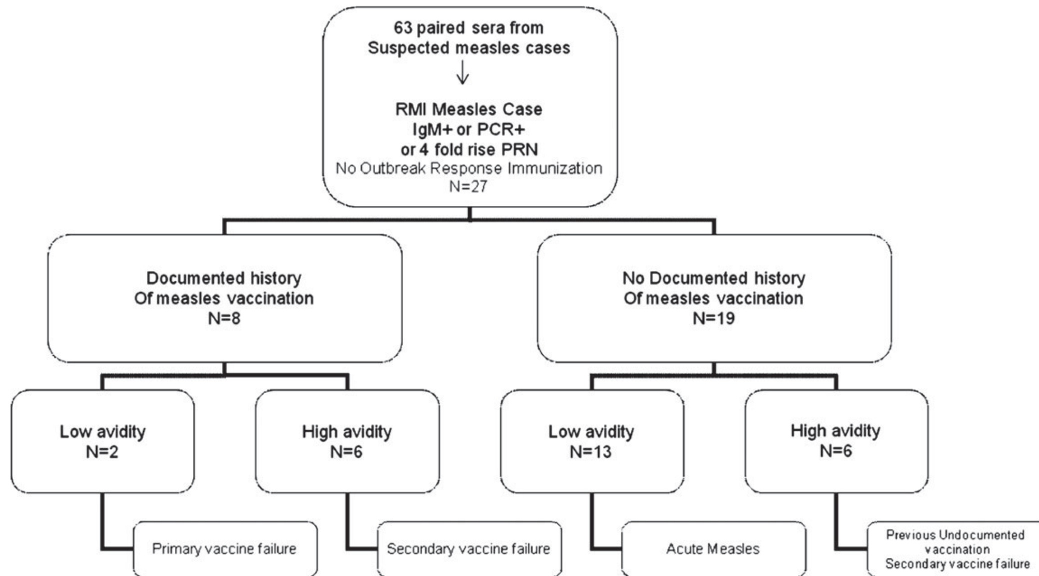

Figure 1. Avidity results and vaccination status for laboratory confirmed measles cases in the Republic of the Marshall Islands (RMI). Laboratory-confirmed case patients included case patients who did not receive outbreak response immunization and were immunoglobulin M (IgM) positive or had a 4-fold increase in measles plaque reduction neutralization (PRN) titer or had reverse-transcription polymerase chain reaction (RT-PCR) results that were positive for wild-type measles virus. Vaccination history was obtained from parental or patient recall, personal and medical records, and immunization logs maintained by the local health department. Patients were classified as vaccinated if recall or documentation provided the number and/or dates of vaccinations or were classified as having no history of vaccine if they reported no previous measles vaccination and had no documentation of vaccination.

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(ages 10, 10, 11, 14, 15, and 19 years) had high-avidity antibody, which demonstrated that they likely had SVF. Of the cases with no reported measles vaccine history, the majority (13 of 19) had low-avidity antibody indicative of a primary response to infection. In contrast, 6 of the 19 cases among the group with no reported receipt of vaccine were found to have high- or equivocal-avidity antibody (1–13 days after rash onset), which indicated that they likely had received an undocumented dose of measles vaccine or, less likely, that they had previously experienced measles infection.

Age Distribution of IgM-Positive Patients

When the age distribution of IgM-positive RMI patients in this study was plotted against the results of antibody avidity testing (Figure 2), it was striking that almost all samples containing low-avidity antibody were from acute cases that occurred in children aged <1 year and in adults aged >20 years. Children aged <1 year would not yet have been eligible for vaccination, and adults aged >20 years would have been too old to have received routine vaccination when it began in 1982 and must have missed any supplemental immunization activity (SIA) "catch-up" vaccination efforts. In contrast, the majority of patients with high-avidity antibody were between the ages of 10 and 20 years and had received vaccination 10–15 years earlier. The high-avidity IgG response observed in these individuals indicates that they represent SVFs, because they had been primed by at least 1 dose of vaccine in the past, met the CCD, and were measles IgM positive.

Disease Severity in Acute vs Secondary Vaccine Failure Cases. Disease symptoms for individuals who met the definition for SVF (Table 1) were compared with symptoms for patients who had acute primary measles infection (Table 2). There were fewer complications in those with presumed SVF than in those with acute primary infection. There were 4 hospitalizations, 3 cases of pneumonia, and 6 other reported complications in the 10 patients with acute illness, whereas there were no hospitalizations, no cases of pneumonia and only 2 complications in the 6 patients with SVF. In this subset of cases with paired measles serological testing, all cases of acute primary measles infection that required hospitalization occurred in infants <1 year of age.

Neutralizing Antibody Responses in RMI. The PRN test is an accepted serological measure of protection because it measures functional neutralizing antibody, which is believed to confer immunity. Five of 6 identified SVF cases had paired serum samples for testing by the PRN test (Table 1). Interestingly, the PRN titers in each of the initial samples, obtained 0–28 days after rash onset, were observed to be exceptionally high; they were 6–60 times the mean PRN titer seen after routine measles vaccination (1 or 2 dose) and were markedly higher than the titers observed after acute measles infection in unvaccinated individuals (Table 3). These PRN titers were also compared with

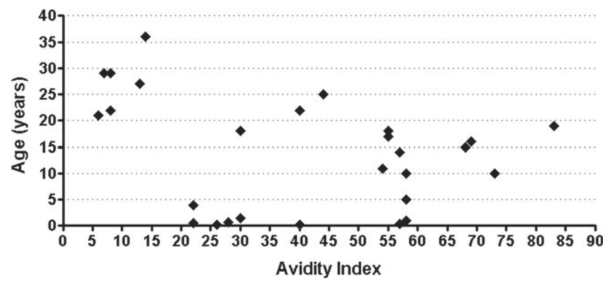

Figure 2. Age distribution and avidity index of laboratory-confirmed cases. Avidity testing was performed using an ImmunoWELL Rubeola assay (GenBio) using purified recombinant N protein. Specimens were tested at 4 dilutions (1:100, 1:1000, 1:5000, and 1:25,000). After incubation with the antigen, samples were washed with either regular assay wash buffer or with avidity reagent. An avidity index was calculated by dividing the optical density value of the well washed with the avidity reagent by the optical density value of the well washed with the kit's regular wash buffer. The linear range was below 1.5 absorbance units. Only the first ratio within the linear range was interpreted. Receiver operating characteristic analysis was performed for blood donors with known measles exposure histories to determine a cutoff value. If the avidity ratio is less than 45% the specimen is likely to contain low-avidity measles antibodies. If the avidity ratio is >50%, the specimen is likely to contain high-avidity measles antibodies. If the avidity index is between 45%–49%, it is considered to be an equivocal response.

those published for individuals who experienced natural infection or were given 1 or 2 doses of measles-containing vaccine. The geometric mean titer (GMT) of 30 unvaccinated children from Venezuela infected with measles was 4764, which is similar to the GMT of 4798 reported previously for 122 women born before 1957 (Table 3). As noted previously and shown in Table 3, PRN titers in vaccinated persons (1 or 2 doses) are considerably lower than those seen in naturally infected individuals.

PRN titers in the second paired serum sample from those with SVF cases were similar to or lower than those seen in the first samples when the pairs were compared, and no 4-fold or greater increases in antibody titer were detected in this group (Table 1). In these laboratory-confirmed cases of measles SVF, very high PRN titers were apparent immediately after rash onset and demonstrate that these individuals, although still susceptible to infection at exposure, were nonetheless capable of rapidly producing an impressive quantity of high-avidity antibody that likely mitigated their symptoms and limited the severity of their disease.

Measles PRN titers in 5 paired serum samples from individuals with acute primary measles infection were also measured (Table 2). As expected, in young infants with acute measles infection, case patient 1 (Table 2), an 8-month-old child, had a low level of measles antibody in the acute sample that was obtained 3 days after rash onset and had a modest increase in measles PRN antibody ~30 days later. If one takes into account the loss of passively acquired maternal antibody over time and compares the observed versus the expected titer in the convalescent sample, case 1 meets the criteria for a 4-fold increase in antibody titer that is consistent with acute measles infection. In contrast, cases 5, 6, and 8, who were 3–6 months of age at the time of infection, had measles antibody titers of 5496, 6640, and 7656 mIU/mL, respectively, in the acute samples obtained within 4 days of rash onset, which was consistent with the presence of passively acquired antibody from mothers previously infected with wild-type measles. Comparisons of measles titers for cases 5, 6, and 8 indicate that seroconversion did not occur even when the natural loss of maternal antibody overtime was taken into account.

Neutralizing Antibody Responses of Secondary Vaccine Failure Cases in the United States. To determine whether the unusually high plaque neutralization results observed for SVF cases in RMI were unique to this population, paired serum samples were obtained from SVF case patients in the United States (Table 4). US case patients had documented receipt of MMR, were laboratory confirmed by IgM and/or RT-PCR, and

Table 1. Self-Reported Disease Severity of Case Patients With Secondary Vaccine Failure

Case patient	Age, years	Rash duration[a]	Cough	Coryza	Conj	Complications	Prev vac	Timing spec 1, days	Timing spec 2, days	PRNT spec 1	PRNT spec 2	Avidity spec 1	Avidity spec 2
1	15	2	Yes	Yes	Yes	Diarrhea	2	28	61	297,070	13,929	68	65
2	14	7	No	No	Yes	None	2	7	N/A	119,228	95,704	57	56
3	11	4	Yes	No	No	Diarrhea	2	0	46	55,715	37,134	54	62
4	10	5	Yes	Yes	No	None	3	2	41	34,124	34,124	73	79
5	19	3	Yes	Yes	No	None	2	4	48	82,196	80,818	65	56
6	10	6	Yes	Yes	Yes	None	1[b]	3	47	N/A	N/A	58	N/A

NOTE. All patients had rash and fever. All patients had documented previous vaccination (case patients 2–5 had recorded dates of vaccination in their immunization logs, whereas donor 1 had a history of receiving 2 vaccine doses but no dates of vaccination), did not receive an outbreak response vaccination, had high-avidity antibodies, and were immunoglobulin M positive or indeterminate. Conj, Conjuctivities; Conv, convalescent; N/A, not available; Prev Vac, number of previous measles-mumps-rubella vaccinations; PRNT, plaque reduction neutralization test; Timing spec 1, days after rash onset that specimen 1 was collected; Timing spec 2, days after rash onset that specimen 2 was collected.

[a] Mean rash duration is 4.56 days (range, 2–7 days).

[b] Patient 6 received 1 dose at age 17 months and a second dose 2 days prior to rash onset.

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Table 2. Self-Reported or Parent-reported Disease Severity of Case Patients With Acute Measles

Case patient	Age, years	Rash duration[a]	Cough	Coryza	Conj	Complications	Prev vac	Timing spec 1, days	Timing spec 2, days	PRNT spec 1	PRNT spec 2	Avidity spec 1	Avidity spec 2
1	0.7	5	Yes	Yes	Yes	D, V, H	0	3	45	84	168	28	25
2	63	14	Yes	Yes	Yes	None	0	21	N/A	N/A	N/A	12	N/A
3	21	4	Yes	Yes	Yes	DE, V	0	24	N/A	N/A	N/A	6	N/A
4	29	7	Yes	Yes	Yes	None	0	28	N/A	N/A	N/A	8	N/A
5	0.25	4	Yes	No	No	P, H	0	4	43	5,496	4,362	40	31
6	0.56	5	Yes	Yes	Yes	P, D, H	0	4	43	6,640	7,345	22	22
7	22	3	Yes	Yes	Yes	None	0	8	N/A	N/A	N/A	8	N/A
8	0.25	3	Yes	No	No	P, H	0	4	39	7,556	8,192	26	34
9	27	3	Yes	Yes	Yes	D	0	2	N/A	N/A	N/A	17	N/A
10	4	N/A	Yes	Yes	Yes	None	0	1	34	427	810	22	22

NOTE. All patients had rash and fever. All patients had no previous vaccination, did not receive an outbreak response vaccination, had low-avidity antibodies, and were immunoglobulin M positive. Data for patients with acute cases who had missing clinical information are not shown. Conj, Conjuctivities; Conv, convalescent; D, diarrhea; DE, dehydration; H, hospitalized; N/A, not available; P, pneumonia; Prev Vac, number of previous measles-mumps-rubella vaccinations; PRNT, plaque reduction neutralization test; Timing spec 1, days after rash onset that specimen 1 was collected; Timing spec 2, days after rash onset that specimen 2 was collected; V, vomiting.

[a] Mean rash duration is 4.8 days (range, 3–14 days).

had high-avidity measles antibodies. Cases 1–3 were part of a well-documented multistate measles outbreak associated with an international youth sporting event [10]. As seen with SVF case patients in the RMI, US laboratory-confirmed SVF case patients also had extremely high PRN titers in their paired serum specimens, which suggested that this high PRN response may be representative of immune responses to MeV in those individuals who were previously primed by measles vaccination.

Immunoprecipitation

To further characterize PRN responses observed following natural measles infection in 2-dose vaccinees, serum specimens were evaluated using immunoprecipitation. As expected, the antibody response was primarily directed against measles H protein; even when diluted 1 to 10,000, anti-hemagglutinin activity was still detected (data not shown).

DISCUSSION

The importance of waning immunity as an impediment to measles elimination remains an open question. Concern exists because measles seroprevalence rates appear to decrease as time since vaccination increases, and the proportion of the population possessing only vaccine-induced immunity continues to grow. Likewise, opportunities for boosting caused by wild-type measles exposure are becoming increasingly rare, and waning antibody titers could, over time, result in an accumulation of measles-susceptible individuals in the population. Recent outbreaks of measles in highly vaccinated populations and among individuals with 2 age-appropriate MMR vaccinations in the US and elsewhere contribute to these concerns [8, 11].

Waning of measles antibody, however, does not necessarily equate to waning immunity because cell-mediated responses are known to play an important role in protection. In addition, the incidence of measles in adults has not increased and measles attack rates, even 10 or more years after vaccination, remain low and consistent with primary vaccine failure rates [41–43]. Furthermore, among US residents during the period 2001–2008, the highest incidences of measles disease were in children ages 6–11 months and 12–15 months, and 65% of the total cases reported were considered to be preventable (eligible for vaccination but unvaccinated), which suggests that failure to vaccinate still plays a greater role in current US measles cases than does vaccine failure. More importantly, secondary spread from measles-infected 2-dose vaccinees to other susceptible individuals has not been documented [8, 10, 26]. No evidence of viral shedding was seen in a laboratory study of asymptomatic or mildly ill vaccinated contacts of persons with measles [44]; however, additional studies using existing, more sensitive laboratory techniques are needed. Disease in those with SVF is frequently muted, and the presence of virus, viral RNA and measles specific IgM are often difficult to detect; therefore, it is likely that transmission is limited and that spread of virus to other susceptible individuals occurs rarely, if at all. Such cases are not likely to be epidemiologically important with respect to transmission. Careful surveillance of vaccinated adolescents and adults, as well as a more thorough understanding of the clinical and laboratory presentation of measles disease in SVF, are needed to better identify cases and to investigate measles transmission capacity.

In this study, we identified and characterized measles SVF case patients using paired serum samples obtained from a large measles outbreak in the RMI (2003) and from recent outbreaks in the United States. We used only laboratory-confirmed cases and compared laboratory diagnostic results and reported clinical symptoms for acute primary measles cases versus vaccine failure

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Table 3. Plaque Reduction Neutralization (PRN) Results Following Vaccination or Wild-type Infection Based on Exposure History

Study group	Vaccination history	Exposure to wild-type measles	Geometric mean titer plaque neutralization	95% CI	Reference	Comments
1	Unvaccinated	Yes	4,764 (*n* = 30)	3,467–1,547	Present study	Unvaccinated Venezualen children
2	Unvaccinated	Yes	4,798 (*n* = 122)	3,945–5,835	Markowitz et al, 1996 [39]	Women born before 1957
3	Unvaccinated	Yes	1,559 (*n* = 312)	…	LeBaron et al, 2007 [33]	Kindergarten children who received MMR between 12 and 24 months
4	Vaccinated with 1 dose	No	757 (*n* = 309)	…	LeBaron et al, 2007 [33]	Middle school children who received MMR between 12 and 24 months of age
5	Vaccinated with 1 dose	No	1,162 (*n* = 7)	…	Wong-Chew et al, 2003 [40]	Adult health care workers (age, 26–40 years) who received first dose of MMR as infants
6	Vaccinated with 2 doses	No	2,814 (*n* = 304)	…	LeBaron et al, 2007 [33]	One month after second MMR dose
7	Vaccinated with 2 doses	No	1,368 (*n* = 6)	…	Wong-Chew et al, 2003 [40]	Adult health care workers 4 weeks after receiving second MMR dose
8	Vaccinated with 1 dose	Yes	53,014 (*n* = 3)	12,313–219,721	Chen et al, 1990 [28]	Measles outbreak at Boston University; Preexposure PRN titers were <16, 80, and 86
9	Vaccinated with 2 doses	Yes	20,501 (*n* = 3)	3,837–540	Chen et al, 1990 [28]	Measles outbreak at Boston University; Preexposure PRN titers were 98, 118, and 120
10	Vaccinated with 2 doses	Yes	32,141,245,580	32,141–245,580	Present study	RMI measles outbreak, 2004

NOTE. PRN tests were performed using low-passage Edmonston MeV on Vero cell monolayers. CI, confidence interval; MMR, measles-mumps-rubella vaccine; RMI, Republic of the Marshall Islands.

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cases. Additionally, laboratory results from SVF cases in the United States were compared with those observed in the RMI. Several interesting properties of the measles immune response emerged during this analysis that may help to characterize SVF cases.

First, the age distribution of IgM-positive laboratory confirmed cases reflected the history of MMR vaccination in the RMI. The RMI started routine single-dose measles vaccination in 1982, administering MMR vaccine routinely to individuals at 9 months of age. In 1998, a 2-dose MMR vaccine schedule was implemented (administered at 12 and 13 months of age), and 3 SIAs were conducted during the period 1994–2002 [6]. In the 2003 outbreak, IgM-positive cases clustered among children <5 years of age who would have received 0 or 1 dose of measles

Table 4. Case Patients With Secondary Vaccine Failure in the United States

Case	Age, years	Cough	Coryza	Conj	Prev vac	Timing spec 1, days	Timing spec 2, days	PRNT spec 1	PRNT spec 2	Avidity spec 1	Avidity spec 2	RT-PCR	IgM
1	33[a]	No	Yes	No	2	1	6	1573	207,954	N/A	N/A	+	+
2	19[b]	No	No	Yes	2	5	10	1858	119,287	High	High	+	–
3	19[b]	No	No	Yes	2	N/A	7	N/A	217,812	N/A	High	+	+
4	34	N/A	N/A	N/A	2	0	49	248,686	152,734	High	High	N/A	+
5	45	Yes	Yes	No	1	0	6	30,208	21,730	High	N/A	N/A	+

NOTE. All patients had rash and fever. Conj, conjuctivities; Conv, convalescent; Ig, immunoglobulin; N/A, not available; Prev vac, no. of previous measles-mumps-rubella vaccinations; PRNT, Plaque reduction neutralization test; RT-PCR, reverse-transcription polymerase chain reaction; Timing spec 1, days after rash onset that specimen 1 was collected; Timing spec 2, days after rash onset that specimen 2 was collected.

[a] Airport worker from Michigan involved in the outbreak reported in Guris et al [41].

[b] College students involved in the outbreak reported in Guris et al [41].

vaccine, representing the cohort of unvaccinated children and those with primary vaccine failure.

Second, a plot of the age distribution of IgM-positive patients with laboratory-confirmed cases versus their avidity measurement also reflected the RMI MMR vaccine recommendations. The majority of low-avidity antibody (acute) cases were seen, as expected, in unimmunized children <1 years of age but also, somewhat surprisingly, in adults > age 20. The low-avidity antibody observed in adult cases suggested that these individuals had not been primed for an immune response to measles and likely missed opportunities to receive measles vaccine when the program was initiated in 1982, may have missed subsequent catch-up vaccination via SIA, or may have been primary vaccine failures. This finding illustrates that pockets of susceptible individuals can accumulate in populations because of changes in vaccination policy and can, in some circumstances, provide a sufficient base to maintain transmission, particularly in densely crowded populations [45]. Recent measles outbreaks among one-dose vaccine recipients and unvaccinated adults in Boston, Sao Paulo, Ukraine, and Australia further highlight this point and demonstrate that this scenario is not unique to RMI [25, 45, 46]

An interesting third characteristic noted was that the majority of patients who were identified with high-avidity antibody were between the ages of 10 and 20 years, which would mean that they had received MMR vaccination 10–15 years earlier. These vaccinated individuals would likely never have been exposed to wild-type virus, because RMI had been free of measles for the previous 14 years. These individuals were IgM-positive and met the CCD, yet they demonstrated high-avidity IgG responses, indicating that they had been primed by vaccine at least once in the past. Past priming appeared to afford protection from severe disease, because these patients reported milder symptoms and experienced fewer complications than did those with acute measles.

The fourth and most striking observation was the magnitude (PRN titers >30,000) of the neutralizing antibody response in SVF cases, which has not, as a rule, been observed following vaccination or primary acute measles. Although these individuals lacked sufficient protective neutralizing antibody to completely inhibit measles infection at the time of exposure, they rapidly mounted an impressive neutralizing response that likely mitigated extensive viral replication and resulted in mild measles with minimal symptoms and few complications. When these high-titered serum samples were examined using immunoprecipitation, it was apparent that, as expected, the PRN titers reflected a predominant anti-H antibody response. Additional study is needed to determine whether elevated PRN titers can be used as a biomarker for SVF in cases that cannot be confirmed using traditional laboratory methods.

Although we do not know the initial PRN titer or immune status of the RMI or US SVF case patients, it is significant that a few were RT-PCR positive for wild-type measles virus, which suggests that these cases have the potential to shed and spread virus to susceptible contacts. It is important to note, however, that there was no known or documented transmission from the SVF cases in RMI. Likewise, an investigation of 2 US SVF cases did not reveal secondary spread of measles to other students within their college community [10]. Similarly, measles in 2 fully immunized (2-dose recipient) siblings exposed during an airplane flight [26] was mild, and these children did not subsequently transmit measles even though a nasal swab sample from 1 of the children was positive for measles virus RNA by RT-PCR. Together, these data suggest that viral transmission from those with SVF cases to other susceptible individuals may be very limited or may not occur at all.

In conclusion, we have characterized measles SVF in 2 populations: in the RMI, which represents a fairly typical international setting as countries move from enhanced control and mortality reduction to regional elimination, and in a highly vaccinated US population with broad 2-dose MMR coverage. SVF in both situations was characterized by documentation of prior measles vaccination, the presence of high-avidity anti-measles IgG, and markedly elevated levels of PRN antibodies. Elevated PRN titers appear to represent a biomarker for SVF, and additional studies are needed to determine whether elevated titers persist and whether they can be used to identify SVF cases within a highly vaccinated society. The duration of MMR vaccine-induced immunity in the absence of circulating virus is not well understood and may be significantly impacted by age at first vaccination, as well as by the timing of the second dose. Consistent implementation of a 2-dose schedule is also needed to maintain high population immunity against measles, to minimize disease, and to prevent subsequent outbreaks. Because SVF cases are generally mild, they may be missed unless they are seen within an outbreak setting and linked to an acute, severe measles case. Until the transmission capacity of SVF cases has been fully established, the presence of measles disease in twice-vaccinated persons illustrates the need to (1) closely monitor levels of measles antibodies in adolescents and adults in the US population, (2) be vigilant of modified disease presentation during outbreaks, and (3) evaluate vaccinated close contacts when investigating sporadic unknown source cases that have no apparent link to importation. Based on accumulating evidence, and as reported by Rota et al [48] in this supplement, patients with SVF cases do not appear to efficiently transmit virus, and their occurrence will likely not impede measles eradication efforts [8, 10, 26, 44, 47].

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A Measles IgG Avidity Assay: Use in Classification of Measles Vaccine Failure Cases in Elimination Settings

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1 **ABSTRACT**

2 In regions where endemic measles virus has been eliminated, diagnostic assays are
3 needed to assist in correctly classifying measles cases irrespective of vaccination status. A
4 measles IgG avidity assay was configured using a commercially available measles-specific IgG
5 enzyme immunoassay by modifying the protocol to include three 5-minute washes with
6 diethylamine (60mM, pH 10.25) following serum incubation; serum was serially diluted and
7 results were expressed as end-titer avidity index. Receiver Operating Characteristic analysis was
8 used for evaluation and validation, and to establish low (≤ 30%) and high (≥ 70%) end-titer
9 avidity thresholds. Analysis of 319 serum specimens expected to contain either high or low
10 avidity antibodies per clinical and epidemiological data indicated that this assay is highly
11 accurate with an Area Under the Curve of 0.998 (95% CI: 0.978-1.000), sensitivity of 91.9%
12 (95%CI: 83.2%-97.0%) and specificity of 98.4% (95%CI: 91.6%-100%). This assay is rapid (<2
13 hours) and precise (SD: 4%-7%). In 18 samples from an elimination setting outbreak, the assay
14 identified 2 acute measles cases with low avidity results; both were IgM positive samples.
15 Additionally, 11 patients (15 samples) with modified measles that were found to have high
16 avidity IgG results were classified as secondary vaccine failures; one sample with an
17 intermediate avidity result was not interpretable. In elimination settings, measles IgG avidity
18 assays can complement existing diagnostic tools in confirming unvaccinated acute cases and, in
19 conjunction with adequate clinical and epidemiologic investigation, aid in the classification of
20 vaccine failure cases.

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24 **INTRODUCTION**

25 Although measles was declared eliminated from the United States in 2000, sporadic
26 outbreaks have continued to occur due to importations from endemic areas (30). Most cases have
27 occurred in unvaccinated individuals presenting with the classic clinical picture of descending
28 macular papular rash, fever, and either cough, coryza or conjunctivitis; consequently they can be
29 readily diagnosed. Suspected cases can be easily laboratory confirmed following timely
30 collection of specimens, usually a serum specimen assayed for the presence of measles specific
31 IgM, and concordant epidemiological information (7-9, 24, 30).

32 A limited number of cases, however, have occurred within the vaccinated population. In
33 contrast to measles in unvaccinated individuals, measles in vaccinated persons may present as a
34 spectrum of symptoms ranging from classic to modified measles, the latter being a less severe
35 disease with milder rash and/or fever, and none, some or all other typical measles symptoms
36 (12). Furthermore, IgM test results in these suspect cases may be falsely negative because
37 vaccinated persons may not make measurable measles IgM antibody in response to infection, and
38 specimens may be collected at suboptimal times due to poor symptom recognition in mild cases
39 of modified measles (19, 21, 34). New measles assays or modified applications of currently
40 available assays which confirm and classify active cases irrespective of vaccination status will be
41 required to certify and maintain measles elimination.

42 Avidity enzyme immunoassays differentiate early (primary) from distant (secondary)
43 antibody responses. Avidity describes the net force by which multivalent antibodies bind to
44 multivalent antigens (18, 23). In the naive host, low avidity IgG antibodies are elicited at the first
45 immunological challenge. Somatic hypermutation of antibody binding sites in the presence of
46 limiting antigen leads to the selection of high affinity antibodies (affinity maturation), and over

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47 time antibody matures from low to high avidity (13, 35). In measles, IgG avidity maturation is a
48 dynamic process that begins with the first encounter with antigens from wild type or vaccine
49 virus (14, 27, 37).

50 Traditionally, measles IgG avidity assays have been used to classify primary (PVF) and
51 secondary (SVF) vaccine failures (2, 16, 31, 33). PVF are vaccinated individuals that never
52 responded to the vaccine and present with classic measles upon measles virus infection. In serum
53 collected within 4 weeks of rash onset, measles IgM is present and measles IgG is of low avidity.
54 Low avidity results classify PVF and help confirm suspect cases even with a record of prior
55 vaccination. In contrast, SVF are individuals with documented IgG antibody responses to
56 vaccination, whose antibody has waned over time making them susceptible to measles virus
57 infection. SVF produce high avidity antibodies upon challenge with measles virus (31, 32). High
58 avidity results classify SVF and can be used to verify secondary immune responses in modified
59 measles cases with elevated plaque reduction neutralization (elevated PRN titers > 30,000
60 mIU/mL), a newly proposed biomarker to confirm SVF that otherwise would not be confirmed
61 by routine serology or molecular-based tests (19, 34).

62 Less traditionally, a measles avidity assay would be valuable in specific situations
63 encountered in the elimination setting. Many laboratories are uneasy about reporting IgM
64 positive results from single serum specimens obtained from sporadic cases of rash illnesses,
65 particularly in elimination settings where tests are rarely used. Additionally, regarding quality of
66 detection, declining levels of IgM antibodies in samples collected more than 4 weeks after rash
67 onset may lead to false negative results (4). In this context, detection of low avidity IgG
68 antibodies could be used to rule in these measles cases and provide assurance that a costly
69 investigation is not initiated based on a false IgM positive result (36, 38). Many public health

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70 laboratories do not have the resources to maintain stocks of reagents for sporadic measles
71 serology and often samples are tested in commercial laboratories. Furthermore, because of the
72 low prevalence of measles, there are fewer companies in the United States selling measles-
73 specific IgM assays. In addition, the availability of well-documented measles IgM containing
74 serum specimens needed to validate both commercial and "home brew" IgM assays has declined.
75 In these situations, measles IgG avidity testing may be a relatively less expensive and a more
76 reliable choice to confirm suspect measles cases (36).

77 A well validated measles avidity assay is needed for use as a complement to IgM testing
78 in the serological confirmation of clinically ambiguous cases, and to aid in the classification of
79 measles vaccine failures. This study describes the development, evaluation, and applicability of a
80 measles avidity assay. A commercially available measles IgG enzyme immunoassay was adapted
81 for wider use.

82 **MATERIALS AND METHODS**

83 **Samples**

84 Assay development: Serum samples and low to high avidity controls used to develop the
85 assay contained measles-specific IgG antibodies as determined by the Captia^{TM} Measles IgG
86 enzyme immunoassay (Trinity Biotech, Jamestown, NY) (Table 1). Samples from healthy adults
87 exposed to measles infection, or vaccination, more than a year before collection were assumed to
88 have high avidity antibodies. Samples collected within 3 months from measles rash onset or first-
89 time vaccination from IgM positive children 9 months and older (to avoid maternal antibody
90 interference) were expected to have results of low and intermediate avidity antibodies, depending
91 on the timing of sample collection (14, 37). IgM was detected using the CDC measles capture
92 IgM enzyme immunoassay (20).

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93 Assay validation: A total of 319 serum samples were retrospectively gathered from
94 specimen collections acquired from diverse locations in the Americas and Africa from the years
95 1988 to 2004 and archived at the Centers for Disease Control and Prevention in Atlanta, GA
96 (United States). Institutional Review Board approval was obtained for outbreak and vaccine
97 study samples. Specimens were also obtained from blood donated by healthy individuals under a
98 CDC Institutional Review Board approved protocol, and from individuals involved in outbreaks
99 who previously had natural measles or had received at least one dose of measles vaccine.
100 Samples were distributed into Group A and Group B (Table 1).

101 **Avidity assay for measles virus-specific IgG antibodies**

102 The protocol of the Captia™ Measles IgG enzyme immunoassay (Trinity Biotech,
103 Jamestown, NY) was modified for use with the denaturant agent diethylamine (DEA) to develop
104 a measles avidity assay. The antigen of the Captia™ assay is whole measles virus extracts.
105 Briefly, serum avidity controls (15µL) and test samples (15µL) were diluted into Captia™ serum
106 diluent (135µL) and were 10-fold serially diluted in two single-well dilution series by combining
107 diluted serum (30µL) into serum diluent (270µL). One dilution series started at 1:100 (or at 1:10
108 with serum specimens with known low levels of measles-specific IgG) and was washed with the
109 manufacturer's suggested wash buffer (WB). The other dilution series started at 1:10 and was
110 washed with 60mM DEA in WB and adjusted to pH 10.25 (± 0.1) with 1.0 M hydrochloric acid.
111 Three washes were performed for 5 minutes each, at room temperature. Next, plates were
112 washed three times with WB without soaking. All other steps of the assay were performed as
113 described by the manufacturer. Incubation times were 20 minutes for serum and conjugate, and
114 10 minutes for chromogen, tetramethylbenzidene. A run was accepted per kit's quality control
115 criteria, and per avidity control criteria as defined by the precision estimates (see *Assay*

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116 *diagnostic performance and precision evaluation*, and Table 2). End-titer avidity index
117 percentages (etAI%) were obtained using the formula graphically described by Jenum *et al.*, and
118 modified as described in supplemental material (Fig.S1) (22). Result classification was low
119 avidity if etAI% ≤ 30% and high avidity if etAI% ≥ 70%. An etAI% between 30% and 70% was
120 considered an equivocal result and sample was retested. If the result after retest was still between
121 these values, the sample was considered to be of intermediate avidity and not interpretable.
122 Samples at 1:10 dilution with undetectable IgG after DEA treatment were classified as low
123 avidity.

124 **Assay development**

125 The effect of DEA on the antigen was studied washing the plates with WB or with DEA
126 solutions at 30mM, 60mM and 80mM each at pH values of 10.00, 10.25, 10.50, 10.75 (± 0.1).
127 Then, a serum containing high avidity IgG antibodies was 10-fold serially diluted and incubated
128 on the plate. The assay was completed per insert instructions. Results were the end-titer of each
129 curve calculated as explained in Fig.S1. The effect of DEA on bound serum antibody was
130 examined using sera expected to contain either high or low avidity antibodies (See *Samples*
131 section). Then, washes were performed with WB or with the DEA solutions described above.
132 Results were etAI% (Fig.S1).

133 **Assay diagnostic performance and precision evaluation**

134 Standard EP05-A2 was followed to evaluate the precision of the assay and calculate the
135 within-device precision standard deviation estimate (S_T). S_T is a global precision estimate that
136 considers estimates of repeatability, and between-day and between-run standard deviations of the
137 assay (10). All 319 validation samples were randomized and tested in 28 separate runs using

138 preliminary thresholds established during a pilot study (data not shown). For the precision
139 experiment, five controls ranging from low to high avidity values were tested by two operators.

140 **Statistical analysis**

141 Receiver Operating Characteristic curve (ROC) analysis was used to evaluate assay
142 accuracy, establish avidity thresholds, and estimate sensitivity and specificity (15). The null
143 hypothesis for sample size calculation was an area under the curve (AUC) ≤ 0.75 (fair diagnostic
144 accuracy), and standard error of the AUC ≤ 5% (17). A gold standard for measles avidity does
145 not exist. Instead, measles clinical and epidemiological information were used. Accuracy was
146 evaluated in a two-step analysis: 1) using well-defined samples (Group A) and 2) outbreak
147 samples with uncertain epidemiological information (Group B) (Table 1) (42). Three
148 assumptions were made to use some of the samples in group B: 1) serum from cases with
149 vaccination records contained high avidity IgG antibodies; 2) serum collected from confirmed
150 measles cases in highly vaccinated elimination settings contained high avidity IgG antibodies
151 from past vaccination, and 3) serum collected in endemic settings up to 12 weeks of rash onset
152 contained low avidity IgG antibodies typical of acute measles. In the absence of date records,
153 samples were assumed to have been collected late (4-18 weeks). Paired samples were accounted
154 for during ROC analysis; SUDAAN was used to estimate the variance of sensitivity and
155 specificity, while the Taylor series linearization method was used to estimate the additional
156 covariance due to repeated measures. ROC analysis and graphs were performed with MedCalc
157 for Windows, version 8.1.1.0 (MedCalc Software, Belgium).

158 **RESULTS**

159 **Avidity assay development**

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160 The pH of DEA increases with concentration, and both DEA concentration and final pH
161 influenced the stability of antigen on the microtiter plate matrix and the stability of antibody-
162 antigen complexes. A solution of 60mM DEA adjusted to pH 10.25 (±0.1) was effective in
163 eluting low avidity antibodies with minimal effect on binding of high avidity antibodies and
164 coating antigen (Fig. S2). The original pH of 60mM DEA was greater than 11.5 and resulted in
165 loss of optical density signal indicating damage to either bound antibody or bound antigen. A
166 similar effect was observed when 6M to 8M urea in WB was used (data not shown).

167 **Assay diagnostic performance and precision evaluation**

168 The assay is highly precise with global precision estimate values (S_T) below 7% (Table 2)
169 and highly accurate, with AUC values greater than 0.9, even when samples with uncertain
170 epidemiological information (Group B) were included to challenge the assay's accuracy (Fig. 1,
171 A and C). When the group B samples were analyzed alone, accuracy was moderate to high
172 (AUC= 0.8) (Fig.1,B) (15, 29, 43). Avidity thresholds were established where sensitivity and
173 specificity were highest, at ≤ 30% for low avidity and at ≥ 70% for high avidity (Table 3). As
174 expected, measles avidity increased with time of exposure or immunization (Fig. 2). However, a
175 postulated low avidity sample among the well-defined samples had a high avidity result (Fig. 2,
176 A). This was an outbreak sample collected 5 days after rash onset from a 9-month–old infant. A
177 follow-up sample collected at day 11 resulted in low avidity. Low avidity results were not
178 obtained among the distant group.

179 **Intermediate avidity results within the well-defined group of samples (Group A)**

180 Among samples collected within the first 3 weeks of rash onset, seven samples out of 76
181 had intermediate avidity results (Fig. 2, A). Out of these seven samples, three were from reported
182 unvaccinated confirmed cases at least 15 months old that were reclassified as low avidity

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183 samples after retest. The remaining four samples were collected from 9 to 15 month old infants
184 during vaccine studies. Samples 1 and 2 in Table 4 were considered to contain residual maternal
185 antibodies because a paired pre-vaccine sample contained high avidity IgG; these two samples
186 were excluded from ROC analysis. Samples 3 and 4 were included in the ROC analysis. Within
187 the distant exposure group, one sample had an intermediate avidity result (Fig. 2, A).

188 **Assumptions made for samples with uncertain epidemiological information (Group B)**

189 Avidity results for 99 group B samples for which assumptions were made classified 32
190 acute infections, 2 PVF cases, 16 SVF cases, and 29 possible SVF cases, while results from 20
191 samples could not be interpreted. Ten samples collected in endemic settings were from patients
192 with uncertain rash onset date and/or vaccination status, and avidity testing determined that 5 had
193 results consistent with acute infection, 2 had high avidity results, and 3 were not interpretable
194 (data not shown). Table 5 summarizes avidity results for 69 samples with unknown vaccination
195 status. Four samples collected in elimination settings had results of low avidity, two of which
196 derived from persons exposed during two independent measles outbreaks, and easily confirmed
197 by routine methods. However, the other two IgM positive samples belonged to two isolated
198 cases, which were more difficult to classify because they were not linked to other measles cases
199 and the IgM result could have been falsely positive. One case was a woman born in 1954, in the
200 pre-vaccine era, when the majority of persons were infected during childhood, acquiring
201 immunity to the disease. The acute sample for this person, collected on the day of rash onset, was
202 IgG negative and a follow-up sample had to be ordered. This convalescent sample collected 31
203 days after rash onset contained low avidity IgG antibodies, thus confirming the case by
204 seroconversion. The second case was a young international traveler who declared he had never

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205 been vaccinated. His sample was collected 16 days after rash onset and had low avidity IgG
206 antibodies.

207 **Assay applicability**

208 The applicability of this measles avidity assay in an elimination setting was tested with 18
209 samples collected in the United States in 2006 during a confirmed measles outbreak. The assay
210 was able to further confirm 2 acute measles cases in IgM positive samples: one patient with
211 classic measles had received an outbreak response immunization dose one day before rash onset,
212 and in all effects this patient was considered unvaccinated; the other patient had no record of
213 vaccination. In 11 patients with modified measles, avidity testing classified 3 (27%; 4 samples
214 tested) as SVFs and 7 (64%; 11 samples tested) as suspect SVFs since they had high avidity
215 results but no record of vaccination, while one (9%; 1 sample tested) with intermediate avidity
216 and IgM negative results could not be classified.

217 **DISCUSSION**

218 Regions involved in maintaining measles elimination refer specimens from many cases of
219 rash illness and fever for laboratory confirmation of measles, typically by IgM enzyme
220 immunoassay and less frequently by other serological or molecular-based tests (4, 41). However,
221 due to inherent limitations of the methods used in measles diagnostics, not all cases can be
222 resolved (1, 4, 11, 21). For those confirmed cases in vaccinees, vaccine failure classification
223 would normally ensue by determination of IgG avidity. In these regions, the primary use of
224 avidity testing is in the classification of vaccine failures. However, a well-validated avidity assay
225 can also support confirmation of clinically ambiguous cases.

226 As an approach to solving some of the current problems with measles diagnostics, a rapid
227 (<2h) and accurate measles-specific avidity assay was developed by adding a DEA wash step to

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228 the protocol of a commercially available platform. The utility of this measles avidity assay was
229 illustrated with the analysis of samples collected during a measles outbreak in an elimination
230 setting. This outbreak was originally confirmed by the detection of measles-specific IgM, the
231 preferred confirmatory test. However, with the current paucity of commercial IgM assays and the
232 limitations of current confirmatory assays, there is a need to find alternatives to help confirm the
233 diagnosis of measles and help detect and control measles outbreaks. In our study, the majority of
234 low avidity results were obtained in immunologically naive IgM positive individuals during the
235 first 3 weeks after exposure to measles virus. Thus, in the event that IgM assays may not be
236 commercially available, this IgG-based avidity method could help confirm measles in those
237 suspect cases with sufficient measles-specific IgG for testing. Furthermore, because specific
238 antibodies are of low avidity for several weeks after classic rash onset, low avidity control
239 samples should be easier to obtain after recovery from classic measles and are expected to be
240 more readily available than controls for IgM assays.

241 The central role of avidity testing is vaccine failure classification, which is important for
242 both control and surveillance purposes (3, 31, 32). In current elimination settings, where
243 surveillance is case-based, vaccine failure classification can be helpful in characterizing the
244 frequency and the symptoms of modified measles, and in investigating the role of SVF cases in
245 measles transmission. Additionally, cases with unknown vaccination status and high avidity
246 results can be classified as suspect SVFs. High measles IgG avidity and very high neutralization
247 titers appear to correlate with SVF cases upon recent exposure to measles. Together, these two
248 parameters have recently been proposed as a biomarker for the confirmation of SVF cases, and
249 with appropriate validation, it could be used to confirm suspect SVFs cases (19). The everyday
250 application of this diagnostic approach was further investigated with the analysis of suspect cases

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251 referred to our laboratory from 2009 to 2011 (data not shown). Among 15 confirmed cases,
252 avidity testing classified 10 as SVF and 5 as suspect SVF. Of the 10 SVF cases, three had PRN
253 titers ≥ 81,916 mIU/mL, or 54 to 163 times the mean (1,525 mIU/mL) observed in unexposed
254 vaccinated individuals (mean calculated by averaging the PRN geometric mean titer values of
255 four previously described studies in unexposed vaccinated (1 or 2 doses) individuals) (19). One
256 of these three SVF had not been confirmed by IgM or RT-PCR assays, only by epidemiological
257 link, and is an example of the potential application of elevated high avidity IgG titers as a
258 biomarker for SVF. Interestingly, none of these 3 cases transmitted measles to others (34).
259 Furthermore, although widespread 2-dose coverage has made PVFs a rarity, identifying PVF
260 cases in young children vaccinated once can expedite control measures.

261 In the measles laboratory diagnostic toolbox, the measles avidity assay is a specialized tool
262 that can be used to help confirm suspect measles cases when information from routine assays is
263 inconclusive (Table 6). Avidity results can be applied as follows to rule in cases. First, low
264 avidity results provide support to confirm measles cases, similar to the identification of recent
265 primary infections with rubella virus, cytomegalovirus, human herpes virus 6 and 7, and HIV (5,
266 26, 39, 40). In measles, low avidity results are especially useful to rule in sporadic cases
267 appearing outside of an outbreak and when a false IgM positive result is suspected. For example,
268 two cases were described with IgM positive and low avidity IgG results. They involved a person
269 born before the vaccine era that would generally be assumed immune, and a person born
270 internationally and who was unvaccinated. Moreover, low avidity results are helpful when a
271 serum specimen is collected late and a false IgM negative result is suspected. The high
272 sensitivity of this measles avidity assay in the determination of primary infections provide
273 reassurance in IgM positive results obtained from sporadic cases, especially those suspect cases

274 of children presenting with febrile rash illness, unknown exposures to confirmed cases, unknown
275 vaccination status, and absence of international travel. Second, high avidity results support that
276 elevated PRN titers in modified measles cases derive from the activation of memory responses to
277 measles virus. In the future, high avidity results, together with elevated measles antibody titers,
278 could assist in confirming SVFs (19). In investigating SVF cases, intermediate avidity results
279 could be used to support the confirmation of some SVF cases (See *Supplemental Material*). In
280 contrast to congenital rubella, cytomegalovirus and toxoplasmosis diagnostics, high avidity
281 results cannot be applied to rule out cases (5, 25, 26). In our laboratory, high avidity results have
282 been observed in a SVF case with classic measles disease, and in an unvaccinated confirmed
283 case with modified measles (data not shown) (19, 34).

284 To correctly interpret the results of measles avidity assays, it is critical to have good
285 records of vaccination status with the number of doses and dates of administration, date of birth,
286 time of rash onset and sample collection, and symptoms. This information is especially useful
287 when investigating cases with modified measles presentations. Additionally, results obtained
288 from young children can be misinterpreted due to the presence of maternal IgG, which is
289 generally of high avidity. For example, avidity results for a nonimmune infant infected with
290 measles can initially be of high avidity. As the infant's immune response to measles virus
291 progresses, maternal high avidity IgG antibodies will be replaced with low avidity antibodies
292 from the infant, which will likely be detected in a second sample collected later. In this report,
293 this was observed in a 9 month-old measles case with a high avidity result in a sample collected
294 5 days after rash onset and a low avidity result in a sample collected 6 days later. It was also
295 observed in two infants recently vaccinated with their first measles-mumps-rubella (MMR) dose
296 (Table 5).

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297 The described measles avidity assay has limitations. First, a minimum level of measles-
298 specific IgG is required; samples must be IgG positive by the Capita^{TM} assay. Second, the results
299 of the assay cannot be used in isolation; the interpretation of the results relies on accurate
300 medical history and epidemiological information, and should be considered together with other
301 laboratory results. Third, during our validation, low avidity results were observed in sera
302 collected up to 9 weeks after vaccination with a first dose of MMR, but were not observed later
303 (data not shown). Therefore, low avidity results are difficult to interpret if vaccination has
304 occurred recently and results cannot be used to distinguish vaccine from wild-type infections.
305 Fourth, intermediate avidity results are complex to interpret and more data is needed to
306 understand their diagnostic relevance and ultimate value. Finally, the assay cannot rule out false
307 IgM positive results; the presence of high avidity measles IgG does not rule out measles as a
308 diagnosis.

309 The evaluation of the assay was limited in that samples were selected per the clinical
310 course of measles alone and did not consider underlying conditions, as in HIV infection, that
311 appear to have slower measles-specific IgG avidity maturation (28). A low avidity result in
312 timely collected samples from HIV-infected unvaccinated individuals would still confirm
313 measles (6, 28). Because an evaluation was not performed on known HIV-infected measles
314 cases, this avidity assay should not be used in the classification of vaccine failures in HIV-
315 infected, measles-vaccinated cases.

316 In conclusion, this paper introduces a new measles avidity assay that is ready to be used in
317 elimination settings. It is highly accurate, precise, and reproducible, as well as sensitive and
318 specific. The assay is able to detect measles-specific antibody maturation over time in line with
319 previous observations (14, 37). Besides vaccine failure classification, avidity testing can provide

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320 most valuable information in confirming those cases with RT-PCR negative results, or with
321 questionable IgM results. It must be emphasized that this assay can only be used to help rule in
322 measles cases, but not to rule them out. With appropriate assay evaluation, it may be possible to
323 adapt other commercially available measles IgG platforms to avidity testing by using the DEA
324 elution method presented here. This avidity method worked well with another commercial plate
325 coated with whole measles virus antigen (data not shown). Finally, the end-point titration method
326 was used because it is independent from IgG concentration and it is considered superior to the
327 single dilution method. However, in those situations where time or resources are limited, this
328 avidity assay could be performed using a single dilution (See *Supplemental Material*).

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464 **TABLES**

465 **TABLE 1** Characteristics of sera used in the validation of a measles avidity assay

Samples	Weeks after vaccination or rash onset (IgM result)			
	0 to 3 (+)	4 to 12 (+ or −)	13 to 18 (not tested)	> 1 year (+ or −)
Group A[a]				
Confirmed measles cases	45	14	0	33
MMR1 recipients	31	50	0	31
Total	76	64	0	64
Group B[b]				
MMR1 recipients[c]	0	0	8	0
Unvaccinated case, age younger than 9 months[c]	3	2	0	0
Outbreak immunization response recipient[c]	2	1	0	0
Collected during outbreaks in endemic settings[c]				
Unknown vaccination status	21	13	0	0
Unknown date of rash onset[d]	0	4	0	0
Unvaccinated, unreliable information	6	0	0	0
Vaccinated with symptoms[e,f]	0	0	0	20
Collected during outbreaks in elimination settings[e]				
Unknown vaccination status[g]	0	0	0	29
Unknown date of rash onset[h]	0	0	0	4
Unknown date of collection (both vaccinated)	0	0	0	2
Total	32	20	8	55

466 [a] Samples for ROC analysis were distributed into: a) a recent exposure group with previously
467 unvaccinated measles IgM positive persons aged ≥ 9 months collected 0 to 12 weeks after their
468 first dose of measles, mumps and rubella vaccine (MMR1), or after rash onset: samples collected
469 0 to 3 weeks were used to select the low avidity threshold, and samples collected 0 to 12 weeks
470 were used to select the high avidity threshold, and b) a distant exposure group with samples
471 collected from IgM negative adults more than a year after natural measles or vaccination and
472 used to select both avidity thresholds.
473 [b] Samples for ROC analysis were distributed into: a) a recent exposure group with samples
474 described in [c]: samples collected 0 to 3 weeks were used to select the low avidity threshold, and
475 samples collected 0 to 18 weeks were used to select the high avidity threshold and b) a distant
476 exposure group with samples described in [f] was used to select both avidity thresholds.
477 Assumptions were made to use samples with uncertain epidemiological information. Groups A
478 and B together make group C.
479 [c] Recent exposure group for group B.
480 [d] Vaccination status was unknown for three individuals and one was unvaccinated.
481 [e] Distant exposure group for group B.
482 [f] Samples were collected 0 to 50 days after onset of rash.
483 [g] Samples were collected -1 to 38 days after onset of rash.
484 [h] Vaccination status was unknown for three individuals and one was vaccinated.

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485 **TABLE 2** Evaluation of precision of a diethylamine-based measles IgG avidity assay

Control serum	Avg etAI%[a]	Days[b]	Lots[c]	S_r[d]	CV%[e]	S_T[f]	CV%[g]
High	88%	20	5	5.76%	6.55%	6.24%	7%
Intermediate 1	47%	8	3	5.41%	11.51%	6.51%	14%
Intermediate 2	46%	8	2	3.48%	7.56%	6.98%	15%
Low-Intermediate	28%	13	3	3.32%	11.85%	4.62%	17%
Low	17%	20	4	1.97%	11.62%	4.23%	25%

486 [a] End-titer avidity index %.

487 [b] Number of days that the control was tested, in duplicates; two runs were performed per day.

488 [c] Number of lots used.

489 [d] Estimate of repeatability standard deviation or within-run precision.

490 [e] Coefficient of variation of Sr (CV%=Sr/Mean x100).

491 [f] S_T is global precision estimate.

492 [g] Coefficient of variation of S_T (CV%=S_T/Mean x100).

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493 **TABLE 3** Diagnostic performance indicators of a diethylamine-based measles avidity assay

Sample group (avidity threshold)	Sensitivity[a]	Specificity[b]
A[c] (≤30%)	91.9 (83.2-97.0)	100 (94.4-100)
C[d] (≤30%)	84.9 (76.6-91.1)	95.0 (89.3-98.1)
A (≥70%)	99.3 (96.0-100)	98.4 (91.6-100)
C (≥70%)	96.5 (92.9-98.6)	86.6 (79.1-92.1)

494 [a] Sensitivity is the percentage of samples with low or intermediate avidity results among all
495 samples collected 0 to 18 weeks after exposure (95% confidence intervals).
496 [b] Specificity is the percentage of samples with high avidity results among all samples collected at
497 least 1 year after exposure (95% confidence intervals).
498 [c] Sample group A are 202 samples with good epidemiological records from persons aged ≥ 9
499 months. Sample collection after exposure was 0 to 12 weeks after first measles vaccine or rash
500 onset, or >1 year when collected from healthy donors.
501 [d] Sample group C include sample group A and 115 samples with uncertain epidemiological
502 information from persons aged ≥3 months. Sample collection ranged 0 to 18 weeks.

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503 **TABLE 4** Intermediate avidity results in sera collected from 9 to 15 month-old infants

504 within 3 weeks of their first dose of a measles-containing vaccine

Sample	Days after vaccination	etAI%[a]	Result
1	0[b]	71%	High
	6 to 22	55%	Intermediate
	28 to 45	27%	Low
	> 120	64%	Intermediate
2	0	78%	High
	6 to 22	54%	Intermediate
	28 to 45	44%	Intermediate
	> 120	74%	High
3	0	NT[c]	NT
	6 to 22	64%	Intermediate
	28 to 45	78%	High
4	0	NT	NT
	14	51%	Intermediate

505 [a] etAI% is end-titer avidity index

506 [b] Pre-vaccine collection

507 [c] Not tested; negative IgG result

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508 **TABLE 5** Measles avidity results for outbreak samples collected in endemic and elimination

509 settings with unknown vaccination status

Result	Endemic settings				Elimination settings			
	Weeks after rash onset			Total (%)	Weeks after rash onset			Total (%)
	≤ 3	4-6	Unknown[a]		≤ 3	4-6	Unknown	
Low	14	9	1	24 (65)	3	1	0	4 (12)
Intermediate	4	4	2	10 (27)	6	0	1	7 (22)
High	3	0	0	3 (8)	17	2	2	21 (66)
Total	21	13	3	37 (100)	26	3	3	32 (100)

510 [a] Unknown date of rash onset.

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511 **TABLE 6** Interpretation and application of measles avidity results obtained in the absence of

512 vaccination within 10 days of rash onset, and samples collected within the first 8 weeks.

Serology		Clinical and epidemiological information			Application
Avidity	IgM	Symptoms	Exposure	Infection history	
Low[a]	P[b] or N[c]	Classic	Wild-type	Unvaccinated; no history	Confirm as measles
Low	P or N	Classic	Wild-type	Unvaccinated; history[d]	Confirm as measles
Low	P	Classic	Unknown	Vaccinated[e]; no history	Confirm as measles
Low	P	Classic	Wild-type	Vaccinated	Classifies as PVF
High[f]	P or N	Classic	Wild-type	Vaccinated	Classifies as SVF
High	P or N	Modified	Wild-type	Vaccinated	Classifies as SVF
High	N	Fever; rash	Recent MMR2[g]	Vaccinated	Confirm previous exposure
High	P or N	Modified	Wild-type	History[h]	Confirm previous exposure

513 [a] Low avidity is interpreted as primary immune response. Diagnostic for acute classic measles.

514 [b] Positive result. IgM is usually positive from day 3 to 28.

515 [c] Negative result. Avidity testing extends up to 8 weeks the opportunity to identify cases.

516 [d] May have been misdiagnosed initially. Consider contagious.

517 [e] Vaccination in the distant past, at least 1 year.

518 [f] High avidity is interpreted as secondary immune response.

519 [g] Symptoms are side effects from second MMR vaccine dose.

520 [i] Rare event. Do not consider contagious.

521

522 **FIGURE LEGENDS**

523 **FIG 1** ROC analysis of a diethylamine-based measles avidity assay using (A) samples with good
524 epidemiological records, collected from persons aged ≥ 9 months either 0 to 12 weeks, or greater
525 than one year after vaccination or rash, (B) samples with uncertain epidemiological information,
526 collected from persons aged ≥3 months; some assumptions were made for 99 samples, and (C)
527 combined samples described in (A) and (B). AUC is area under the ROC curve and is plotted as
528 a solid line. Diagonal line is AUC=0.5, interpreted as random guess. 95% confidence intervals
529 are in parenthesis and are plotted as dashed lines. Circle indicates point with maximal accuracy.

530

531 **FIG 2** Diethylamine-based measles avidity assay: box-and-whisker analysis of results over three
532 time intervals from (A) samples with good epidemiological records, collected from persons aged
533 ≥ 9 months; (B) samples described in (A) and samples with uncertain epidemiological
534 information, collected from persons aged ≥3 months; some assumptions were made for 99
535 samples. Asterisks indicate outliers within sample group A. Open symbols are values smaller
536 (larger) than the lower (higher) quartile minus (plus) 1.5 times the interquartile range. Solid
537 symbols are values smaller (larger) than the lower (higher) quartile minus (plus) 3 times the
538 interquartile range. Low avidity threshold is 30%. High avidity threshold is 70%.

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Figure 1

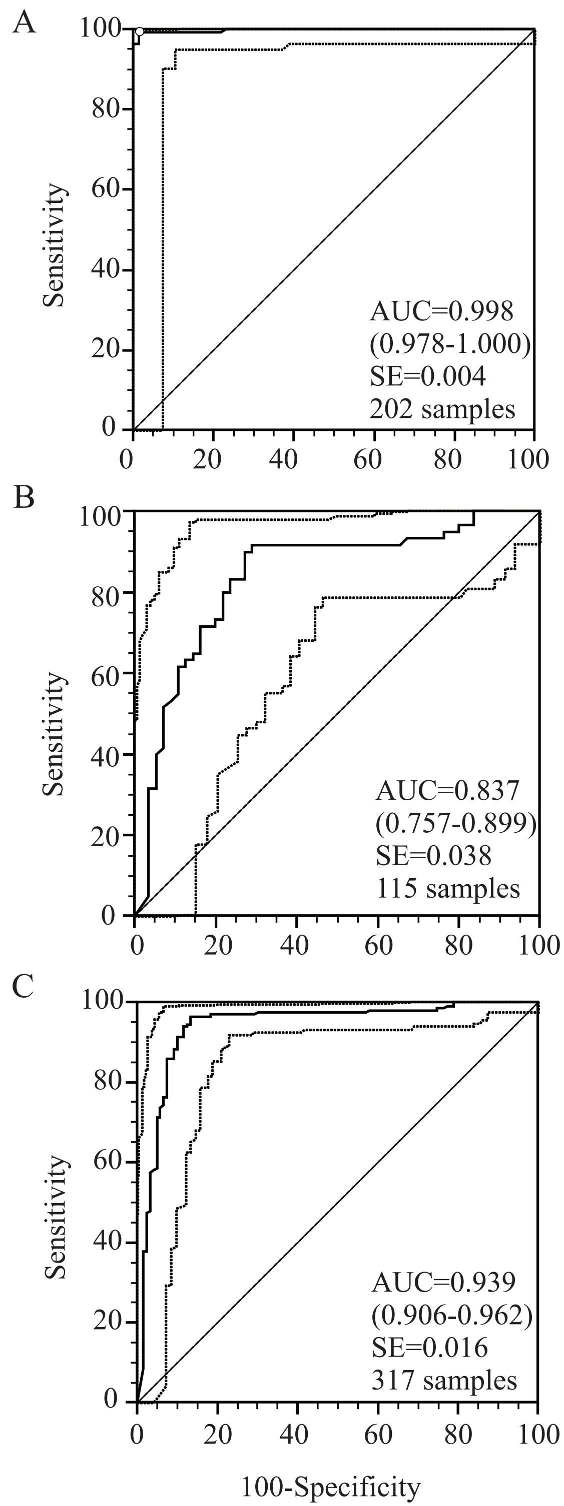

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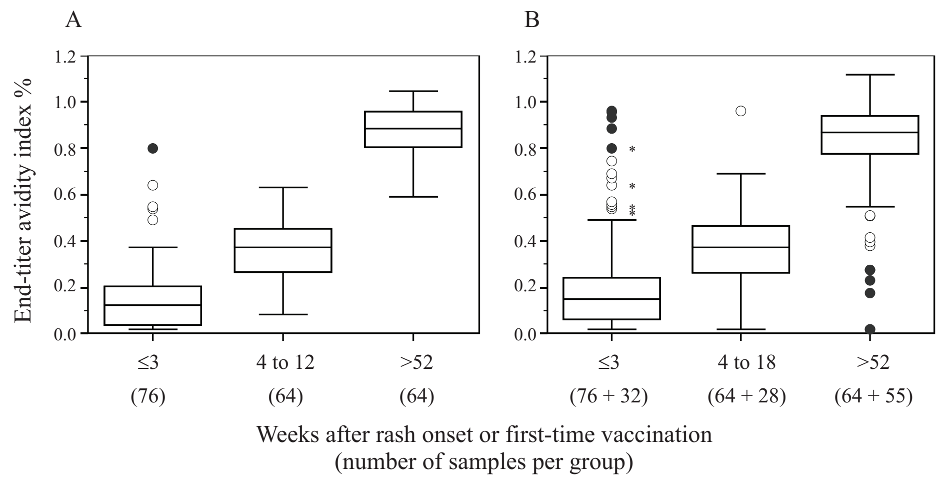
Figure 2



SUPPLEMENTAL MATERIAL

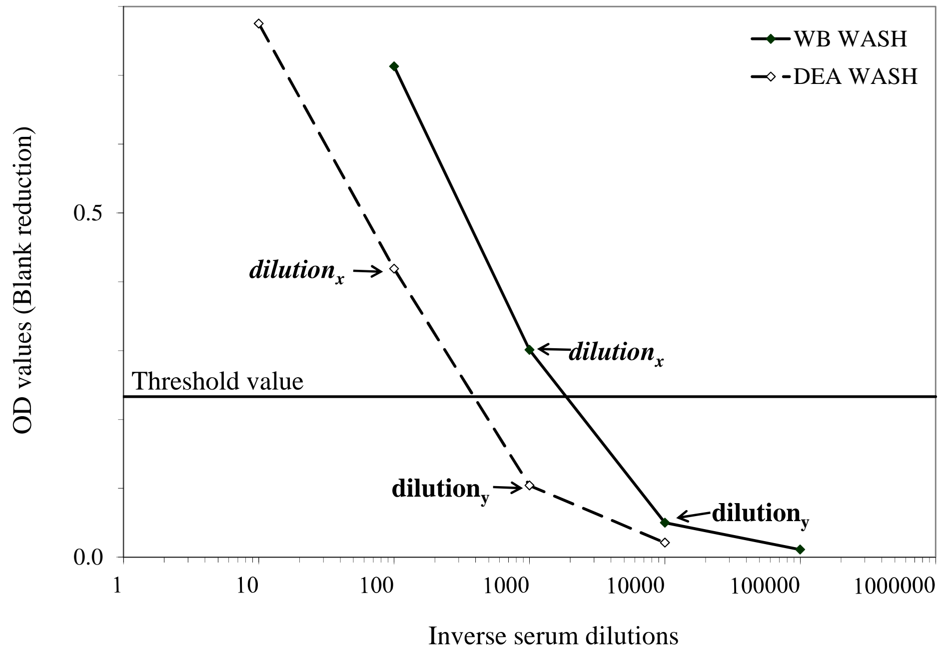


FIG S1 Modified formula to calculate end-titer avidity index percentages (etAI%) (22). Dilution series washed either with wash buffer or diethylamine depicted for reference. OD is optical density value.

etAI% = (*end-titer* diethylamine curve / *end-titer* wash buffer curve) X 100,

where

end-titer= $\text{dilution}_x^{-1} \text{x} 10^{a}$

where

$a = \log 10 \text{ X } (OD_x - OD\ threshold\ value)/OD_x - OD_y)$

where

10 is the dilution factor

OD_x is the optical density value at dilution_x

OD threshold value is 0.9 X calibrator value as described in kit's insert

OD_y is the optical density value at dilution_y

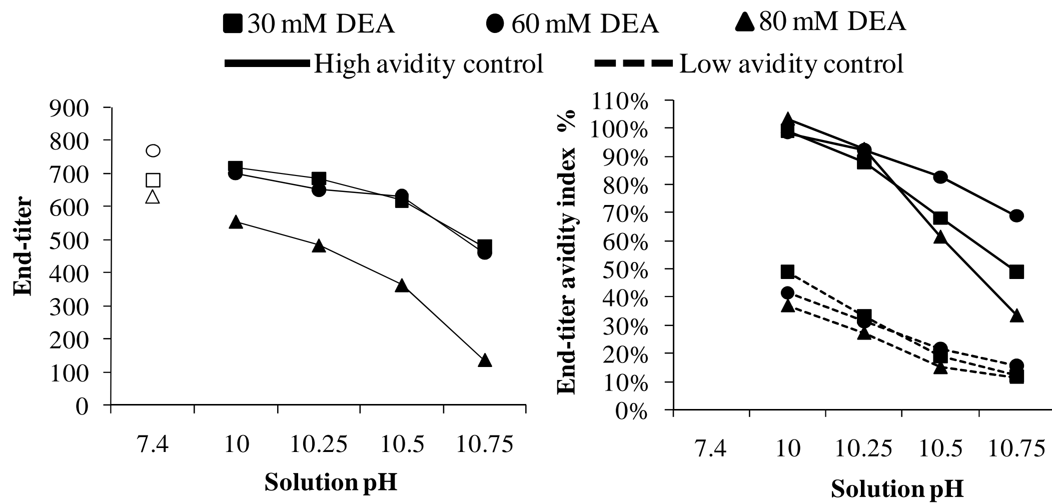


FIG S2 Effect of diethylamine (DEA) molarity and pH on binding of coating measles virus antigen (left panel) and on binding of measles-specific antibodies (right panel). Open symbols are treatment with wash buffer. Solid symbols are treatments with DEA solutions at specified molarities. Original pH for these solutions was >11. The pH of the DEA solution increases with molarity. In preliminary experiments, the effect of higher pH values and higher molarities was already observed. A solution of 8.75mM DEA (pH 9.3) was not effective in eluting antibodies expected to be of low avidity, while a solution of 17.5mM DEA (pH 10.9) consistently washed away high avidity antibodies. A solution of 60 mM DEA adjusted to a pH of 10.25 (±0.1) was selected. In our hands, 6M, 7M, and 8M urea destabilized the antigen and resulted in loss of optical density signal (data not shown).

Avidity results based on a single dilution

Materials and methods: Comparison of single serum dilution and serial serum dilutions was performed using all samples in groups A and B (Table1). Single serum dilution points at 1:100 (n=154) and 1:10 (n=53) were taken out of the serum dilutions curves used to calculate etAI%. Avidity results were calculated as avidity index (AI%):

AI%= (*OD* after diethylamine wash / *OD* after wash buffer wash) X 100,

where *OD* is optical density at the selected dilution (either 1:100 or 1:10)

The AUCs were compared using MedCalc for Windows, version 8.1.1.0 (MedCalc Software, Belgium. Microsoft® Office EXCEL 2003 was used to calculate coefficients of determination (R^2). *Results:* The AUCs were not significantly different (P=0.763). The AUC at dilution 1:100 was 0.959 (95%CI: 0.914 to 0.984). At dilution 1:100, R^2 was 0.889 and at dilution 1:10 R^2 was 0.918. At 1:100, a threshold greater than 80% lead to an assay specificity of 95.08% (95%CI: 86.3-98.9) and sensitivity of 94.62% (95%CI: 87.9-98.2). *Conclusion:* In those situations where time or resources are limited, this avidity assay could be performed using a single dilution. An AI% threshold should be established for the serum dilution (1:20) that is used in the Captia™ assay protocol.

Interpretation of intermediate avidity results

Intermediate avidity results are generally uninterpretable, but can be used in two situations. First, except for underlying conditions that may result in potentially slower IgG avidity maturation (as in HIV-infections, Down syndrome, or organ transplant recipients) intermediate avidity results in samples collected 4 to 8 weeks after rash onset from unvaccinated individuals suggest recent infection with measles virus (S1, S2, 27). To confirm this possibility, high avidity antibodies should be detected in a second sample collected a few weeks later. Second, when investigating SVF with elevated PRN titers, intermediate avidity results can be interpreted as suggestive of secondary immune responses, since low avidity results were not observed in samples collected from persons with long-standing immunity. Noteworthy, intermediate avidity results have been obtained in a healthy individual born during the pre-vaccine era, in recently vaccinated persons with rash and fever illness, and in young infants with classic measles with possible interference of maternal antibody.

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RESULTS AND DISCUSSION

This dissertation includes three papers describing tools that can assist in confirming measles in the elimination setting. The results of these papers relate to I) implementation of DBS use in elimination settings (Paper 1), and II) complementary diagnostic approaches to enhance measles case classification (Papers 2 and 3).

I. IMPLEMENTATION OF DBS USE IN ELIMINATION SETTINGS

The first source of information for the laboratory confirmation of measles is the specimen collected from the suspected case. In fact, the choice of assay and quality of results will depend on type, quality, quantity, and the amount of time that has passed since rash onset of the specimen received at the laboratory. In elimination settings, where surveillance is case-based, both serology and molecular virology are necessary to confirm or discard suspected cases, and to genotype confirmed cases for molecular epidemiology. Single specimens amenable for use in antibody and RNA detection methods are attractive because they would increase the laboratory's capability to classify cases, especially in those areas where obtaining both blood and viral specimens may be challenging. Recently, the WHO has evaluated OF and DBS specimens for use in the confirmation of suspected cases[82, 83]. OF collected with the Oracol® (Malvern Medical Developments, Ltd, Worchester, England) collection device is a good candidate for serology, RNA amplification and genotyping[87]. OF samples are stable at 42°C for up to three days[82]. DBS can be stored longer without refrigeration. Stability studies report that measles IgG in DBS is stable for at least two weeks at room temperature, while measles RNA appears to be stable for two weeks at 45°C, for one

month at 37°C, and for at least six months at room temperature (20-22°C)[86, 128, 129]. Because MeV RNA is detected in only about a fourth of DBS samples (26% and 28% detection has been reported), the utility of DBS to confirm cases based on RT-PCR may be limited. However, DBS is a promising alternative to serum for serology and for tracking transmission pathways[126, 177].

A PubMed literature search was performed to find DBS elution protocols (Paper 1)[177] in preparation to collaborate with WHO in the evaluation of DBS. From this search, 74 articles were identified, where DBS were collected for serodiagnosis of infectious diseases. Overall, the described protocols for DBS elution were different enough to warrant a comparison that would help in the implementation of DBS technology. Paper 1 was designed to provide guidelines to laboratories and researchers in the selection of which DBS elution protocol to use. Of the protocols compared in Paper 1, protocols 3, 4 and 6 were used during the DBS evaluations that led to the WHO recommendation, while protocols 1, 2 and 5 were not used. The paper provides a description of these six protocols as well as a thorough cross-examination based on diverse criteria, and establishes that all protocols have similar performance based on the results obtained in measles-specific IgM and IgG assays. One limitation of the study is the use of a small number of control samples prepared in the laboratory, rather than clinical samples. It could be argued that the results may not be applicable when real samples from measles cases are used. Unfortunately, DBS samples from measles cases were not available to perform an appropriate comparison of the described elution protocols. However, as observed in Appendix I, the performance for IgM detection in DBS eluates extracted using protocols 1, 2, 3 and 6 in independent studies was high, with Se values ranging from 90% to 100% and specificity values ranging from 96% to 99%. Agreement between DBS and serum was at least 97%. Protocol 6 was used to elute IgM

antibodies in the evaluation of DBS as part of the collaborative project with WHO[177]. Paper 1 found that protocol 6 was the most advantageous procedure for outbreak situations, because it featured a rapid removal method with recovery of ample specimen. In elimination settings, the availability of ample volume may become important in differential diagnosis of rash illnesses and in the combination of laboratory procedures for measles diagnosis, e.g., IgM detection followed by IgG avidity testing. In fewer than eight hours, 10 DBS specimens received in the laboratory from remote outbreak areas can be eluted by protocol 6 and assayed by the measles-specific IgM Dade Behring Enzygnost® (Siemens Enzygnost®) EIA. Furthermore, implementation of protocol 6 in serological laboratories should be seamless, because the required equipment is readily available.

Regardless of how antibodies are eluted, DBS are adequate to detect measles IgM and IgG (Appendix I)[82, 83]. In detecting IgM for measles case confirmation, all protocols described in Paper 1 are suitable for measles-specific antibody detection. In Paper 1, statistical differences were detected in IgM detection when passive elution protocols (protocols 1 and 2) were used. However, the comparatively lower optical density values did not lead to misclassification of results. Unfortunately, borderline positive DBS samples were not available for investigation to show whether or not lower optical density values could become a problem in misclassifying results. Nevertheless, protocol 1 was used by Helfand *et al*. and found that IgM detection in DBS eluates agreed 98% with the cognate serum specimen; this agreement level was similar to that observed when active elution protocols (Protocols 3, 4 and 6) were used[132]. Similarly, De Swart *et al.* used protocol 2 and observed Se and Sp values that were in the range of those obtained using active elution protocols (Appendix I)[129]. If equipment can be afforded, protocols 3, 4, and 6 are preferable over protocols based on passive elution, or over protocol 5. Similar results were obtained for IgG

detection, as observed in two studies that used protocols 2 and 3; minimum Se was 98% and minimum Sp was 97%. Therefore, DBS are good serum alternative specimens for measles-specific IgG detection. As an example, detection of measles-specific IgG in DBS eluates was recently used to study seroprevalence in Papua New Guinea as a step toward measles elimination in the region[178]. Although the use of OF and DBS still needs to be evaluated in elimination settings, these collection systems should be considered for assays that are becoming useful in these settings. For instance, avidity and PRN assays could be adapted and validated for use with OF and DBS, in a similar fashion to the attempts made in rubella diagnosis[149]. For now, OF collection has been used to enhance measles diagnosis in Finland, where measles was eliminated in the mid-1990s[179].

II. COMPLEMENTARY DIAGNOSTIC APPROACHES TO ENHANCE MEASLES CASE CLASSIFICATION

The laboratory confirmation of measles in elimination settings and subsequent case classification is like puzzle solving. The complexity of these puzzles varies with each case and depends on the pieces of information that are available in relation to clinical symptoms (e.g., classic versus modified measles), epidemiology (e.g., vaccination and exposure history, timing of sample collection since rash onset, and age), and laboratory results. The majority of cases are straightforward because they occur among unvaccinated exposed persons, who will present with classic measles symptoms: the easily solved puzzles. Such suspected cases with classic measles rash and fever are readily confirmed by detection of IgM antibodies or MeV RNA in good quality samples that have been collected in a timely manner. Diagnosis becomes complicated when the pieces of information are not available or are unreliable. In clinical diagnosis, modified disease with very mild fever or uneven or atypical rash distribution may not be recognized as measles, and samples may not be collected for confirmation, or may be collected too late (Appendix II)[180]. In terms of epidemiologic data,

vaccination or exposure status may not be known or the information simply cannot be gathered. In terms of laboratory results, the selected assay(s) may have inherent limitations that may lead to false positive or negative results (IgM, PCR assays), or may not help resolve every single case. Therefore, a combination of assays may be needed to provide reassurance about the final resolution of the suspected case. Typically, these assays would include a combination of EIAs to detect measles-specific IgM and IgG antibodies, and conventional and real time RT-PCR[91, 92]. However, these recommended assays are not sufficient to resolve all suspected cases, especially suspected cases presenting with modified disease or sporadic cases with unknown exposures and unknown vaccination status.

Papers 2 and 3 introduce the use of PRN and IgG avidity testing as additional specialized tools to diagnose measles in elimination settings. Paper 2 establishes high avidity PRN titers greater than 30,000 mIU/mL as a possible biomarker to identify SVF cases. These characteristically high PRN titers were in some cases six to 60 times higher than the PRN titers typically observed in convalescent serum, or in healthy vaccinated persons, and were observed in vaccinated measles cases with modified disease in the Republic of Marshall Islands and in the United States. Although the PRN titer before MeV infection was not known, detection of high avidity antibodies demonstrated that a secondary immune response rapidly amounted to levels much higher than expected. However, the PRN titer threshold of 30,000 mIU/mL is based on the observed data during the outbreaks described in Paper 2 and should be considered as a temporary PRN threshold until fully validated. Rota *et al.* described three SVF cases with modified disease and PRN titers that ranged from 168,640 to 248,628 mIU/mL, results similar to those observed in Paper 2. One was IgM negative for measles, while the other two were IgM positive. The serum of these cases contained high avidity antibodies measured by the assay described in Paper 3 and Appendix II[180]. Additionally,

Chen *et al*. described two previously vaccinated cases with PRN titers of 101,339 and 44,661 who had milder measles disease with three days of rash, fever and three measles symptoms[57]. One of these cases was IgM positive with an IgM PRN titer of 662, while the other had an IgM PRN titer of <48, but it is not clear in the information provided in that paper whether the latter was positive or not. Others in highly vaccinated settings have detected elevated IgG titers by EIAs based on whole MeV particles as antigen[181, 182]. IgG detected by these assays is targeted to the MeV's internal and surface proteins (H and F); therefore, a direct comparison with PRN titers cannot be made. Van den Hoek *et al.* detected IgG titers of 36,000 and 140,000 mIU/mL in two IgM negative SVF cases with modified measles, and De Serres *et al.* found that 10 of 12 SVF cases with modified disease had IgG titers that were ≥13,000 mIU/mL; unfortunately, IgG titers for these cases were not presented. It is important to note that avidity testing was not used to classify vaccine failures[57, 181, 182]. Instead, SVF cases were identified on the basis of epidemiological link, IgM and RNA detection, or IgG titer increase in paired samples. Detection of high avidity antibodies would have verified the development of secondary immune responses and provided reassurance in the SVF classification. In contrast to the observations in Paper 2, Chen *et al.* also describe two vaccinated cases with PRN titers of 35,363 and 39,268 who had more severe measles disease with six days of rash, fever and six and five measles symptoms, respectively. Again, detection of high avidity antibodies would have verified the development of secondary immune responses and classified these as SVF cases, while detection of low avidity antibodies would have classified the cases as PVF. Although these two cases could in fact still be SVF cases, the possibility that they were not classified as such further proves the need to gather more information about new suspected SVF cases in order to validate the diagnostic performance and utility of high avidity elevated PRN titers as a tool to identify SVF cases. Similar evaluations on the use of elevated EIA IgG titers could also be performed. The identification of SVF would be

expedited if detection of elevated EIA IgG titers was found to be superior to detection by the PRN assay. ROC analysis could help in the determination of cut-off values and the estimation of accuracy, Se and Sp. A sufficiently large number of serum specimens would have to be analyzed to distinguish SVF from uninfected vaccinated individuals. Sample size to perform the ROC analysis could be calculated based on data from a pilot study[164]. This pilot study could consist of two groups of serum specimens: a measles SVF group consisting of specimens from exposed vaccinated individuals with modified measles and confirmed by routine laboratory methods, and an uninfected vaccinated group consisting of healthy individuals with a record of two-dose vaccination. All serum specimens would then be analyzed by avidity, PRN and IgG EIA testing, appropriate cut-offs established for avidity determination, and for PRN and EIA IgG titers. However, the number of measles cases in elimination settings is extremely low, and the number of suspected SVF cases is even lower, making it difficult to gather a sufficient number of cases for the analysis. A way to circumvent this problem would be to establish collaborations with regions where measles has been eliminated and modified measles has been observed.

The avidity assay presented in Paper 3 differentiates primary from secondary immune responses. Next is a discussion of the development and validation of this avidity assay, followed by a discussion on its applications in elimination settings.

1. DEVELOPMENT OF A MEASLES AVIDITY ASSAY

During development, DEA and urea were considered to be possible denaturing agents for the assay. Treatments with urea were at concentrations of 6M, 7M and 8M for 10 minutes followed by three washes with wash buffer, all at room temperature. Results indicated that urea caused damage to the coated antigen since detection of optical density signals was

negative after urea treatment, but not after treatment with wash buffer. Apparently, a solution containing 6M urea would distinguish between low and high avidity antibodies. However, damage to the coated antigen appeared to be partially responsible for the loss of signal in samples collected recently after infection. Therefore, a decision was made not to use urea (Figure 9). Use of DEA was explored because of previous experience with a rubella avidity assay, and because DEA had been used by others at concentrations 20mM and 70mM[53, 183].

The antigen of the measles avidity assay in Paper 3 is whole MeV particles in which the most abundant and antigenic protein is the N protein, which is also the most antigenic protein. The pI of N is 7.2 in denaturing conditions, and between 6 and 7 in non-

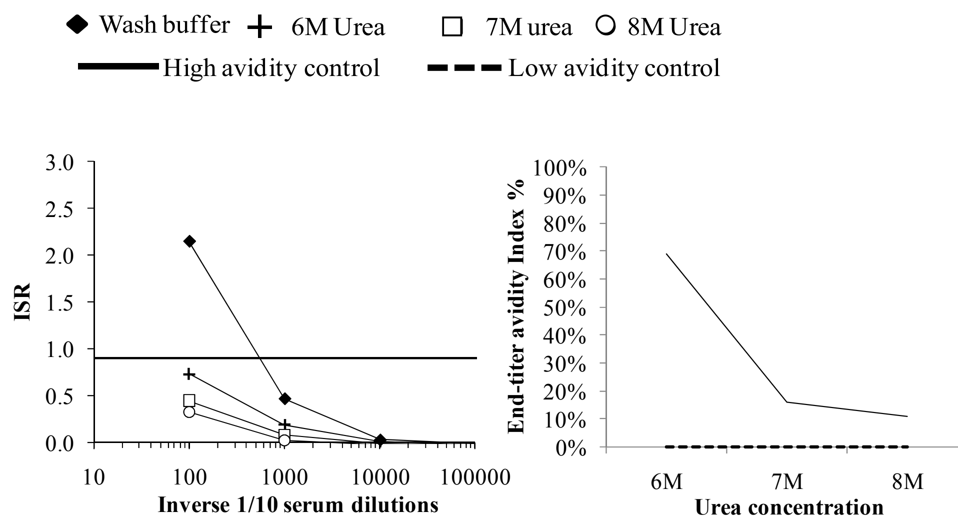

Figure 9: *Effect of urea molarity on binding of coated measles virus antigen (left panel) and on binding of measles-specific antibodies (right panel). A high avidity control serum was used for detection. Urea at the specified molarity. ISR is calculated per manufacturer's instructions to normalize results. Horizontal line is threshold for IgG positivity (left panel).*

denaturing conditions[184, 185]. Because of this pI, the nucleoprotein has more negative charges at pH 11 than at pH 7, and this difference may interfere with binding on the microtiter plate, resulting in lower optical density values. This effect is already observed at pH 10.75 (Paper 3, Figure S2). It was hypothesized that a DEA solution adjusted with a pH between 10.00 and 10.75 would not disrupt antigen interactions to the plate. This hypothesis was tested and a solution of 60mM DEA in wash buffer at pH 10.25 (±0.1) was selected.

2. EVALUATION OF DIAGNOSTIC PERFORMANCE

Paper 3 describes a highly sensitive and specific measles IgG avidity assay (Table 3 in paper). Regarding Se, the assay was able to detect low avidity antibodies in 68 of 74 (92%) samples collected from acute measles cases (or first time vaccinees) during the first three weeks after rash onset (or vaccination). One sample with a high avidity result was included in the estimation of Se. This was an outbreak sample collected five days after rash onset from a 9-month–old infant. This sample was considered to contain maternal IgG antibodies because a result of a low avidity was obtained in a follow-up sample collected at day 11. Additionally, three intermediate avidity samples that had a low avidity result upon retest (Table 4 in paper) were included. The Se of the assay within the first three weeks increased to 97% (71/73) when the one sample with maternal antibodies was excluded from analysis and the results of low avidity were considered for the three retested samples. The assay did not detect high avidity antibodies in 139 of 140 (99%) samples collected within the first 12 weeks after rash onset (or vaccination). This Se increased to 100% (139/139) when the one sample with high avidity maternal antibodies was excluded.

Regarding assay Sp, the assay did not detect low avidity antibodies in any of the samples collected from persons who were exposed to measles in the distant past; assay Sp

was 100% at the low avidity cut-off. The assay detected high avidity antibodies in all but one sample; assay Sp was 97% at the high avidity cut-off. Therefore, the Sp of the assay to detect secondary immune responses is high, although intermediate results are possible. At this point, intermediate avidity results are considered generally uninterpretable. There are not sufficient data for a full understanding of the meaning and ultimate value of an intermediate avidity result. However, intermediate avidity results may help in the investigation of SVF with modified disease and elevated PRN titers. Because the Sp at the low avidity cut-off is 100%, an intermediate result in this context indicates with assurance that a secondary (and not a primary) immune response is observed (Paper 3, Supplemental Material). Finally, during the performance validation of the DEA-avidity assay, a ROC analysis was performed to include samples from cases that were not easily classifiable into the recent and distant exposure groups (Group B samples). This validation helped realize that the assay loses diagnostic power if pieces of clinical or epidemiological information are not available.

There is at least one commercial measles avidity assay (Euroimmun, EUROIMMUN AG, Germany) that is available in Europe and the United States, but a literature search was not fruitful in finding an external evaluation for this assay or any others. Commercially available measles avidity assays could easily be introduced to laboratories that routinely perform EIAs, thus eliminating the need to ship specimens for further testing. To this end, the Wampole measles IgG EIA (Wampole Laboratories, Inc., Princeton, NJ, USA) was compared to the Captia measles IgG EIA following the avidity method presented in Paper 3. Wampole avidity results correlated with those obtained using the Captia kit (Figure 10). However, avidity result thresholds would have to be established and diagnostic performance evaluated on the Wampole-based measles avidity assay before use.

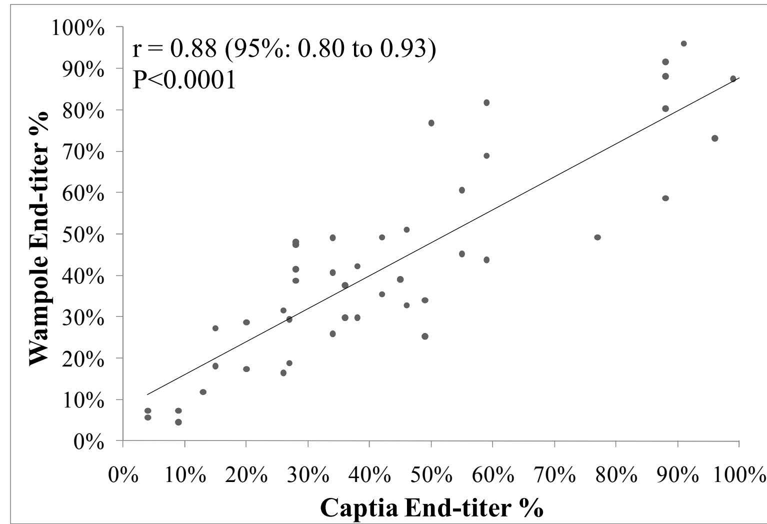

Figure 10: *Comparison of the determination of measles avidity by the adapted Wampole measles IgG EIA and Captia measles IgG EIA, using DEA as a denaturant. A total of 47 serum samples were analyzed following the protocol described in paper 3. Pearson correlation coefficient estimated using MedCalc for Windows, version 8.1.1.0 (MedCalc Software, Mariakerke, Belgium).*

III. MEASLES AVIDITY TESTING IN ELIMINATION SETTINGS

The significance of avidity testing in the confirmation of suspected measles cases depends on the patient's age, vaccination status, and level of exposure; the timing of sample collection; and the overall symptoms of the patient. The three main uses of avidity testing are**:** (a) Classifying vaccine failures; (b) Providing reassurance in results obtained by IgM assays; and (c) Resolving cases when an IgM assay is not available. Following are examples based on samples received at the CDC measles reference laboratory for verification of results and tested with a capture measles IgM EIA, an indirect measles IgG EIA, a DEA-based avidity assay (described in Paper 3), a real-time RT-PCR assay, and a PRN assay[81, 105, 109, 186].

1. CLASSIFYING VACCINE FAILURES

Avidity testing allows distinguishing between PVFs (low avidity detection) from SVF cases (high avidity detection). This is important for two reasons: a) to verify secondary immune responses in SVF cases with elevated PRN titers; and b) to understand outbreak dynamics:

a) To verify secondary immune responses in SVF cases with elevated PRN titers

Paper 2 describes a new approach to identifying SVF cases through the detection of high avidity elevated PRN titers, which makes it possible to confirm cases that otherwise would not have been confirmed with current laboratory methods (Tables 1 and 4 in paper). Specific examples of this approach can be found in Rota *et al.* with the description of an outbreak that occurred in Pennsylvania in 2009 (Table 7, cases 4 and 5) (Appendix II)[180]. During this outbreak, it was possible to confirm a SVF in a suspected case with modified disease, high avidity results, and PRN titers as high as 248,628 and 206,580 mIU/mL (Table 7, case 5). With routine methods, this case would have been missed because IgM and RT-PCR results were inconclusive.

Interestingly, this case involved the physician who examined the index case. Because the physician had previously received three doses of vaccine and the symptoms were not those of classic measles, the (infected) physician continued visiting patients during the most infectious stage. However, MeV was not transmitted to any of the patients that were visited, including many unvaccinated infants.

Table 7: *Characteristics of measles cases in an elimination setting, Pennsylvania (United States) 2009 (Appendix II)*[180].

Case	Age	Symptoms	Vaccine[1]	Days	IgM[2]	Avidity[3]	PRN[4]	RT-PCR[5]
1	11	Classic	Unknown	24	P	L	3,644	nd
2	2	Classic	No MMR	3	P	IgG N	2,332	P
3	5	Classic	No MMR	7	P	L	9,503	P
4	34	Modified	1 MMR	4	P	H	168,640	Nd
5	37	Modified	3 MMR	6	I	H	248,628	Nd
				20	N	H	206,580	
6	4	None	Unknown	no rash	N	H	15,035	Nd
7	6	None	Unknown	no rash	N	H	2,173	Nd
8	8	None	Unknown	no rash	N	H	853	Nd

[1] Vaccine is vaccine record: MMR is measles, mumps and rubella vaccine;

[2] Measles-specific IgM results: P is positive; N is negative

[3] Avidity results: H indicates high avidity; L indicates low avidity

[4] PRN is Plaque Reduction Neutralization titer.

5 RT-PCR results: P is positive; nd is not done

b) To understand outbreak dynamics

The ability to classify vaccine failures has allowed for an understanding of the dynamics of outbreaks, and to identify the need to have a second dose of vaccine. About 2-5% of the population vaccinated with a single dose of measles vaccine will not seroconvert[50,102,150,187,188]. PVF can also occur if, for instance, there is a problem with a vaccine batch, if the cold chain was broken, or if there was a problem during manufacture. Today, the two-dose measles vaccine schedule makes PVF a very rare event. However, PVF

cases may still occur and avidity testing can help identify them and alert of possible spread. For example, low avidity IgG antibodies were detected in a 2-year-old boy with a rash, fever, cough and anorexia who was originally suspected of having Dengue fever due to travel to a country endemic for this disease. Measles-specific IgM and IgG seroconversion were detected in serum, thus confirming the case. A result of low avidity IgG provided reassurance that this was an acute case in a previously vaccinated person, a PVF. This case highlights the need to classify vaccine failures in persons vaccinated with only one dose of measles vaccine. This is especially important in young children, who may be attending childcare centers and may spread disease among infants who are too young to be vaccinated. On the other hand, the ability to better identify SVF cases will provide better data to investigate the role of SVF in spreading measles during outbreaks (Paper 2).

1. PROVIDING REASSURANCE IN RESULTS OBTAINED BY IgM ASSAYS

Avidity testing can provide reassurance in positive IgM results with the use of a) low avidity results; and b) avidity maturation:

a) Use of low avidity results

Low avidity results provide reassurance that IgM positive results are true. This is especially important in areas with extremely low measles prevalence, because many laboratories are uncomfortable confirming cases on the basis of IgM results only. For instance, a man born in 1946 presented to the clinic with fever, maculopapular rash and coryza. Measles was suspected and a sample was collected 16 days after rash onset. Serology results were IgG and IgM positive. Because of measles high transmissibility, immunity to measles is assumed in persons born before the pre-vaccine era. A false IgM positive result was suspected. A result of low avidity provided reassurance that the IgM positive result was

true, thus helping confirm this sporadic case of measles. For another example, see case 1 in Table 7.

b) Use of avidity maturation

Avidity maturation could be used to investigate unvaccinated suspected cases with samples collected four weeks after rash, when the IgM result may be falsely negative. In two independent reports, Tuokko and El Mubarak described the timing of transition from low to high avidity occurring within the first three months after exposure; their evaluations used measles IgG avidity assays based on urea and/or assay formats different from the avidity assay described in Paper 3[101, 153]. Because the assays were different, avidity maturation was also studied using the DEA avidity assay and data from Paper 3 groups A and B samples (N=223). Samples excluded from the analysis were three samples with demonstrated maternal antibody, 37 samples from SVF cases, 55 samples with insufficient epidemiological information, and three samples from individuals who had had a vaccine dose administered within 35 days prior to rash onset. Timing of sample collection was weeks 1 to 11 after measles rash onset, and weeks 2 to 18 after the first dose of measles-containing vaccine. Avidity of measles-specific IgG gradually increased with timing of sample collection (Figure 11). Therefore, the DEA-avidity assay is able to detect avidity maturation. Among outbreak cases, Se and Sp were high at the low and high avidity cut-offs (Table 8). All samples collected within the first 18 weeks resulted in low or intermediate avidity results. After the first three weeks of exposure, the percentage of samples with an intermediate avidity result was higher (Chi-square for the comparison of two proportions; $P<0.0001$) in vaccinees (83%, 48/58) than in infected individuals (10%, 2/20) (Table 9). Twenty samples with undetectable IgG after DEA treatment (20/220) were all collected within the first three weeks after rash onset (9 samples) or vaccination (9 samples), except for two samples that were obtained from

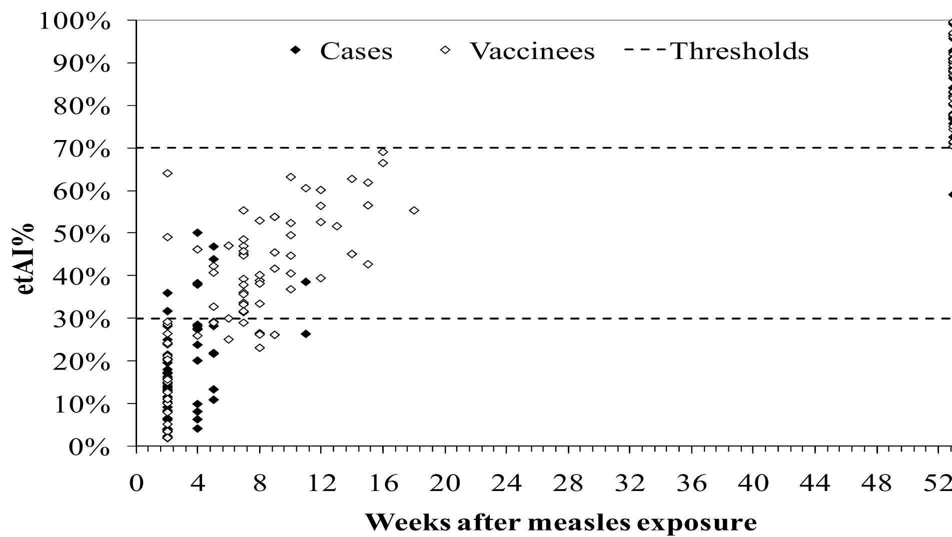

Figure 11: *Avidity maturation of measles specific IgG in measles cases and vaccinees as detected per a DEA-based avidity assay for measles. etAI% is the end-titer avidity index %. Weeks 0 to 3 are shown as a single group at week 3. Samples collected > 1 year after exposure are shown as a single group at week 52.*

a 3-month-old (week 5) and an 8-month-old (week 7) infant. The observation has been made that 6-month-old infants have deficient antibody responses to measles vaccine compared to older infants[156, 189].The apparently slower avidity maturation of these two infants could be related to immaturity of humoral responses to measles antigens. Another explanation would be that there are underlying conditions in these infants that could have caused a delay in IgG avidity maturation. For instance, persons with certain conditions such as HIV infection or Down syndrome, or recipients of an organ transplant, have slower IgG avidity maturation[190-192]. However, data on underlying diseases were not available; therefore, it was not possible to investigate further.

Table 8: *Sensitivity and specificity in acute measles cases*

Timing of collection	≤ 3 weeks	3 to 11 weeks
Nr[1]	49	20
Nd[2]	32	32
$Se^{3}_{\leq 30\%}$ (95% CI)	94% (83%-99%)	90% (63%-99%)
$Sp^{4}_{\leq 30\%}$ (95% CI)	100% (89%-100%)	100% (89 %-100%)
$Se_{\geq 70\%}$ (95% CI)	100% (93%-100%)	100% (83%-100%)
$Sp_{\geq 70\%}$ (95% CI)	97% (84%-100%)	97% (84%-100%)

[1] Nr: Number of samples from recent measles cases in unvaccinated individuals
[2] Nd: Number of samples from healthy individuals with a distant history of measles
[3] Se: Sensitivity at the indicated cut-off. Probability of detecting low avidity (≤30%) or intermediate avidity (≥30% and ≤70%) in recently infected naive individuals.
[4] Sp: Specificity at the indicated cut-off. Probability of detecting high avidity (≥70%) in healthy individuals with a history of measles.

The study in Paper 3 was designed to evaluate the overall performance characteristics of the measles avidity assay, rather than evaluate the time frame when antibodies acquire high avidity, or explore the differences in avidity maturation after wild-type infection or vaccination. However, the data confirm previous findings indicating that high avidity occurs sometime after the third month of exposure, possibly after the fourth month[153, 193]. It is important to note that low avidity antibodies were detected up to 12 weeks after rash onset, opening the possibility of confirming IgM negative cases in samples collected late. For instance, an IgM negative serum from an unvaccinated 8-month-old suspected case was

Table 9: *Avidity results before and after week 3 of rash onset or vaccination.*

		Avidity				
		Low		Intermediate		
Group	Weeks	n	%	n	%	Total
Cases (n=69)	≤ 3	46	94%	3[1]	6%	49
	3 to 11	18	90%	2	10%	20
Vaccinees (n=88)	≤ 3	28	93%	2[2]	7%	30
	3 to 11	10	22%	36	78%	46
	3 to 18	10	17%	48	83%	58

[1] Of these three samples, one had a paired sample obtained 5 days earlier (7 days after rash onset) with an avidity value of 26%.
[2] Paired pre-vaccine samples were IgG negative.

collected six weeks after rash onset as part of an outbreak investigation. A low avidity result confirmed acute measles. In a second example, avidity maturation was applied to resolve a suspected case that occurred in a childcare center. The 17-month-old patient presented with rash and fever, and had been vaccinated for the first time three months earlier. The patient's serum sample collected five days after rash was positive for IgM and for IgG measles-specific antibodies at the local laboratory. The serum was IgM positive by two different assays. At CDC, the IgM result was negative and the avidity result was intermediate avidity

(etAI values of 54% and 67%, duplicate test), indicating that the child did not have measles. Based on the maturation of measles IgG per the avidity assay, the intermediate avidity result was concordant with measles IgG maturation from immunization three months earlier. It also indicated that the results obtained by the other assays were falsely positive. Because this suspected case attended childcare, the ability to discard the case using information from avidity testing together with a negative IgM result provided reassurance that there was no need to initiate a costly measles outbreak investigation. No further suspected cases were reported from that childcare center.

1. RESOLVING CASES WHEN AN IgM ASSAY IS NOT AVAILABLE

Commensurate with the dwindling number of measles cases in elimination settings, commercially available measles IgM assays as well as the means to validate them may decline. For instance, only two measles IgM assays are approved for *in vitro* diagnosis in the United States. The cost per kit is $450, with a typical shelf-life of six months; therefore, it is very expensive to maintain stocks of kits. On the other hand, because of the decline in the number of cases, it has become very difficult to collect measles-specific IgM positive sera as a source of controls for IgM assays. Therefore, there is a need to have a backup plan to confirm suspected cases in the absence of IgM assays, since current alternatives to IgM testing have limits of their own:

1) *Cell culture to isolate MeV from clinical specimens*: appropriate specimens need to be collected zero to five days after the appearance of rash. Samples often need to be transported in the cold to a laboratory with suitable facilities for isolation.

2) *RT-PCR to detect MeV genomes*: appropriate specimens need to be collected within one week after rash onset. Often, specimens are not collected. There are no commercially available products. Laboratories may not be set up to perform RT-PCR.

3) *Detection by EIA of a four-fold IgG rise in paired samples*: difficulty in collecting the convalescent sample (second sample).

4) *Detection of IgM by IFA*: IFA is a subjective and less sensitive technique.

Avidity testing can confirm acute measles cases and can assist in confirming SVF cases. In unvaccinated populations, detection of low IgG avidity antibody can confirm unvaccinated suspected measles cases in those situations in which IgM testing is not available. Two measles outbreaks, one described in Paper 3 and the Pennsylvania outbreak (Table 7), offered the possibility to study this idea using the avidity test described in Paper 3 and AppendixII[180]. The first outbreak was originally confirmed by IgM EIA and RT-PCR. Low avidity antibodies were detected in two IgM positive samples. The Pennsylvania outbreak was confirmed by IgM detection and RT-PCR amplification in two unvaccinated siblings (Table 7: cases 2 and 3). Avidity testing was able to further confirm one IgM positive case (Table 7: case 3); the other was IgG negative. In the absence of a measles-specific IgM assay, avidity testing can help confirm IgG positive cases with primary immune responses. Furthermore, measles avidity testing has the advantage that the window of opportunity to identify cases is extended for several weeks beyond rash onset in unvaccinated persons. However, avidity testing has its own limitations (Paper 3). For instance, sufficient measles-specific IgG is required to perform avidity testing (Table 7: case 2).

In conclusion, the diagnostic approaches presented in this dissertation, when incorporated into the current measles diagnostic tool box, will help improve case identification in elimination settings. As described, the process to identify measles cases in elimination settings is flexible in the sense that the tests performed will depend on each particular suspected case and available specimens. Because of the importance of identifying SVF cases, measles surveillance would greatly benefit from rapid assays to detect neutralizing antibodies as alternatives to the PRN[194]. The PRN assay currently in use takes about a week to perform. Appendix III describes an antigen that is used in a prototype cellular EIA for the detection of MeV-specific anti-H antibodies and shows that cells expressing recombinant MeV H could be used to detect neutralizing antibodies in an EIA format. It is reasonable to think that the current measles diagnostic algorithm will eventually be updated to include new diagnostic approaches to confirm acute measles cases and SVF cases, fulfilling a need in measles case identification. New algorithms should also include serology based on serum alternative specimens, DBS and OF, for use in those areas where these collection systems have already been implemented.

CONCLUSIONS

The main conclusions that can be derived from this study are:

1. A combination of laboratory diagnostic tools beyond detection of measles-specific IgM and RT-PCR is needed to identify all measles cases, regardless of vaccination status, due to the specific challenges encountered in measles diagnosis in the elimination setting.

2. Measles-specific antibodies can be extracted from DBS samples by at least six different elution protocols; all protocols performed well. Differences were only observed in logistical aspects.

3. A measles avidity assay based on elution of low avidity IgG using DEA at pH 10.25 is highly accurate, precise, reproducible, sensitive and specific. This avidity assay is able to detect measles-specific antibody maturation over time.

4. The DEA-based avidity assay distinguishes between primary and secondary immune responses and assists in vaccine failure classification and case confirmation.

5. A high avidity result and PRN titer greater than 30,000 mIU/mL can confirm some SVF cases.

6. A low avidity result can confirm measles cases, if the individual is unvaccinated or if vaccination was in the distant past in PVFs.

7. In order to optimize case identification in elimination settings, an update in the current measles diagnostic algorithm is needed to include measles avidity and PRN testing to assist in the confirmation of acute and SVF cases.

RESUM EN CATALÀ

INTRODUCCIÓ

I. EL DIAGNÒSTIC DE LES MALALTIES INFECCIOSES

El diagnòstic de les malalties infeccioses, o dit d'altra manera, la capacitat de poder identificar quin és el patògen causant d'una malaltia que es transmet, és un fenòmen relativament recent[1, 2]. Fins que al segle XIX Henle i Pasteur van formular la teoria dels gèrmens, el diagnòstic es basava en coneixements poc científics. El descobriment que cada malaltia infecciosa és causada per un patògen en concret va permetre l'avanç del camp de la microbiologia i de la immunologia. El descobriment de les bactèries va ser necessari per a poder descobrir el anticossos i d'aquí el desenvolupament de les primeres proves de serologia[4, 5]. Des d'aquest moment, les proves de diagnòstic de les malalties infeccioses han evolucionat per tal de poder determinar amb precisió el patògen responsable de la malaltia i així proveir d'una informació essencial, no només per al tractament del malalt sinó també per al control i la vigilància epidemiològica. En aquesta tesi es presenten eines de diagnòstic per ajudar a millorar el control i la vigilància del xarampió dins del marc de l'eliminació d'aquesta malaltia, és a dir, allà on la circulació del virus del xarampió (VX) endèmic ha estat interrompuda.

II. PERSPERCTIVA GENERAL DEL XARAMPIÓ

A. LA MALALTIA

El xarampió és una malaltia respiratòria altament infecciosa que típicament té lloc durant la infantesa i que es transmet de persona a persona. El VX és el causant del xarampió i

pertany a la família dels *Paramixovirus* (Taula 1). El xarampió dura de 15 a 21 dies i comença per una invasió per mitjà de gotetes aerosòliques infectades[7]. Els símptomes inicials del xarampió són la febre, la tos, la conjuntivitis, la coriza, el malestar i l'aparició de les taques de Koplick a les mucoses de la cavitat bucal. Aquesta fase prodròmica ve seguida de l'aparició d'una erupció cutània característica que comença a la cara i que s'estén centrífugament cap a les extremitats (Figura 1). Aquest exantema desapareix al cap de 3 o 4 dies, coincidint amb l'inici de la resposta immunològica específica contra el VX. El xarampió pot anar acompanyat de complicacions devastadores com a resultat de la immunosupressió provocada pel virus. El VX també pot causar encefalitis i la mort. Els efectes més destructius del xarampió s'observen en infants menors de 5 anys en àrees empobrides, especialment on hi ha desnutrició i manca de vitamina A[7, 11-15].

Impacte en la salut en l'era pre-vacunal

El xarampió és una malaltia antiga, causant d'epidèmies importants cada 2-3 anys i infectant a tothom que n'era susceptible. S'estima que el xarampió era responsable mundialment de 135 milions de casos i de 8 milions de morts anuals, resultant en una mortalitat estimada del 30%. El primer document sobre el xarampió ens arriba de Rhazes de Baghdad (segle IX)[16-20]. Les observacions detallades de Peter Panum sobre el xarampió durant una epidèmia a les illes Faroe han estat importants per a establir les característiques epidemiològiques de la malaltia (segle XIX)[21]. Al segle XVIII, Francis Home va demostrar la naturalesa infecciosa del xarampió, però no va ser fins 150 anys després que Ludvig Hektoen va descobrir que l'agent etiològic era un virus[24, 25]. A l'any 1954, Enders i Peebles van aïllar el VX per primera vegada donant peu al creixement *in vitro* del virus i a la creació de la primera vacuna[26-28].

B. EL XARAMPIÓ A L'ERA DE LA VACUNA

1. LA VACUNA DEL XARAMPIÓ

La primera vacuna contra el xarampió es va desenvolupar el 1963. Actualment es disposa d'una vacuna viva atenuada que és segura i efectiva, i que s'administra sovint en combinació amb les vacunes de la rubèola i de les galteres (Figura 2)[29, 30]. L'ús de la vacuna del xarampió ha permès la reducció dràstica de casos i morts anuals deguts al VX. L'any 2008 es va estimar que mundialment hi va haver 278,358 casos i 164,000 morts[32]. Per tal d'interrompre la circulació del VX d'una àrea geogràfica determinada i eliminar la malaltia és imprescindible aconseguir i mantenir una cobertura vacunal del 92% al 95%[42]. Actualment el xarampió ha estat eliminat de la regió de l'Organització Mundial de la Salut (OMS) de les Amèriques i d'altres zones geogràfiques. Malgrat es considera que el xarampió podria ser eradicat l'any 2020, tal objectiu encara no s'ha establert (Taula 2)[34-41].

2. EL XARAMPIÓ EN EL MARC DE L'ELIMINACIÓ DE LA MALALTIA

En territoris on s'ha aconseguit eliminar el xarampió la majoria de la població ha rebut dues dosis de la vacuna del xarampió i el nombre de casos notificats és extremadament baix. Tot i així, casos esporàdics i brots petits de xarampió poden tenir lloc a causa d'importacions des d'altres zones on el VX és encara endèmic. Els Estats Units d'Amèrica i Catalunya són exemples d'aquesta situació[38, 44]. En aquest context d'eliminació del xarampió, els brots tendeixen a involucrar principalment casos dins de la població no vacunada. Ara bé, també s'observen casos de xarampió dins de la població vacunada degut a fallades vacunals, les quals es classifiquen en fallada vacunal primària (FVP) i fallada vacunal secundària (FVS)[45-48]. Les FVP són casos de xarampió en persones vacunades que mai van respondre immunològicament a la vacuna. Les FVS són vacunats que sí que van fer una resposta immunològica a la dosi de vacuna del xarampió, però, o bé han perdut immunitat al llarg del

temps, o bé la resposta que van fer era incompleta[48, 50, 51]. Des d'un punt de vista de salut pública és important que cada cas de xarampió sigui correctament identificat i classificat per tal de proporcionar les mesures de control necessàries.

III. EL DIAGNÒSTIC DEL XARAMPIÓ

A. L'EVOLUCIÓ DEL DIAGNÒSTIC DEL XARAMPIÓ

El diagnòstic del xarampió ha evolucionat amb la vacunació. Aquesta evolució es podria explicar en tres fases. La primera fase seria la que va tenir lloc abans que hi hagués una vacuna per al xarampió, la malaltia era endèmica i el reconeixement simptomàtic per part dels metges era suficient per al diagnòstic[52]. La segona fase tindria lloc en regions que estan treballant per a eliminar el xarampió i que tenen una cobertura vacunal elevada. En aquest context, els metges estan menys familiaritzats amb els símptomes clínics del xarampió i esdevé fàcil confondre el xarampió amb altres malalties exantemàtiques. Per aquest motiu s'implementa la vigilància cas a cas, on els metges envien al laboratori les mostres de tots els pacients que encaixen amb la definició clínica de cas per a ser confirmats amb la detecció d'anticossos específics o la detecció del genoma del VX[54, 55]. La tercera fase tindria lloc en territoris on el VX endèmic ha estat eliminat i, per tant, la prevalència de la malaltia és extremadament baixa. En aquest context, els metges poden tenir encara més dificultats per a reconéixer el xarampió, ja sigui per la poca familiaritat amb la malaltia, com també perquè el xarampió en casos de FVS es presenta amb una simptomatologia diferent, sovint més lleu, fins al punt que no es reconeixen les FVS a fora d'un brot de xarampió (Taula 3)[51, 56-58]. La vigilància cas a cas continua en aquesta fase, amb una clara dependència en la confirmació al laboratori dels casos sospitosos, independentment del seu estat de vacunació.

B. EL DIAGNÒSTIC DE LABORATORI DEL XARAMPIÓ

Les proves utilitzades al laboratori per a classificar el casos sospitosos de xarampió s'han desenvolupat en base a la virologia, immunologia, bioquímica i epidemiologia del VX, i s'han avaluat amb mètodes estadístics.

1. VIROLOGIA DEL XARAMPIÓ

El virus del xarampió i el seu cicle infecciós

El virió i el genoma del VX estan representats gràficament a la Figura 3[61-65]. El cicle infecciós del VX comença amb la interacció de l'hemaglutinina (H) amb un dels receptors del VX. Hi ha dos receptors reconeguts pel VX salvatge: SLAM i Nectin 4. La interacció de la proteïna H amb el receptor promou l'acció de la proteïna de fusió (F), la qual fusionarà la membrana de l'embolcall del virus amb la membrana de la cèl·lula a infectar. També pot crear cèl·lules gegants multinucleades si es fusiona la membrana d'una cèl·lula infectada amb la d'una cèl·lula per infectar[7, 11, 66]. Al citoplasma, la ribonucleoproteïna, constituïda per ARN genòmic i les proteïnes N, L i P, transcriu i replica el genoma[64, 67, 70]. La proteïna N és la més transcrita, abundant, i antigènica, per això s'empra com a antigen en les proves serològiques (Figura 3)[68, 69]. Després de la replicació, els virions es formen per co-localització de les proteïnes H, F, M i de la ribonucleoproteïna a la membrana cel·lular des d'on eixiran[71-73].

2. MÈTODES DE DIAGNÒSTIC I VIGILÀNCIA BASATS EN LA VIROLOGIA DEL XARAMPIÓ

a) El cultiu cel·lular

El cultiu cel·lular és rellevant i necessari per a l'epidemiologia i vigilància molecular del virus, però és rarament utilitzat per a confirmar els casos sospitosos de xarampió ja que hi ha mètodes més ràpids (Figura 4, consultar C*ellular culture*)[74, 75].

b) Amplificació del genoma mitjançant la reacció en cadena de la polimerasa en transcriptasa inversa

El mètode de la reacció en cadena de la polimerasa en transcriptasa inversa (RT-PCR, en l'acrònim en anglès) s'utilitza per a detectar l'ARN del VX en mostres clíniques i per a amplificar els gens N i H abans de genotipar (Figura 4, consultar V*irus detection*)[76-79]. La RT-PCR pot ajudar a classificar casos quan els mètodes serològics són inconclusius. Actualment amb la RT-PCR en temps real es poden detectar fins a 10 còpies de genoma[81]. Malgrat els avantatges importants d'aquesta tècnica, la RT-PCR pot presentar alguns problemes ja sigui perquè pot originar resultats falsos negatius si la mostra clínica ha estat recollida massa tard o si ha estat manipulada incorrectament, o bé originar resultats falsos positius si el laboratori no té les instal·lacions adequades per a impedir la contaminació creuada. Un dels majors desavantatges és la no obtenció d'especímens per a emprar la RT-PCR[75, 195].

c) Genotipatge per a la vigilància virològica

La RT-PCR seguida de la seqüenciació permet identificar els genotips que estan circulant i, per tant, aporta informació sobre les vies de transmissió del VX, a la vegada que ajuda a discriminar les soques salvatges de les vacunals. Això és important quan se sospita que hi ha hagut reaccions adverses a la vacuna[84, 85].

d) Especímens per a la virologia molecular del xarampió

Els especímens principals que s'utilitzen són l'orina i el frotis faringi obtinguts en els 7 primers dies després de l'aparició de l'exantema. Els especímens de sang assecada en taques (SAT) i de fluid oral obtingut amb l'estri Oracol també es poden utilitzar (Figura 4, consultar *Virus detection*)[75, 76, 78, 86, 87].

3. IMMUNITAT AL XARAMPIÓ

La resposta immunològica a la infecció pel VX involucra les dues branques del sistema immunològic, la innata i l'adquirida[21, 88, 89]. La protecció específica contra el VX s'obté amb els elements humorals i cel·lulars del sistema immunològic adquirit activats pel contacte amb el VX, tant si és la soca salvatge o com la vacunal. La immunitat que se'n genera protegeix contra totes les soques del VX. El xarampió només genera un sol serotip. En general, es considera que la protecció a la reinfecció amb el VX es manté de per vida. El diagnòstic de laboratori del xarampió es basa només en el component de la immunitat humoral, és a dir, en la detecció d'anticossos específics al VX.

a) Anticossos importants per a la serologia del xarampió

(1) Anticossos IgM

Les immunoglobulines IgM antixarampionoses es poden detectar al sèrum i a la saliva. La Figura 4 presenta la cinètica de detecció de les IgM durant el xarampió clàssic[80]. La detecció de les IgM indica que hi ha infecció amb el VX i és un criteri important per a la confirmació del xarampió en persones no vacunades o en casos de FVP. Tot i que es poden produir anticossos IgM durant la resposta immunitària secundària, les IgM esdevenen un marcador menys fiable per a diagnosticar els casos de FVS, atès que la resposta d'IgM, si es dóna, és de curta durada i pot ser, o no, detectada[90-92].

(2) Anticossos IgG

Les immunoglobulines IgG antixarampionoses es poden detectar en tots els fluids corporals, incloent-hi el sèrum i la saliva. La Figura 4 presenta la cinètica de detecció de les IgG durant el xarampió clàssic[93]. La detecció de les IgG és important per a 1) determinar la seroconversió, 2) identificar el xarampió agut si s'observa un increment de 4 o més vegades del títol d'IgG entre una mostra aguda i una de convalescent, o bé si es detecten anticossos de

baixa avidesa, 3) avaluar la protecció amb la detecció d'anticossos neutralitzants, i 4) classificar les fallades vacunals.

b) Serologia del xarampió: propietats útils dels anticossos

(1) Avidesa de l'anticòs

Avidesa és el terme que s'utilitza per a descriure la força global antigènica en la qual anticossos multivalents s'enllacen amb antígens multivalents. Després del primer contacte amb el VX, els primers anticossos IgG que es produeixen són de baixa avidesa i estableixen enllaços febles amb els epítops específics de l'antigen. La força de la interacció antigen-anticós s'incrementa amb el temps, de tal manera que les IgG adquireixen alta avidesa, podent així fer enllaços més potents amb l'antigen. El procés de maduració de les IgG antixarampionoses després del primer contacte amb el virus salvatge, o amb la vacuna, té lloc durant els primers 3 mesos. Val a dir que els anticossos de baixa avidesa correlacionen amb l'exposició recent al VX en persones mai infectades ni vacunades, o en persones amb FVP, mentre que els anticossos d'alta avidesa es correlacionen amb l'exposició al VX llunyana en el temps o en casos de FVS[100-102, 103 Polack, 2003 #98].

(2) Anticossos neutralitzants

Els anticossos neutralitzants s'enllacen a les proteïnes H i F impedint que el VX sigui infecciós. La detecció d'IgG contra les proteïnes H i F correlaciona amb la neutralització del VX, i, per tant, amb la protecció immunitària. S'ha demostrat que cal un nivell mínim d'anticossos neutralitzants (títol ≥ 1:120 mIU/mL) per a protegir una persona del xarampió clàssic[57, 105-107].

4. LA CAIXA D'EINES DEL SERODIAGNÒSTIC DEL XARAMPIÓ

a) Proves immunoenzimàtiques basades en el sèrum

Les proves confirmatòries del xarampió que generalment es realitzen als laboratoris actuals són els assaigs immunoenzimàtics sobre suport sòlid (ELISA en l'acrònim en anglès) específics per a la detecció d'IgM i d'IgG antixarampionoses. La Figura 5 presenta un esquema dels passos típics dels formats ELISA de captura i indirecte. L'antigen utilitzat pot consistir d'extractes de cèl·lules infectades amb VX o de proteïna N recombinant. En comparació amb mètodes anteriors, les ELISA IgM tenen una alta sensibilitat (83% al 89%) i especificitat (95% al 100%) diagnòstiques, i són fàcils de realitzar i d'estandarditzar (Taula 4). Endemés, les ELISA IgM donen un bon marge de temps (4 setmanes) per a recollir la mostra de sang i detectar-hi les IgM antixarampionoses (Figura 4, veure IgM)[55, 108-110].

Limitacions de les ELISA IgM en el marc de l'eliminació de la malaltia

Les següents limitacions són degudes a que tant la circulació del VX com la prevalència de la malaltia són extremadament baixes en la població:

1) Es poden originar resultats falsos positius degut a la reactivitat creuada amb d'altres patògens que també ocasionen febre i exantema, i, a vegades, per la competició amb el factor reumatoide[53, 120].

2) Es poden donar lloc a resultats falsos negatius si la mostra de sèrum s'ha recollit a fora del temps adequat, ja sigui massa aviat o massa tard en mostres de xarampió agut, o massa tard en casos de FVS[58].

3) Es poden obtenir resultats falsos positius a causa de la forta influència de la prevalència de la malaltia (<1%) en el valor predictiu positiu. A la pràctica, s'acostumen a analitzar per la prova d'IgM un gran nombre de mostres de persones que presenten símptomes de febre i exantema, la qual cosa augmenta la probabilitat de detectar reaccions positives falses.

4) És probable que es considerin sospitosos de ser falsos els resultats d'IgM positiva obtinguts en casos aïllats que típicament es pensaria que són immunes. Per exemple, en casos sospitosos nascuts abans de que la vacuna fos disponible i que s'assumeix que són immunes a la malatia per infecció natural.

5) Amb la important reducció del nombre de casos de xarampió és possible que hi hagi cada vegada menys proves comercials per a confirmar la malaltia a nivell de laboratori.

6) La detecció d'IgM no pot distingir entre la soca salvatge i vacunal en persones que tenen símptomes i han rebut la vacuna recentment. Aquesta distinció es pot fer per genotipatge.

Les ELISA IgG es poden fer servir per a completar la informació obtinguda amb les proves d'IgM, i ajudar així a reconfirmar aquells casos dels que se sospita que el resultat d'IgM pot ser fals, especialment en mostres de persones no vacunades. Tot i així, la prova d'IgG no és útil per a distingir la resposta immunitària primària i secundària. Una altra limitació és que pot requerir una segona mostra recollida més tard, la qual pot ser difícil d'obtenir.

b) ELISA per a espècimens alternatius al sèrum

El fluid oral (saliva) i les mostres de SAT són espècimens alternatius a l'ús del sèrum i ajuden a superar algunes de les limitacions d'aquesta mostra tradicional (Veure una comparació de les característiques respectives a la Taula 5).

Robert Guthrie va inventar la recollida de mostres de SAT, el qual és un mètode àmpliament utilitzat en el diagnòstic precoç en nounats[123, 124]. La recollida de mostres de SAT és un mètode simple de recollida de sang total. Per a obtenir la mostra de SAT es deixa caure unes gotes de sang dintre d'un cercle imprès sobre un paper de filtre especial i la sang es deixa assecar (Figura 6). Les IgM i IgG antixarampionoses i l'ARN del VX són estables en

les mostres de SAT[86, 125-129]. Les mostres de SAT no es trenquen ni saliquen, són lleugeres, fàcils d'enviar per correu, de manipular i d'emmagatzemar[124, 130].

Com en el cas de la venipunció, el sèrum es pot recuperar de les mostres de SAT i pot ser emprat per a la detecció d'anticossos. Al llarg dels anys s'han fet servir diversos mètodes per a l'extracció del sèrum de les mostres de SAT. Una comparativa dels mètodes disponibles per a eluir el sèrum d'aquestes mostres facilitaria la seva implementació als laboratoris serològics del xarampió durant la fase posterior a l'eliminació del VX endèmic.

Per a la detecció d'anticossos antixarampionosos en mostres eluïdes de SAT, la OMS recomana l'ús de les proves d'ELISA indirecte Enzygnost® d'IgM Anti-VX i d'IgG Anti-VX (Siemens, Germany)[75]. Aquestes proves d'ELISA es van dissenyar originalment per a analitzar-hi sèrum, però actualment es disposen de protocols que les adapten a l'anàlisi de mostres eluïdes de SAT[125, 128]. En estudis d'avalució s'ha demostrat que la detecció dels anticossos antixarampionosos en eluïts de mostres de SAT correlaciona extremadament bé amb la detecció en sèrum derivat de venipunctura, tant si s'utilitzen proves ELISA de format indirecte com de format de captura (Apèndix I)[125, 128, 129, 131-133].

c) Prova d'avidesa

El principi bàsic de les proves d'avidesa juga amb la qualitat de les interaccions establertes entre l'anticòs i l'antigen al llarg del temps. Les proves d'avidesa diferencien la resposta d'anticossos recent de la resposta distant en el temps mitjançant l'ús de reactius caotròpics. Aquestes substàncies trenquen els enllaços no covalents, i per tant poden trencar el complex antigen-anticòs (Figura 7)[134-138]. El protocol de referència de les proves d'avidesa es basa en l'anàlisi de mostres diluïdes en sèrie seguit del càlcul de la relació dels títols en extinció de les dues corbes de dilució establertes, tal com es descriu a la Figura 7[144-146]. La

determinació de l'avidesa dels anticossos del xarampió s'ha utilitzat principalment per a avaluar el paper de les fallades vacunals en els brots de xarampió i per a estudiar la maduració dels anticossos després de la infecció (o vacunació) amb el VX[50, 101, 150-156]. Malgrat s'han descrit vàries proves d'avidesa específiques per anticossos del xarampió, no existeix un protocol estandarditzat ni tampoc hi ha avaluacions externes descrites de les proves disponibles comercialment. Actualment hi ha la necessitat de tenir una prova d'avidesa que estigui avaluada adequadament per a ser utilitzada com a complement de la prova d'IgM en la confirmació dels casos sospitosos de xarampió i com a eina classificadora de les fallades vacunals.

d) Proves per a mesurar la immunitat al xarampió

La prova *in vitro* de neutralizació per reducció del número de plaques (PRN en l'acrònim en anglès) específica per al VX es va desenvolupar als anys 1980 (Taula 4)[105, 157, 158]. La PRN és la prova que ofereix la millor correlació amb la protecció immunitària al xarampió i s'ha adoptat com a tècnica referent per a mesurar la immunitat generada pel VX, tant natural com vacunal. La Taula 6 presenta els nivells dels títols segons la PRN en relació a la protecció a la infecció pel VX i en relació al desenvolupament del xarampió[57].

IV. EINES ESTADÍSTIQUES PER A L'AVALUACIÓ DE PROVES DE DIAGNÒSTIC

A. EL RENDIMENT DIAGNÒSTIC

L'anàlisi de la característica operacional del receptor

L'anàlisi de la característica operacional del receptor (ROC en l'acrònim en anglès) és un mètode estadístic àmpliament emprat per a descriure i comparar el rendiment diagnòstic de proves mèdiques [161-166]. Els avantatges que ofereix sobre altres mètodes utilitzats són: 1) la independència de la prevalència de la malaltia, 2) la possibilitat de comparar dues o més proves, i 3) el rendiment diagnòstic global de la prova es pot copsar en un sol índex a través de l'estima de l'àrea sota la corba ROC (AUC en l'acrònim en anglès)[167, 168]. La corba ROC és la representació gràfica de la relació entre la sensibilitat i l'especificitat a tots els possibles punts de tall segons la mostra de resultats de la prova: cada punt a la corba ROC està associat a un punt de tall (Figura 8). Les qualitats diagnòstiques de la prova en estudi s'avaluen en comparació a una prova de referència. Els valors de l'AUC poden anar del 0.5 al 1 (Figura 8)[167]. L'AUC es pot utilitzar fàcilment per a comparar el rendiment diagnòstic de dues o més proves analítiques[174]. L'anàlisi ROC també es fa servir per a establir el punt de tall de proves amb mesures contínues, com la densitat òptica. El punt situat a la part més alta i a l'esquerra de la corba correspon al punt de tall on la prova té la millor sensibilitat i especificitat[167].

B. LA PRECISIÓ

Una prova de diagnòstic útil és precisa, i per tant es pot repetir al llarg del temps amb el mateix nivell de qualitat dels resultats. La precisió és l'estimació de la proximitat dels valors obtinguts en mesures independents i repetitives en unes condicions determinades[175]. La precisió s'expressa en termes de desviació estàndard. Una desviació estàndard baixa indica una precisió elevada[176].

OBJECTIUS

L'objectiu general d'aquesta tesi doctoral és el d'avaluar eines per al diagnòstic de laboratori per al seu ús dins del marc de l'eliminació del xarampió, i es divideix en els següents objectius específics:

- Comparar protocols per a l'elució de mostres de SAT
- Analitzar i descriure dades analítiques i clíniques de mostres obtingudes de FVS
- Trobar biomarcadors per a la confirmació de casos de xarampió de FVS
- Desenvolupar i validar una prova d'avidesa específica per a xarampió a fi de:
    1. Classificar fallades vacunals
    2. Reconfirmar casos de xarampió en persones no vacunades

RESULTATS I DISCUSSIÓ

Aquesta tesi inclou tres articles que descriuen eines que poden ajudar a classificar casos sospitosos de xarampió dins del marc de l'eliminació d'aquesta malaltia. Els resultats d'aquests articles fan referència a A) la implementació de l'ús de les mostres de SAT en el marc de l'eliminació del xarampió (article 1), i B) estratègies de diagnòstic complementàries per a la millora de la classificació dels casos de xarampió (articles 2 i 3).

I. LA IMPLEMENTACIÓ DE L'ÚS DE LES MOSTRES DE SAT EN EL MARC DE L'ELIMINACIÓ DEL XARAMPIÓ

La OMS ha avaluat l'ús d'especímens alternatius al serum, és a dir, el fluid oral i les mostres de SAT, els quals poden solventar alguns dels problemes logístics del serum (Taula 5)[82, 83]. En preparació per a col.laborar en l'avaluació de la OMS, es va cercar a PubMed protocols per a l'extracció d'anticossos de les mostres de SAT. Els protocols que es van trobar eren suficientment diferents com per iniciar-ne una comparació que permetés copçar les qualitats de cadascun d'ells. L'article 1 es va dissenyar per a proporcionar una guia per ajudar a escollir el protocol adequat per a l'elució de les mostres de SAT.

L'estudi descrit en l'article 1 demostra que amb els sis protocols d'elució estudiats els anticossos antixarampionosos IgM i IgG són detectables en els eluïts de mostres de SAT. És a dir, tant els mètodes d'elució activa (protocols 3, 4, 5 i 6) com d'elució passiva (protocols 1 i 2) podrien servir per a la serologia del xarampió utilitzant mostres de SAT. En referència als protocols 1 i 2, es va detectar diferències significatives en la detecció de la IgM antixarampionosa, però en cap cas va causar un canvi de classificació del resultat, de positiu a negatiu. Una de les limitacions de l'estudi és que es va analitzar un número limitat de

controls, enlloc d'analitzar mostres clíniques. Tanmateix, com es pot comprovar en l'Apèndix I, la sensibilitat i l'especificitat diagnòstica per a la detecció tant d'IgM com d'IgG antixarampionoses són molt altes, independentment del protocol utilitzat. L'elució activa ha estat utilitzada en estudis independents per a avaluar l'ús de mostres de SAT per al diagnòstic del xarampió en zones amb VX endèmic. És a dir, l'observació de que no hi ha diferències en la classificació dels resultats utilitzant protocols d'elució activa o passiva (Article 1) es tradueix també en la seva aplicació al camp amb l'anàlisi de mostres clíniques (Appendix I).

Si en el futur s'avaluen totes les proves d'ús en el marc d'eliminació del xarampió i es considera que la mostra de SAT continua sent una mostra vàlida, llavors un aspecte que sí que caldrà considerar és el volum de mostra eluïda que es recupera. Dins del marc de l'eliminació del xarampió es pot preveure que es necessitaran varies proves per a la confirmació de determinats casos sospitosos. Per exemple en la identificació de FVS caldrà la detecció d'IgM, la determinació del títol d'anticossos neutralitzants per la prova de PRN i la verificació per la prova d'avidesa. És aquí on les diferències observades entre els protocols poden esdevenir importants. La major quantitat de volum eluït es va obtenir seguint el protocol 6, fent aquest protocol el més avantatjós per a l'ús en el marc de l'eliminació del xarampió.

II. ESTRATÈGIES DE DIAGNÒSTIC COMPLEMENTÀRIES PER A LA MILLORA DE LA CLASSIFICACIÓ DELS CASOS DE XARAMPIÓ

La confirmació a nivell de laboratori del xarampió dins del marc de l'eliminació d'aquesta malaltia és com resoldre un trencaclosques, la dificultat del qual depèn de les peces d'informació que es tenen a mà. La informació epidemiològica, clínica i analítica s'integra per a resoldre cada cas sospitós de xarampió. La resolució dels casos sospitosos de xarampió esdevé un major repte de la ciència diagnòstica quan es tracta de casos vacunats infectats amb

el VX, ja que poden presentar una simptomatologia i un perfil serològic modificats. Alguns casos de FVS no poden ser classificats per varies raons: a) les dades epidemiològiques són incompletes, b) la mostra s'obté fora del temps òptim, degut a un reconeixement dels símptomes tardà, i el resultat obtingut és d'IgM negativa, c) les proves d'IgM i RT-PCR específiques del xarampió tenen limitacions intrínseque (AppendixII)s[180]. Per tant, es necessita una combinació de proves diagnòstiques per a assegurar que els resultats obtinguts són veritables[91, 92]. Els articles 2 i 3 suggereixen l'ús de les proves de PRN i d'avidesa com a eines especialitzades addicionals per ajudar a resoldre determinats casos sospitosos. L'article 2 descriu la detecció de títols elevats d'anticossos neutralitzats (> 30,000 mIU/mL) d'alta avidesa com a biomarcador dels casos de FVS. Aquesta estratègia es va utilitzar per a classificar casos que altrament no haurien pogut ser classificats. Aquests exemples estan inclosos en l'article 2, l'article de Rota *et al.* i a la taula 7 de la tesi (Appendix II)[180]. D'interès, títols elevats d'IgG han estat observats per Chen *et al.*, que descriu dos casos de xarampió vacunats amb símptomes relativament lleus que presentaven títols de PRN superiors a 44,600 mIU/mL, i per De Serres i Van den Hoek, que descriuen títols d'IgG antixarampionosa mesurats amb ELISA superiors a 36,000 mIU/mL i 13,000 mIU/mL, respectivament. D'altra banda, Chen *et al.* descriu dos casos addicionals de xarampió també vacunats que presentaven un simptomatologia greu i títols de PRN superiors a 35,000 mIU/mL[57, 181, 182]. Malhauradament, en cap d'aquests estudis es va classificar les fallades vacunals. L'ús de la prova d'avidesa hauria ajudat a fer aquesta classificació. L'estratègia diagnòstica de confirmar casos de FVS amb la detecció de títols elevats d'anticossos neutralitzats d'alta avidesa hauria de ser validada adequadament per a poder estimar la seva rendibilitat diagnòstica, sensibilitat i especificitat a l'hora d'identificar aquests casos. Per a això caldria determinar el punt de tall òptim del títol d'anticossos neutralitzants, així com el punt de tall de la prova d'avidesa. També caldria avaluar l'ús de la prova d'ELISA d'IgG

antixarampionoses com a alternativa ràpida a la prova de PRN. Per a realitzar aquestes valoracions, és necessari un número estadísticament adequat de mostres[164]. Degut a que el nombre de casos amb FVS és molt baix, seria beneficiosa la col.laboració entre regions on el VX ha estat eliminat, i així proporcionar el suficient número de casos amb FVS per a l'anàlisi.

DESENVOLUPAMENT I AVALUACIÓ D'UNA PROVA D'AVIDESA ESPECÍFICA DE XARAMPIÓ

L'article 3 presenta el desenvolupament, avaluació i aplicació d'una prova d'avidesa de la IgG antixarampionosa. Durant el desenvolupament de la prova, l'agent desnaturalitzant dietilamina (DEA) va ser escollit per sobre de la urea per la seva major eficàcia (Figura 9). La DEA utilitzada a una concentració de 60mM i al pH de 10.25 és una solució suficientment desestabilitzadora per a eluir els anticossos IgG de baixa avidesa sense afectar les interaccions establertes pels anticossos IgG d'alta avidesa.

La prova d'avidesa per a xarampió es va avaluar mitjançant l'anàlisi ROC establint dos punts de tall òptims per a determinar la presència d'IgG de baixa avidesa al nivell de 30% i per a determinar la presència d'IgG d'alta avidesa al nivell de 70%. Els resultats obtinguts en la prova es classifiquen com a avidesa baixa, intermèdia i alta. La sensibilitat de la prova és del 97% i l'especificitat de la prova és del 100% al punt de tall de baixa avidesa, un cop excloses mostres amb anticossos maternals i mostres amb un resultat inicial d'avidesa intermèdia que es van reclassificar amb un resultat de baixa avidesa. Aquesta alta sensibilitat i especificitat diagnòstica proporciona una gran seguretat en la determinació de les respostes immunitàries primàries en mostres obtingudes en les 3 primeres setmanes després de l'aparició de l'exantema i en l'absència de possibles anticossos maternals d'alta avidesa. La sensibilitat de la prova al punt de tall per a la detecció d'alta avidesa és del 100% en mostres

obtingudes dins de les 12 setmanes després de l'inici de l'exantema. Aquest fet permet la detecció de casos amb respostes immunitàries primàries en mostres obtingudes després dels 28 dies recomanables per a la detecció d'IgM, quan hi ha la possibilitat d'obtenir resultats falsos negatius. Ara bé, l'especificitat de la prova al punt de tall d'alta avidesa és del 97% indicant que és possible obtenir resultats d'avidesa intermèdia. Exceptuant els casos on està documentat que el cas no està vacunat i exceptuant les investigacions de FVS, els resultats d'avidesa intermèdia no es poden interpretar per ara.

El protocol de la prova d'avidesa per al xarampió basat en l'ús de la DEA es va aplicar a una altra plataforma comercial (Wampole Laboratories, Inc., Princeton, NJ, USA) i els resultats indiquen que el protocol també funciona fent servir un producte comercial que on s'empren com a antigen virions integrals de xarampió (Figura 10). Els punts de tall i la qualitat diagnòstica de la prova basada en el producte Wampole no es van avaluar i per tant això caldria fer-ho abans de ser implementada. La possibilitat d'aplicar el protocol d'avidesa a un altre producte comercial és important ja que amplia les possibilitats d'ús del protocol.

UTILITZACIÓ DE LA PROVA D'AVIDESA PER A XARAMPIÓ EN EL MARC DE L'ELIMINACIÓ DE LA MALALTIA

Els resultats de la prova d'avidesa per a xarampió s'interpreten juntament amb la informació epidemiològica, clínica i analítica que es té del cas sospitós. Aquesta integració de dades permet: 1) classificar les fallades vacunals i així ajudar per una banda a verificar les FVS en casos amb títols d'anticossos neutralitzants elevats (Taula 7, cas 5), i, per l'altra, a entendre millor la dinàmica dels brots (Appendix II)[180], 2) donar confiança en la interpretació dels resultats d'IgM positius en casos aïllats o esporàdics, i ajudar a confirmar els casos sospitosos on la mostra s'ha recollit massa tard, atès que la prova descrita en l'article 3 pot detectar la maduració de l'avidesa de les IgG antixarampionoses (Figura 11, Taules 8 i 9); i

3) confirmar casos documentats com a no vacunats quan no hi ha una prova d'IgM disponible.

En conclusió, les estratègies diagnòstiques presentades en aquesta tesi poden ajudar a millorar la classificació de casos de xarampió dins del context de l'eliminació d'aquesta malaltia. El tàndem de les proves de PRN i d'avidesa té potencial per a ser inclòs a la caixa d'eines del serodiagnòstic del xarampió per al seu ús en aquest context. Atesa la importància d'identificar les FVS, la vigilància del xarampió es podria beneficiar d'una prova ràpida per a detectar anticossos neutralitzants[194]. L'Apèndix II presenta els resultats de la caracterització d'un prototipus d'ELISA per a la detecció d'anticossos neutralizants. És raonable pensar que l'algoritme actual de classificació de casos sospitosos de xarampió serà actualitzat per a incloure noves estratègies de diagnòstic per a confirmar els casos aguts de xarampió i les FVS, omplint així un buit en la identificació dels casos de xarampió. Els nous algoritmes també haurien d'incloure la serologia basada en mostres alternatives al sèrum, mostres de SAT i de fluid oral, per a ser emprades en aquelles àrees on aquests sistemes de recollida de mostra ja s'han implementat.

CONCLUSIONS

Les principals conclusions derivades de l'estudi presentat són les següents:

1. Per tal d'identificar tot els casos de xarampió dins del marc de l'elimimació del xarampió és necessària una combinació d'eines de laboratori que vagi més enllà de la detecció de les IgM antixarampionoses i de la RT-PCR.

2. Els anticossos antixarampionosos poden ser extrets de mostres de SAT mitjançant com a mínim 6 protocols d'elució diferents; tot els protocols analitzats van funcionar bé. Les diferències es van observar en aspectes de caire logístic.

3. Una prova d'avidesa basada en l'elució d'IgG antixarampionosa de baixa avidesa amb DEA a molaritat 60mM i pH10.25 té un rendiment diagnòstic elevat i és altament precisa, reproduïble, sensible i específica. Aquesta prova d'avidesa pot detectar la maduració dels anticossos d'IgG contra el VX.

4. La prova d'avidesa basada en la DEA distingeix les respostes immunitàries primàries de les secundàries i ajuda en la classificació de les fallades vacunals i en la confirmació de casos sospitosos.

5. Un resultat d'alta avidesa juntament amb un títol d'anticossos neutralitzants elevat pot confirmar alguns casos de FVS.

6. Un resultat de baixa avidesa pot confirmar casos sospitosos de xarampió si l'individu no ha estat vacunat o si és un cas de FVP, on la data de vacunacio és en el passat llunyà.

7. Per tal d'optimitzar la identificació dels casos de xarampió en el marc de l'eliminació d'aquesta malaltia, és necessari fer una actualització de l'algoritme de diagnòstic del xarampió per a incloure l'anàlisi de mostres per les proves d'avidesa i de PRN de xarampió, ja que aquests poden ajudar en la confirmació de casos aguts i de FVS.

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APPENDIX I

Comparison of measles-specific IgM and IgG enzyme immunoassays using dried blood spots

	Format	Protocol	N[a]	%A.[b]	Cor.[c]	Se[d]	Sp[e]	PPV[f]	NPV[g]	Reference
IgM assay										
Behring	Indirect	6	567	99%	-	94	99	100	97	177
Behring	Indirect	3	88[j]	98%	0.93[h]	100	97.1	90	100	133
Behring	Indirect	3	216	97%	0.91[h]	90	99	96	97	133
CDC	Capture	1	119	98%	0.99[i]	-	-	-	-	132
In-house & RT-PCR	Capture	2	117	-	-	99	96	99	-	129
In-house	Capture	2	117	-	-	95	96	99	-	129
IgG assay										
In-house	Indirect	2	227	99%	-	-	-	-	-	128
Behring	Indirect	3	499	98%	0.96[h]	98	97	98	97	125
Melotest		2	165	98%	0.93[i]	100	90	97.8	100	131

[a] Number of samples
[b] %Agreement between dried blood spot and serum
[c] Correlation
[d] Sensitivity
[e] Specificity
[f] Positive and Negative Predictive Values
[g] Kappa value
[h] Correlation Coefficient
[j] Samples stored less than 6 months

APPENDIX II

Jennifer S. Rota, Carole J. Hickman, Sun Bae Sowers, Paul A. Rota, Sara Mercader, and William J. Bellini Two Case Studies of Modified Measles in Vaccinated Physicians Exposed to Primary Measles Cases: High Risk of Infection But Low Risk of Transmission. *Journal of Infectious Diseases (2011), 204 (suppl 1):* S559-S563

Paper is attached in pages 157 to 161

SUPPLEMENT ARTICLE

Two Case Studies of Modified Measles in Vaccinated Physicians Exposed to Primary Measles Cases: High Risk of Infection But Low Risk of Transmission

Jennifer S. Rota, Carole J. Hickman, Sun Bae Sowers, Paul A. Rota, Sara Mercader, and William J. Bellini

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In 2009, measles outbreaks in Pennsylvania and Virginia resulted in the exposure and apparent infection of 2 physicians, both of whom had a documented history of vaccination with >2 doses of measles-mumps-rubella vaccine. These physicians were suspected of having been infected with measles after treating patients who subsequently received a diagnosis of measles. The clinical presentation was nonclassical in regard to progression, duration, and severity. It is hypothesized that the 2 physicians mounted vigorous secondary immune responses typified by high avidity measles immunoglobulin G antibody and remarkably high neutralizing titers in response to intense and prolonged exposure to a primary measles case patient. Both of the physicians continued to see patients, because neither considered that they could have measles. Despite surveillance for cases among contacts, including unvaccinated persons, no additional cases were identified.

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In the United States, limited measles outbreaks continue to occur after importation of measles, and the cost of conducting follow-up investigations and case containment can be substantial [1]. Prior to a diagnosis of measles, a patient may be seen in multiple health care facilities, resulting in numerous exposures of patients and health care workers. In hospitals, there are often immunocompromised patients and other persons for whom infection with measles can have severe consequences. For this reason, health care workers born after 1957 are generally required to have documentation of having received 2 doses of measles-mumps-rubella (MMR) vaccine and/or demonstrate immunity to measles by serological testing.

Potential conflicts of interest: none reported

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The laboratory plays a critical role in case classification when rash and fever develop in persons who have possibly been exposed to measles. To complicate matters, nonclassic cases of measles in vaccinated persons may be identified, which must be investigated. Often the symptoms are mild and resolve rapidly and, outside of the context of an outbreak or known exposure to a measles case patient, the nonclassic presentation might not raise suspicion of measles [2–4]. However, the consequences of possible spread from such cases, and particularly from cases among health care workers, puts tremendous pressure and demands on those who are responsible for outbreak control.

In this report, we describe 2 instances in which physicians developed rash and fever following treatment of a confirmed measles case despite a history of receipt of >2 doses of MMR vaccine. The laboratory findings from the 2 suspected cases were consistent with a secondary immune response (SIR) to measles. The relevance of immunoglobulin (Ig) M detection for case confirmation in such circumstances and the implications for outbreak investigations are discussed. The absence of spread cases from the 2 physicians suggests

that such cases, although not asymptomatic, have a very low potential for infecting others, compared with cases in fully symptomatic individuals.

CASE REPORT: MEASLES OUTBREAK WITH EMERGENCY DEPARTMENT EXPOSURE TO PHYSICIAN, PENNSYLVANIA, MARCH 2009

A 37-year-old female emergency department physician in Pennsylvania with mild symptoms was suspected of being infected with measles in the course of an outbreak investigation in which the initial case of measles was not immediately recognized. The physician had a history of having received 3 doses of MMR vaccine, 2 of which were documented, with the most recent dose being administered in 2003. The reason given for the additional dose was failure to show immunity to rubella. The physician had evaluated a 10-year-old child in the emergency department on 10 March 2009 who was suspected to have Kawasaki disease. The child had arrived from India on 8 March and had developed a rash on 9 March. A review of medical records led the investigators to reconsider the rash illness in the 10-year-old child as a possible measles case following the laboratory confirmation of measles by IgM testing on 30 March in 3 family members who had been in the same emergency department on 10 March for an unrelated complaint. The family members, comprising 2 unvaccinated male siblings (23 months of age and 4 years of age) and their father (33 years of age) had developed rash illnesses 13–16 days after the emergency department visit. Viral samples collected from the 2 children were positive for measles by reverse-transcriptase polymerase chain reaction (RT-PCR), and genotype D8 was identified, which was consistent with importation of the disease from India [5]. The clinical sample obtained from the father, who reported having received 1 dose of MMR vaccine as a child, had negative RT-PCR results. The description of his symptoms included a rash on the face and the trunk, cough, and coryza, but the highest recorded temperature was 99°F (37.22°C). Because of the positive IgM result, the father was confirmed as having a measles infection but no additional cases occurred, even though the father continued to work while potentially infectious. The 10-year-old child from India was determined to be the index case patient after a serum sample that was collected 24 days after rash onset had test results that were positive for measles IgM (Table 1). One additional case was confirmed in an 11-month-old infant (who was unvaccinated) who had also been in the same emergency department on 10 March.

After measles was diagnosed among the secondary cases, the physician recalled having flu-like symptoms, including myalgia, cough, and fever that lasted 4–5 days, prior to the appearance of a rash on the abdomen, which spread to the neck. The rash appeared on 26 March, 16 days after contact with the index case patient, and had resolved within 24 h. The physician continued to work and saw >100 patients during her infectious period, including unvaccinated infants; however, no spread cases were detected. The laboratory results are summarized in Table 1.

CASE REPORT: SPORADIC MEASLES CASE WITH ACUTE CARE FACILITY EXPOSURE TO PHYSICIAN, VIRGINIA, APRIL 2009

In contrast to the first case report, the exposure of a 39-year-old male physician in Virginia to measles was recognized within a few days after seeing the patient. However, the physician had a history of having received 5 doses of MMR vaccine (2 in childhood and 3 in medical school), and his immunity to measles had been verified in December 2004. The reason given for receipt of the multiple doses was failure to demonstrate seroconversion to ≥1 of the antigens. On 14 April 2009, the

Table 1. Centers for Disease Control and Prevention Laboratory Results for 6 Measles Cases Including an Emergency Department Physician in Pennsylvania, March 2009

Case description, rash onset date	Vaccine history	Interval, rash onset to serum collection	IgM result	IgG result	PRN titer	Avidity
Index case patient, 9 March	Unknown	24 days	Positive	Positive	3644	Low
Sibling 1, 23 March	No MMR	7 days	Positive	Positive	9503	Low
Sibling 2, 26 March	No MMR	S1: 5 days S2: 7 days	S1: Positive S2: Positive	S1: Negative S2: Positive	S1: 2332 S2: 10,564	Not done
Father, 26 March	1 MMR dose (no record)	4 days	Positive	Positive	168,640	High
11-Month-old infant, 27 March	No MMR	6 days	Positive	Positive	2395	Not done
Physician, 26 March	3 MMR doses	S1: 6 days S2: 20 days	S1: Ind S2: Negative	S1: Positive S2: Positive	S1: 248,628 S2: 206,580	High

NOTE. The S1 (physician) was determined to be immunoglobulin (Ig) M positive at the Pennsylvania Bureau of Laboratories. Ind, indeterminant; MMR, measles-mumps-rubella vaccine; PRN, plaque reduction neutralization.

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physician had examined a 25-year-old patient who presented to his clinic with fever and rash. Blood samples were collected from the 25-year-old patient on 17 April and again on 20 April, at which time the diagnosis of measles was confirmed by IgM detection. Because there was no recent travel and no other cases had been identified, the IgM results for the 25-year-old patient were confirmed by additional laboratory tests. Because the first serum sample collected had test results that were negative for measles IgG, seroconversion was demonstrated (Table 2). Also, viral samples collected on 17 April were positive for measles by RT-PCR (data not shown).

On 29 April, 15 days after seeing the measles case patient, the physician noticed that he had developed a rash. However, he had removed a tick from himself 2 days earlier and attributed the rash either to the tick bite or to taking doxycycline. A blood sample was drawn on 29 April, which was tested at a commercial laboratory and found to be IgM negative and IgG positive for measles. The physician continued to see patients during his infectious period because he considered himself to be protected from measles. Because the first blood sample collection date was the first day of the rash (and therefore possibly yielded a false-negative result), a second blood sample was obtained on 8 May. No viral samples were collected. The second blood sample was sent to a different commercial laboratory, and the sample was found to be IgM positive and IgG positive. The symptoms reported by the physician were temperature to 103°F (39.44°C) and headache prior to rash, but no coryza, conjunctivitis, or cough were reported. The physician also had an unvaccinated 3-month-old child at home who remained well, as did the child's mother (whose vaccination status was unknown). No additional cases were reported. Another serum sample was collected on 12 May. The blood samples collected on 8 May and 12 May were submitted to the Centers for Disease Control and Prevention (CDC) for testing and were found to be IgM negative, at which time the physician was ruled out as a case patient. However, a second aliquot of the serum, dated 12 May, arrived later and a positive result for measles IgM was obtained. The laboratory results are summarized in Table 2.

DISCUSSION

Physicians are often exposed to patients at a very infectious stage of measles disease, during the prodrome when fever is present or at onset of rash [6]. Persons with preexisting antibody levels that would be clinically protective against disease (or would asymptomatically boost) after a lesser or "casual" exposure, may be mildly to moderately symptomatic upon prolonged exposure in close quarters, such as an examination room. The vaccination history of the 2 physicians and the high avidity antibody were consistent with designation of these cases as having a SIR. Furthermore, the symptoms reported were modified or nonclassic; they were less severe and/or of shorter duration than what is typically observed in a primary infection. In the absence of a known exposure to a measles case patient, the possibility of measles would likely not have been considered.

Laboratory testing of serum samples from asymptomatic or mildly ill contacts of a measles case patient can detect an immunologic response to measles infection [3, 7]. As reported by Helfand et al [3], many persons who were exposed to a measles case patient on a 3-day bus trip had a detectable IgM response, regardless of having received previous vaccination or, for some, having a history of natural measles infection. In addition, the microneutralization titers measured from the exposed persons on the bus in which the measles case patient traveled were significantly higher than those obtained from persons who traveled on the second bus in the caravan. The clinical presentations of the exposed persons with detectable antibodies and/or measles-neutralizing antibodies, however, did not meet the measles clinical case definition [3].

In addition to IgM testing and IgG avidity testing, the serum samples collected from the 2 physicians and the index case patients (and other case patients in the Pennsylvania outbreak) were tested using a plaque reduction neutralization (PRN) assay [7]. The magnitude of the titers obtained from the PRN test from acute-phase serum samples collected from the primary measles case patients (who were identified as having low avidity or having initial IgG-negative test results) did not exceed 10,564 (Tables 1 and 2). In contrast, at comparable intervals after rash (4–9 days), PRN titers from the 2 physicians (as well as from the father in the Pennsylvania outbreak) were 10–168 times higher, reflecting a robust booster response. Concomitant with high

Table 2. Centers for Disease Control and Prevention Laboratory Results for the Index Case Patient and Exposed Physician in Virginia, April 2009

Case description, rash onset date	Vaccine history	Interval, rash onset to serum collection	IgM result	IgG result	PRN titer	Avidity
Index case patient, 14 April	Unknown	S1: 3 days S2: 6 days	S1: Positive S2: Positive	S1: Negative S2: Positive	S1: 341 S2: 1472	Not done
Physician, 29 April	5 MMRs	S1: 9 days S2.1: 13 days S2.2: 13 days	S1: Negative S2: Negative S2: Positive	S1: Positive S2: Positive S2: Positive	S1: 81,916 S2: 129,424 S2: 128,043	High

NOTE. Two serum samples (S2.1, S2.2) were received with collection date of 12 May (13 days after rash onset). The second sample that was received (S2.2) tested positive for immunoglobulin (Ig) M. MMR, measles-mumps-rubella vaccine; PRN, plaque reduction neutralization.

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PRN titers in acute phase serum samples, a very strong reaction in the IgG enzyme immunoassay was observed, compared with that obtained from the primary case patients (data not shown). High levels of IgG can interfere with IgM assays because of insufficient removal of the IgG from the serum, giving rise to false-positive results, as well as to false-negative results [8].

Intensified surveillance for rash illnesses in an outbreak setting has frequently presented dilemmas for outbreak control when vaccinated persons with modified illness are identified as suspected case patients. Although detection of IgM is the recommended method for measles confirmation, it is an unreliable marker for measles infection in persons with an SIR. The father of the 2 unvaccinated siblings in the Pennsylvania outbreak (who had a history of having received 1 MMR vaccine dose in childhood) was confirmed as a measles case patient by IgM testing performed on serum samples collected 4 days after rash. However, the IgM was weakly positive, and 3 replicates of the serum run in the same test were IgM indeterminate (data not shown). Similarly, inconsistent results for IgM were obtained from serum samples obtained from the 2 physicians (Tables 1 and 2), possibly attributable to the very high levels of IgG and/or relatively low levels of IgM [8].

The ability to detect IgM among persons with an SIR following an exposure to measles will depend on the magnitude and kinetics of the individual immune response (current and previous), the timing of the serum sample collection, and the sensitivity of the assay [9]. In addition, because of the rapid boosting of IgG, it may not be possible to demonstrate a 4-fold rise in titer among SIR cases. However, when clinical samples are collected in a timely manner, real-time RT-PCR testing may detect virus in persons with modified illness. For example, during a measles outbreak in 2007 [10], 2 vaccinated college students (cases 6 and 7 in [10]) were identified in the course of follow-up investigations of contacts of an acutely ill measles case patient. Both of the students had some rash and fever, but neither of the students presented with cough, coryza, or conjunctivitis. Only 1 of the students had a detectable IgM response; the other case was confirmed by virus detection using RT-PCR [10]. The PRN titers obtained from the students were very high (119,287 and 217,812), and the avidity was also high, consistent with a SIR (cases 2 and 3 in [7]). No spread cases from the 2 students were identified.

Modified measles infections may also resemble other rash illnesses, including rubella, which is a situation that can be confusing, because serum from measles-infected persons can cause interference in rubella IgM assays, producing false-positive results [11]. This occurred during a measles outbreak in 2006, when several persons with a mild rash illness were identified. Because the serum samples were negative for measles IgM (and the case patients had symptoms that were suggestive of rubella), the samples were tested for rubella IgM. Although 3 of 4 serum samples sent to the CDC for confirmatory testing were weakly positive for rubella IgM, the avidity index for 3 of the samples (1 sample was IgG negative) was either intermediate or high and, therefore, was inconsistent with a current rubella infection. In addition, 2 of the case patients with viral samples available for testing had positive results when later tested for measles by real-time RT-PCR, including the 1 sample with results that were negative for rubella IgG, and could not be ruled out by avidity testing (CDC, unpublished data).

As suggested by Chen et al [12], immunity to measles may not be absolute but, depending on the levels of preexisting antibody, reflect a continuum of clinical illness. In addition to the level of preexisting antibody, the intensity of exposure (ie, the dose of virus received) is an important risk factor for breakthrough infection and one that could not be quantified in studies that retrospectively determine the protective titer against symptomatic infection. The absence of circulating virus and the periodic boosting that may have provided additional protection from infection may alter the paradigm of lifelong (asymptomatic) immunity after vaccination or disease. As pointed out by Helfand et al [3], the rate of nonclassic infection is likely to increase as measles control improves in a population, because boosting from exposure to wild-type measles virus will be rare. This may also occur among older persons who have a history of natural disease, although prior disease is difficult to document. One such case occurred in 2008 in a 55-year-old man who was born outside of the United States and who claimed to have had measles in childhood. He had traveled to his home country and was exposed to children who had measles. Initially, the case was not strongly suspicious for measles because of the nonclassic presentation and disease progression. However, the case was confirmed as measles by IgM detection and by an RT-PCR result positive for measles. The avidity was high, and the PRN titer was >160,000 (CDC, unpublished data).

Despite ample opportunities for transmission of virus, the 2 physicians in this report did not infect any patients, including many patients who were unvaccinated. The determination of whether a vaccinated individual who is exposed to measles (who develops symptoms that are suggestive of measles) represents a case patient and therefore a potential source of infection for others often hinges on a laboratory test result as the deciding factor. Reliance on the absence of IgM to rule out a case may be unjustified under these circumstances. In the future, more of these difficult cases will be confirmed by detection of measles RNA. Additional studies are needed to determine whether persons with modified measles can infect others. The absence or reduced severity of respiratory symptoms, particularly a cough, may result in lower infectivity relative to a classic measles infection [13, 14]. The ability to discern measles infection in persons with an SIR, however, is valuable for surveillance purposes in support of measles eradication efforts.

The absence of spread cases from the 2 physicians in this report suggests that there may be limited replication of virus in

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vaccinated persons with mild or short-lived symptoms. Although this report may raise questions regarding case classification for persons with a mild rash illness detected during a measles outbreak (eg, should positive laboratory results trump the clinical case definition?), the limitations of standard methods for confirmation (ie, IgM detection) in cases of modified or nonclassic measles may be better appreciated. The collection of viral samples in addition to serum samples is strongly recommended. An investigation into the timing of the rise and fall of neutralization titers in previously vaccinated persons with modified measles is underway.

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APPENDIX III

Sindbis-expressed measles virus hemagglutinin at the surface of whole cells:

a novel antigen to evaluate immunity

Partly based on Sara Mercader' s Master in Science Thesis

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ABSTRACT

To evaluate measles immunity, an easy-to-perform alternative to the currently used plaque reduction neutralization assay is needed. Recombinant measles virus hemagglutinin and fusion proteins on whole cells could serve as the protective antigen. A SIN-H virus, a Sindbis virus that expresses the hemagglutinin, was engineered. Immunofluorescence, radioimmunoprecipitation and fusion assays show that an authentic hemagglutinin was produced in 90% of SIN-H infected cells. Fixed whole cells infected with SIN-H were used in a prototype enzyme immunoassay to detect neutralizing antibodies. Four sera with increasing plaque reduction neutralization titers (134, 384, 2190, and 4096), but not a negative serum (titer of 8), reacted with the expressed hemagglutinin (optical signal increased at least three-fold). This proof of concept encourages the development of detection systems based on the co-expression of recombinant measles glycoproteins on whole cells. Cellular enzyme immunoassays are promising as simpler alternatives to evaluate measles immunity.

INTRODUCTION

Measles virus (MeV) is a highly transmissible human virus in the genera *Morbillivirus* in the family Paramyxoviridae. MeV causes measles, a childhood disease associated with serious complications, including death [21]. An effective measles vaccine became available in 1963. Worldwide, a two-dose vaccine schedule has reduced the estimated number of annual measles cases from 852,937 in 2000 to 278,358 in 2008 (a 67% decline), and the estimated number of deaths from 733,000 in 2000 to 164,000 in 2008 (a 78% decline) [9]. Measles vaccination has interrupted circulation of endemic measles in the Americas and other parts of the world [12, 25, 34, 37]. These accomplishments have been achieved in part through political will, epidemiological vigilance, and laboratory support. Laboratory support is critical in the detection and rapid confirmation of clinically defined measles and in providing requisite population-based data on measles immunity to evaluate protection for purposes of vaccination strategy and distribution [5]. Furthermore, in elimination settings, the measles diagnosis of cases presenting with vaccine-modified measles can be specially challenging. Vaccinated persons with waning immunity, or secondary vaccine failures (SVF), are difficult to diagnose due to their modified presentation of clinical symptoms and humoral responses; IgM responses may or may not be detected. In a recent paper, Hickman *et al.* described extremely high titers of measles protective antibodies in SVF. Because these protective titers are about 6 to 60 times higher (> 30,000) mIU/mL than the mean titers observed after routine measles vaccination, high titers of protective antibodies may constitute a biomarker to diagnose SVF [26, 33]. However, no rapid and easy-to-perform assay is yet available to assess measles protective immunity.

Laboratories worldwide routinely use enzyme immunoassays (EIA) in measles serology. Suspect measles cases are confirmed using measles-specific IgM EIAs. Measles seroconversion is determined using measles-specific IgG EIAs [36, 47]. However, these assays are based on either whole virus antigen or recombinant MeV nucleoprotein. The majority of antibodies detected in whole virus-based EIAs are targeted to the nucleoprotein [11, 36, 47]. Antibodies directed to the nucleoprotein, an RNA-associated protein, do not provide information regarding protection to

measles. Plaque reduction neutralization (PRN) assays, which are a measure of antibodies directed to the hemagglutinin (H) and fusion (F) membrane surface glycoproteins of measles virus, are accepted as the best correlate of protective immunity [1, 2, 48]. To detect protective antibodies, Bouche et al. described an H-EIA with an antigen consisting of fragmented cell membrane preparations expressing a recombinant H [7, 8]. When compared with a plaque neutralization test, H-EIA detected neutralizing IgG antibodies better than a whole virus-based EIA. Because F was not included in the antigen, anti-F antibodies were missed by H-EIA resulting in some false negative results; however, those samples were positive by plaque neutralization test and by an F-specific fluorescence activated cell scanner assay [24].

The goal of this project was to develop a widely accessible alternative assay to the PRN. The hypothesis was that whole cell membranes expressing H and F may display a more "native" conformation of the MeV antigen than lysates or purified protein that were chemically altered by attachment processes. To obtain proof of concept, a functional recombinant H was expressed at the surface of whole cells that were attached to EIA microtiter plates. This antigen was then used to detect neutralizing antibodies. For this purpose, a copy of the H gene was introduced between the two open reading frames of a double subgenomic Sindbis virus (SIN) vector [18, 38]. The expression, functional capabilities, and protective antigenicity of the Sindbis-expressed H are described in this report.

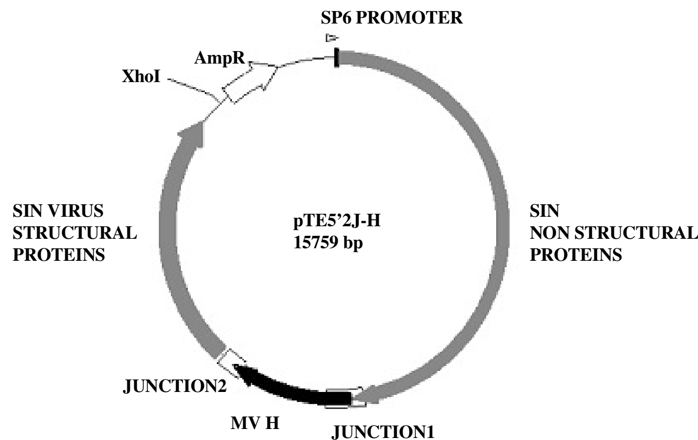
MATERIALS AND METHODS

Cell lines, plasmids, and viruses: Cell lines: Vero E6 (ATCC number: CRL-1586™) were propagated in Modified Eagle Medium containing 5% fetal calf serum, 1% penicillin-streptomycin (PS), and 1% L-glutamine.BHK-21 (ATCC number: CCL-10™) were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 0.1% gentamycin. MA160 (human prostate cells) and MA160/SSPE, which are MA160 cells chronically infected with the Mantooth strain of MeV were maintained in Roswell Park Memorial Institute Media 1640 containing 10% FBS and 1% PS[4]. MA160 served as negative control and MA160-SSPE as positive

control for immunofluorescence and radioimmunoprecipitation experiments. All cell lines were maintained at 37^{0}C in a 5% CO_2 environment. Plasmids: pTM1/MeV-H and pCMeV-H contained the full-length copy of Moraten vaccine strain MeV H gene, and pTM1/MeV-F and pCMeV-F held the full-length copy of Edmonston strain MeV F gene (gifts from Dr. P.A. Rota and B. Newton, CDC, Atlanta, GA); in pCMeV-H and pCMeV-F, the MeV genes were adjacent to a human cytomegalovirus promoter. pTE5'2J contained a modified cDNA copy of the SIN virus genome that allows insertion of heterologous genes (a gift from Dr. C.M. Rice, Washington University, St. Louis) [22, 35]; pS08 contains a copy of the β-galactosidase gene under the bacteriophage T7 promoter (a gift from Dr. B. Moss, National Institutes of Health) [43]. Virus: SIN-Hlp was produced from a pTE5'2J-H clone that had a deletion in the Kozak sequence located upstream from the ATG start codon of the H gene, as determined by PCR plasmid amplification and nucleotide sequencing, resulting in low production (lp) of H; SIN-H had no mutations. MeVA VVT7 is an attenuated vaccinia virus with a copy of the DNA dependent T7 RNA polymerase gene (a gift from Dr. B. Moss) [43]. For SIN-F construction, see *Production of SIN-H recombinant virus*.

Production of SIN-H recombinant virus: Standard protocols with minor modifications for cloning, screening and plasmid purification were followed [32]. First, pTM1/MeV-H was excised at the unique *Cla* I site, and the H gene was amplified (1854 bp) by polymerase chain reaction (PCR) using specific primer sets ($^{5'}$GCG**ACTAGT**ACCATGTCCATCATGGGTCTCAAG$^{3'}$and $^{5'}$CGC**ACTAGT**TTAGAGCGACCTTACATAGGA$^{3'}$). Primers contained flanking *Spe* I sites (bold letters) for insertion into pTE5'2J. PCR was performed with an Ex Taq Polymerase Kit (TaKaRa Shuzo Co. Ltd., Japan) and the following cycling protocol: 20 seconds at 98°C, one minute at 40°C, and three minutes at 70°C for 25 cycles with a final incubation at 0°C. To construct pTE5'2J-H (Figure 1), the *Spe* I- restricted MeV-H amplicons were ligated with T4 DNA Ligase to the Xba I-restricted linear pTE5'2J, which had been adequately prepared for cloning [35]. Competent MC1061 Escherichia coli cells were transformed with pTE5'2J-H ligation mixtures, (or with *Xba* I-restricted control vector pTE5'2J), and ampicillin resistant colonies containing MeV H were selected. Plasmids with correctly oriented MeV H gene were identified by restriction enzyme digestion, purified by

Figure 1 *Schematic diagram of the recombinant pTE5'2J-H construct: SIN open reading frame (ORF) sequences are represented by gray arrows. The inserted MeV H gene is shown as a black arrow, located between the nonstructural and the structural SIN ORFs. The phage SP6 promoter used for the* in vitro *transcription of the SIN and foreign gene sequences is shown as an arrowhead above a small black box. The white arrow represents the plasmid encoded ampicillin resistance gene. XhoI plasmid linearization site is also indicated. SIN: Sindbis virus; MeV: measles virus; AmpR: ampicillin resistance gene.*



CsCl-Ethidium Bromide, linearized through the unique *Xho* I site, and prepared for *in vitro* transcription, which was performed with SP6 DNA-dependent RNA polymerase (Epicentre Technologies, Madison, WI) [32]. Resulting RNA transcripts were transfected into BHK-21 cell monolayers using 8 µl of Lipofectamine, 200 µl of OPTIMEM medium and 10 µl of transcription reaction and an incubation of two hours at 37°C; the negative control (MOCK) consisted of Lipofectamine/OPTIMEM mix without RNA. The following day, SIN-H and SIN virus stocks were harvested and stored for later use at -70°C. Viruses were titrated by plaque assay. Similarly, SIN-F recombinant virus was generated, but, in this case, MeV F (1662 bp) gene was PCR-amplified from circular pTM1/MeV-F using specific primer sets $^{5'}$GCG**ACTAGT**ACCATGTCACCACAACGAGACCGG^{3} and $^{5'}$CGC**ACTAGT**TTATCTGCGATTGGTTCCATC$^{3'}$. The level and location of recombinant H expression in BHK-21 cells was examined in acetone-fixed cells by indirect immunofluorescence 18 hours after transfection using a 1:270 dilution of monoclonal antibody (MAb) 8-II-366 which is specific for a conformational epitope of H (kindly supplied by E. Norrby, Karolinska Institute, Stockholm, Sweden), and rabbit polyclonal anti-SIN antibody (a gift by T.K. Frey, Georgia State

University, Atlanta, GA), and 20 µg/ml of highly adsorbed fluorophore-conjugated secondary antibodies ALEXA 488 anti-mouse IgG and ALEXA 594 anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) [3, 46].

Infections: SIN or recombinant SIN virus were diluted in 0.5 ml of media to achieve the desired multiplicity of infection (m.o.i.), and incubated on cell monolayers at 37°C. One hour later, maintenance media was added and then used the following day at the time post-infection indicated for each experiment. Infection controls were SIN virus derived from pTE5'2J transfection plates (positive control) and MOCK transfection media (negative control).

Surface immunofluorescence: BHK-21 cells were infected with SIN-Hlp (5 p.f.u./cell) and SIN-F (5 p.f.u./cell) viruses. At 20 hours after infection, the cells were scraped off, suspended into 5 ml of PBS, washed three times with ice-cold PBS by low speed centrifugation (4°C for five minutes), and incubated 30 minutes on ice with the H specific MAb 80-III-B2 at a 1:150 dilution in PBS with 1% BSA buffer; 80-III-B2 antibody detects a linear epitope [20, 39]. After two washes, the living cells were incubated in the dark for 30 minutes on ice with Alexa Fluor® 568-conjugated goat anti-mouse IgG antibody (6.67 µg/ml) and 0.5 µM of Hoechst 33342, a nucleic acid stain that can cross the cell membrane. Cells were fixed with 1% paraformaldehyde in deionized H_2O at 4°C for 15 minutes. Paraformaldehyde is a reversible protein cross-linker used to fix and preserve cell structure [17]. Pictures were obtained using a Zeiss Laser Scanning Microscope (LSM) 410 coupled to an Axiovert 135 (Carl Zeiss Microimaging, Inc. Thornwood, NY).

Surface radioimmunoprecipitations: All incubations were performed at 37°C, unless otherwise stated. The m.o.i for each virus was 5 p.f.u./cell. BHK-21 monolayers were infected with SIN-Hlp, SIN, MOCK, or SIN-Hlp plus SIN-F. At 13 hours after infection, the medium was replaced with DMEM (without methionine) containing 2% FBS; cells were incubated for 20 minutes, washed, and incubated again for 20 minutes. Next, cells were pulsed-labeled for one hour with 100 µCi/ml of Tran ^{35}S-Label containing 70% L-methionine (^{35}S) and ~15% L-cysteine (^{35}S) (original specific activity 1175 Ci/mmole; ICN, Costa Mesa, CA) for one hour, washed twice with PBS, and chased for

four hours with 5 ml of maintenance media. MeV H was incubated with MAb 8-II-366 at a 1:40 dilution in PBS for 15 minutes at 4°C. Following two washes with ice-cold PBS, cells were lysed with RIPA buffer with proteinase inhibitors (Complete-EDTA free, Roche Molecular Biochemicals, Indianapolis, IN) and 1 mM EDTA (RIPA /PI/E), and resulting cell lysate was clarified, aliquoted and stored at -70 °C [29]. For immunoprecipitation, 100 µl of the cell lysate and 25 µl of Gammabind G (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in RIPA/PI/E were incubated one hour at 4°C while shaking, then centrifuged (815 x g) for 10 minutes at 4°C; Gammabind G helps purify antibodies by binding to all IgG subclasses. The pellet was washed three times with RIPA/PI/E and once with RIPA alone, and proteins were resolved by SDS-polyacrylamide gel electrophoresis [28]. The gel was fixed with Amplify (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), dried, and autoradiographed using KODAK BioMax film (Eastman Kodak Company, Rochester, NY).

Fusion assay: A previously described method was followed to observe fusion promoted by the recombinant SIN-H protein [6, 43]. As described in Figure 4, cells termed effector cells were Vero E6 infected with SIN-H (5 p.f.u./cell), transfected with 0.5 µg pCMeV-F, and infected with MeVA VVT7. Cells termed indicator cells were Vero E6 transfected with pS08. Included controls were cells co-transfected with pCMeV-F and pCMeV-H plasmids (positive control), and cells infected with SIN-H alone (negative control).

Hemagglutinin cell-based EIA (H-CELISA): Vero E6 cells or BHK-21 cells were grown in 96-well fibronectin-coated tissue culture plates. Vero cells were either infected (2 p.f.u./cell) with SIN (two negative wells; N) or with SIN-H (two positive wells; P). BHK-21 cells were mock-infected or infected (100 p.f.u./cell) with SIN, SIN-Hlp, or SIN-Hlp and SIN-F. Forty hours after infection, plates were washed and fixed with 1% paraformaldehyde for 15 minutes at 4°C. Cells were washed again and incubated at 37°C for one hour with primary antibodies, as follows: BHK-21 with MAb 8-II-366 diluted at 1:150 in PBS with 1%BSA, and Vero E6 cells with sera diluted at 1:100 in appropriate serum diluent; four protective sera had PRN titers of 134, 384, 2190, and 4096, and one non-protective serum had a PRN titer of 8 (PRN titers >120 are considered protective) and served as a

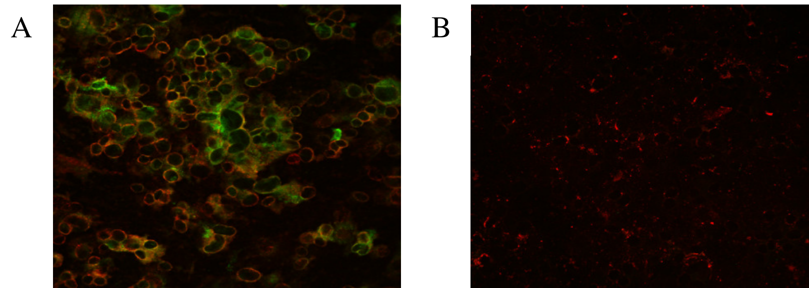
negative control serum [1, 10]. The remainder of the procedure, including calculation of P-N and P/N values, was carried out as previously described [27]. P-N and P/N values at least three times greater than that of the negative control were considered positive. To validate surface antigen detection, a MAb was used to detect actin (a cytoskeletal protein) in cells fixed with paraformaldehyde (non-permeable membranes; control for surface antigen detection) and in cells fixed with acetone (permeable membranes; positive control for actin detection). Quality control passed if detection of actin was negative.

RESULTS

Expression of SIN-derived MeV H

BHK-21 and Vero E6 cells produced recombinant measles H from a double subgenomic SIN vector. BHK-21 cells transfected with pTE5'2J-H and fixed with acetone exhibited more than 90% of the cells co-stained for H and SIN proteins, suggesting an active production of H in nearly all transfected cells. H was not detected in the cytoplasm of transfected cells (Figure 2). Vero E6 cells infected with SIN-H expressed sufficient concentration of functional H at the cell membrane to lead to fusion of as many as 50 contiguous cells in a fusion assay (Figure 3). SIN recombinant H was inserted at the surface of BHK-21 as demonstrated by immunofluorescence and immunoprecipitation experiments under living conditions (Figures 4 and 5). Three-dimensional scanning with a confocal microscope revealed specific fluorescence at the rim of positive cells previously labeled with a MAb targeted to a linear epitope, thus demonstrating that H was exposed at the cell surface (Figure 4). An immunoprecipitation experiment using an MAb specific for a conformational epitope further confirmed that H was localized at the cell surface; a band of 78 kDa migrated with the H protein positive control cells (Figure 5, lane 6), but was not present in lysates lacking H (Figure 5, lanes 2

Figure 2 *Recombinant H synthesis in transfected BHK-21 cells.* ***A*** *BHK-21 cells were transfected with in vitro transcribed RNA from pTE5'2J-H.* ***B*** *Mock-transfected cells. Transfected cells were acetone-fixed and co-stained using a polyclonal anti-SIN rabbit serum (red color) and an anti-H mouse Mab (green color).*



and 7). By H-CELISA using BHK-21 cells, SIN-Hlp infected cells yielded OD values six times higher than mock infected cells and four times higher than SIN infected cells (Figure 6). Protective PRN titers were detected by this H-CELISA. Corresponding P-N values were at least 0.012, three times greater than a negative sample. Samples with PRN titers equal to 384 or greater resulted in P-N values that were at least five times the P-N value of a negative sample. Detection was specific for surface antigens because actin was not detected in cells previously fixed with paraformaldehyde. BHK-21 cells were not used in the H-CELISA because they detached from the plate.

DISCUSSION

To better assess questions pertaining to measles antibody immunity, a more rapid and practical alternative to the currently used plaque reduction neutralization assay is needed. An authentic MeV H was expressed that is fully functional, as it allows membrane fusion to occur in the presence of MeV F and is recognized by neutralizing antibodies in human convalescent serum specimens. The presented findings demonstrate that MeV H expressed at the surface of cells attached to microtiter plates (H-CELISA antigen) is capable of serving as antigen for a cellular EIA.

Figure 3 *Analysis of SIN recombinant H fusion activity:* ***A and B*** *Vero cells infected with SIN-H, co-infected with MeVA VVT7 and co-transfected with pCMeV-F were termed effector cells. Vero cells transfected with pS08 were termed indicator cells. Two hours after infection with MeVA VVT7, effector cells were overlaid with trypsinized indicator cells and the plates were incubated overnight. Effector and indicator cells were fixed with 0.2 % glutheraldehyde/2 % formaldehyde and stained with X-Gal 48 hours after infection with SIN-H and 5 hours after cells were overlaid with indicator cells. Resulting multinucleated cells were stained with X-Gal and manually counted.* ***C and D*** *Negative control was SIN-H and MeVA VVT7 co-infected cells. Pictures in panels A and C were taken using a 10X objective. Pictures in panels B and D were taken using a 32X objective. Black arrow heads indicate sites of fusion activity. White arrow heads point to groups of nuclei.*

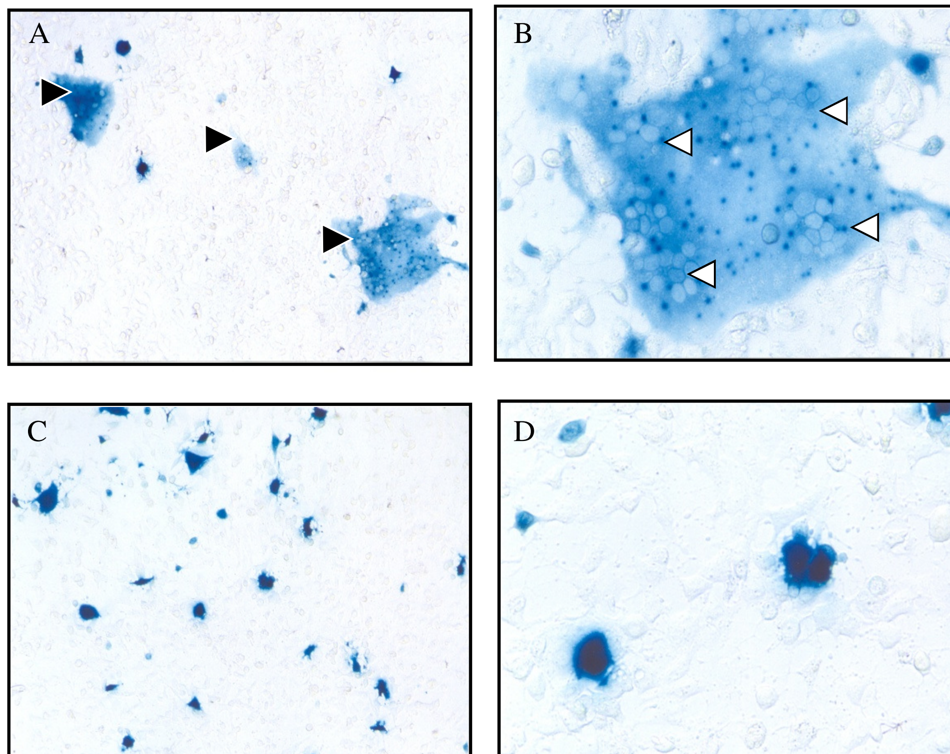
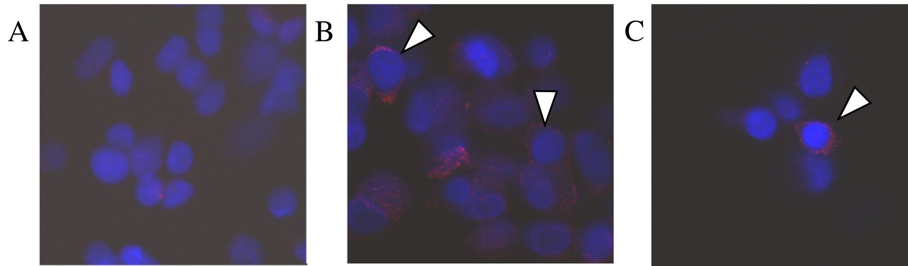


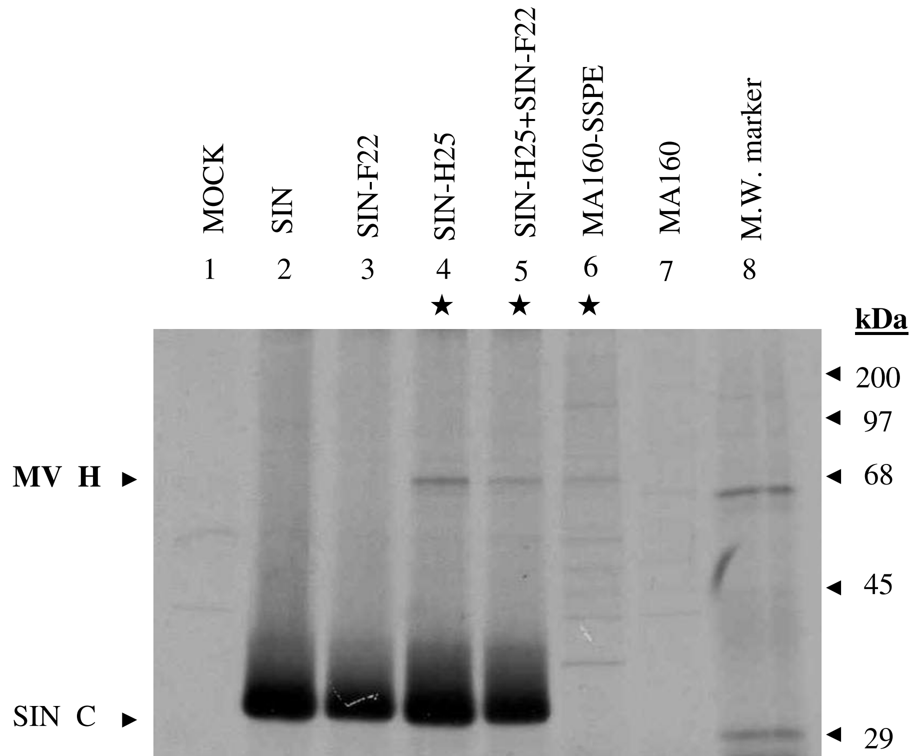
Figure 4 *Recombinant MeV H at the surface of infected BHK-21 cells:* ***A*** *SIN.* ***B and C*** *SIN-Hlp plus SIN-F. Cells were incubated with 80-II-B2 MAb, followed by an anti-mouse IgG conjugated with ALEXA 568 (red color). Cellular DNA was counterstained with Hoechst 33342 (blue color). In this procedure cells were fixed with paraformaldehyde after reaction with primary and secondary antibodies. White arrows point to cells that express H at the cell surface.*



Two features of PRN should be considered in the development of EIAs to measure protective responses to measles to increase assay sensitivity. First, the antigen should consist of H and F to allow detection of all neutralizing antibodies [13]. In whole cells, co-expression with F should assist in exposing some otherwise hidden H epitopes, thus increasing binding of anti-H antibodies [42]. Furthermore, by using recombinant H and F, assay specificity should also increase. Second, assays should detect all classes of measles-specific immunoglobulins (IgG, IgA and IgM) [16, 36].

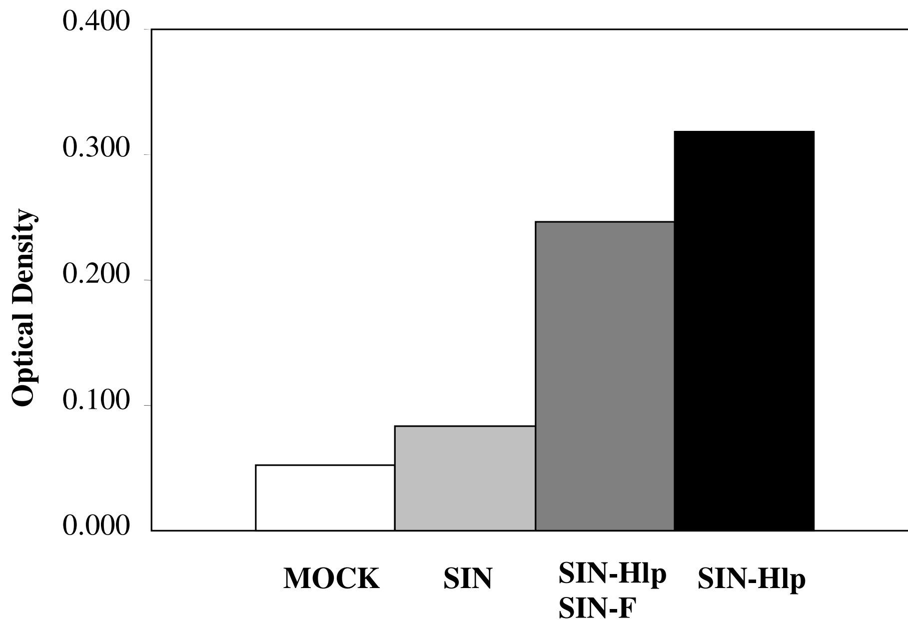
A limitation of this study is the small panel of specimens used to validate the H-CELISA antigen. However, the observed positive relation between anti-H antibody detection by H-CELISA and PRN titers is promising, and thus warrants confirmation with a larger panel of samples. A second limitation is that only the expression of H, not H and F, in Vero cells is reported. Whole-cell based EIAs rely on cell monolayers that are resistant to the multiple washings of the assay [14, 15, 30, 40, 41]. Initially, BHK-21 cells (hamster kidney cells) were selected because SIN can replicate well in

Figure 5 *Immunoprecipitation of surface MeV H protein from SIN-Hlp infected cells: BHK-21 cells were infected with the viruses indicated below. Cells were pulse-chase radiolabelled with ^{35}S-methionine, incubated with 8-II-366 Mab, lysed and immunoprecipitated. The position of the MeV H and SIN C (which bind nonspecifically to protein A agarose) bands are indicated. Samples with immunoprecipitated H protein are indicated with a black star.*



this cell type. Additionally, BHK-21 cells lack MeV receptors. Therefore, co-expression of H and F would not lead to membrane fusion [48]. Unfortunately, BHK-21 proved to be too fragile to endure the rigorous washing procedures, thus precluding further use of this cell type. To ensure cell attachment to the microplates Vero-E6 cells were used, which proved to be wash resistant. Because Vero-E6 cells have MeV receptors, the decision was made to only express the MeV H; the

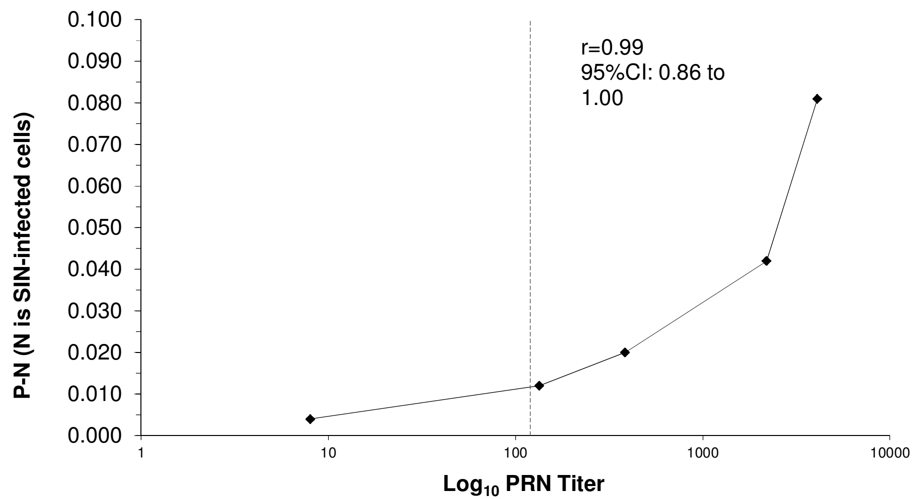
Figure 6 *H-CELISA detection of cell surface recombinant H. Infected BHK-21 cells were fixed with paraformaldehyde. Recombinant H protein at the cell surface was detected using 8-II-366 MAb. Mock infected cells (white bar), SIN infected cells (light gray bar), SIN-Hlp plus SIN-F (dark gray bar), SIN-Hlp infected cells (black bar).*



glycoprotein that generates the majority of neutralizing antibodies. To avoid cell fusion, MeV F was not expressed. A third limitation was that, the authors chose to detect only IgG antibodies to simplify this proof of concept.

Several attempts have been made to simplify the PRN assay by using either fluorescent MeV particles or immunofluorescence-based assays [13, 19, 23]. Additionally, a syncytium inhibition assay and an acetone-fixed PRN method have also been described [31, 45]. Although simpler, and less time-consuming than PRN, these assays are based in cell culture and some may require instrumentation not always available to basic serology laboratories (fluorescence reader, FACS scan). In contrast, EIAs to detect neutralizing measles antibodies could readily be implemented in measles serology laboratories. EIA is already a standard technique that is easy and relatively economical, and is used to process large number of samples. Furthermore, microtiter plates could be prepared in advance with the H-CELISA antigen, thus eliminating the need for cell culture by the end user [44].

Figure 7 *Detection of protective antibodies by H-CELISA: Neutralizing antibodies in sera recognize the SIN-derived MeV H protein expressed at the surface of whole Vero E6 cells infected with SIN-H. Black diamonds represent each serum. Individuals with pre-exposure serum plaque reduction neutralization titers lower than 120 (dashed vertical line) are not protected against measles. Y-axis is P-N values. X-axis is the 10 logarithm of the plaque reduction neutralization titer.*



In conclusion, new assays to measure measles immunity would benefit from employing membrane-embedded recombinant MeV glycoprotein antigens and detection of all classes of neutralizing antibodies (IgG, IgM and IgA), as in PRN. This paper documents proof of concept that attached whole cells expressing recombinant MeV H are a compatible antigen for the detection of IgG neutralizing antibodies using an EIA format. Protective assays based on the EIA technology should allow rapid sample throughput, and easy technology transfer to laboratories worldwide.

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APPENDIX IV

RESUM DELS ARTICLES INCLOSOS A LA TESI

1. *Comparació de mètodes per a l'elució del sèrum de mostres de sang assecada en taques per a ser emprat en el diagnòstic serològic del xarampió*

Es van comparar sis protocols per a l'extracció del sèrum de mostres de sang assecada en taques sobre paper de filtre per tal de fer una avaluació del seu ús en el diagnòstic serològic del xarampió. L'avaluació va incloure els següents criteris: la detecció d'IgM i d'IgG anti-xarampionoses mitjançant els tests immunoenzimàtics de Dade Behring Enzygnost® (Siemens Enzygnost®), el volum recuperat després de l'elució, la reproducibilitat, el temps de processament, el rendiment, la dificultat del protocol, l'equipament necessari, la seguretat i el cost. Els resultats van indicar que en 4 dels protocols estudiats la detecció d'IgM anti-xarampionosa en les mostres eluïdes va ser com en el sèrum, i només es van observar diferències significatives seguint els 2 protocols restants ($p < 0,05$). Tanmateix, la diferència observada no va resultar en un canvi de classificació del resultat. En quant a la detecció d'IgG anti-xarampionosa, no es van detectar diferències en mostres de valors d'IgG positius o negatius. Només es va trobar diferències significatives en una mostra de valor d'IgG indeterminat ($p < 0,05$). En referència al volum de mostra recuperada, 4 dels protocols van resultar en un volum de mostra suficient per a realitzar la prova d'IgM, mentre que tots els protocols van generar suficient volum per a fer la prova d'IgG. Si bé tots els protocols són relativament fàcils de realitzar, només dos es poden finalitzar en menys de 2 hores. En general, els protocols estudiats funcionen bé per a l'extracció d'anticossos IgM i IgG anti-xarampionosos. Les diferències s'observen bàsicament en la recuperació del volum eluït, el temps d'execució, l'equipament i el cost. A l'article es proposa un protocol de fàcil

implantació per a l'extracció ràpida del sèrum per al diagnòstic serològic del xarampió per a ser utilitzat en situacions de brot dins del marc de la Xarxa Global de Laboratoris de Xarampió i Rubèola de l'Organització Mundial de la Salut.

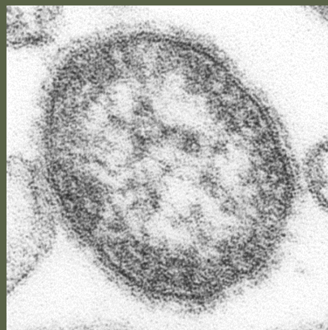
2. Caracterizació de la resposta d'anticossos produïda durant la infecció pel virus del xarampió en pacients prèviament vacunats i en pacients no vacunats

La disminució de la immunitat, o la fallada vacunal secundària (FVS), és considerada per alguns com un possible entrebanc als esforços cap a l'eliminació global del xarampió. Encara que els casos de FVS són poc freqüents, la infecció amb el virus del xarampió pot tenir lloc en individus vacunats si hi ha hagut una exposició intensa i / o perllongada a una persona infectada. En tal cas la malaltia es pot presentar com una malaltia modificada que és irreconeixible com a xarampió fora del context d'un brot. A més, la resposta d'IgM en individus prèviament vacunats pot ser minsa o fugaç, i la replicació viral pot ser limitada. A mesura que avança l'eliminació global, caldran mètodes addicionals per tal de confirmar els casos de xarampió amb presentacions clíniques modificades i per a estudiar si aquests casos de FVS contribueixen a la transmissió del virus del xarampió. En aquest informe es descriuen els símptomes clínics i els resultats de laboratori de persones no vacunades que presenten símptomes de xarampió agut i de persones amb FVS, identificades durant brots de virus del xarampió. Els casos de FVS es van caracteritzar mitjançant paràmetres serològics d'anticossos d'alta avidesa i de nivells clarament elevats d'anticossos neutralitzants. Aquests paràmetres poden representar biomarcadors útils per a la classificació de casos de FVS, els quals no haurien pogut ser confirmats amb les tècniques rutinàries de diagnòstic de laboratori.

3. Test d'avidesa de la IgG anti-xarampionosa: Ús en la classificació de casos amb fallada vacunal dins del context d'eliminació

En regions on s'ha eliminat el virus del xarampió endèmic es necessiten proves de diagnòstic addicionals per a ajudar en la confirmació de cada cas sospitós de xarampió independentment de l'estat de vacunació. Es va modificar un test immunoenzimàtic comercial per a la detecció de les IgG específiques contra el virus del xarampió tot incloent-hi tres rentats de 5 minuts amb dietilamina (60 mM; pH 10,25) realitzats després de la incubació del sèrum, prèviament diluit en sèrie. Els resultats es van expressar com a índex d'avidesa dels títols d'extinció de les senyal òptiques de les corbes de dilució del sèrum tractades amb i sense dietilamina. L'anàlisi de la característica operacional del receptor (ROC, en l'acrònim anglès) va ser emprada per a l'avaluació i la validació del test d'avidesa, i també per a establir els llindars de baixa (≤ 30%) i alta (≥ 70%) avidesa. L'anàlisi de 319 mostres de sèrum, de les quals s'esperava que continguessin anticossos d'alta o baixa avidesa segons les dades clíniques i epidemiològiques, indica que aquesta prova té un rendiment diagnostic molt alt, amb una àrea sota la corba ROC de 0,998 (Interval de Confiança (IC) al 95%: 0,978-1,000), una sensibilitat del 91,9% (IC 95%: 83,2% -97,0%) i una especificitat del 98,4% (IC 95%: 91,6% -100%). Aquest test és ràpid (<2 hores) i precís (desviació estàndard: 4%-7%). La prova d'avidesa es va fer servir per a analitzar 18 mostres d'un brot ocorregut en una regió on el virus del xarampió endèmic ja s'havia eliminat. Els resultats de baixa avidesa van identificar dos casos de xarampió agut en mostres amb resultats positius per a IgM anti-xarampionoses. Els resultats d'alta avidesa van ajudar a classificar com a fallades vacunals secundàries a 15 pacients amb símptomes de xarampió modificat. El cas restant no es va poder interpretar perquè el resultat va ser d'avidesa intermèdia. En un context d'eliminació i en conjunció amb la informacio clínica i epidemiològica adequada, les proves d'avidesa de la IgG anti-xarampionosa poden complementar les eines actuals de diagnòstic per a confirmar els casos aguts de xarampió i ajudar en la classificació de fallades vacunals.

In regions where endemic measles virus circulation has been interrupted, laboratory confirmation of measles is like puzzle solving. The complexity of these puzzles depends on the available pieces of clinical, epidemiological and laboratory information. The main goal of this dissertation is to evaluate diagnostic laboratory tools to aid in suspected case confirmation in these settings. First, protocols to elute measles IgM and IgG antibodies from blood spots dried onto filter paper were compared to propose one that will permit the recovery of the maximum volume of eluted sample in the minimum time, effort and cost. An easy-to-implement protocol is proposed for the rapid extraction of serum for measles/rubella serology in outbreak situations for use in the World Health Organization Global Measles and Rubella Laboratory Network. Second, due to inherent limitations of measles specific IgM enzyme immunoassays and molecular methods used for measles confirmation, not all suspected cases can be resolved. For example, IgM and RNA may not be detected in vaccinated cases with waning immunity (secondary vaccine failures) and presenting with modified measles. The observation is made that serological parameters of elevated titers of high avidity neutralizing antibodies correlate with measles secondary vaccines failures and may be useful biomarkers for confirming secondary vaccine failures that cannot be confirmed otherwise. Third, a highly accurate measles IgG avidity enzyme immunoassay for vaccine failure classification is described. Detection of low avidity antibodies using this highly sensitive and specific avidity assay can complement existing measles diagnostic tools in confirming suspected cases when routine IgM testing may be inconclusive. Therefore, these diagnostic approaches can provide additional laboratory information to resolve suspected cases irrespective of vaccination status. Together, data presented in this dissertation may assist in enhancing measles control and surveillance in elimination settings.



Thin-section transmission electron micrograph of a single measles virus particle
(Source: Centers for Disease Control and Prevention, C. Goldsmith and W. Bellini, Ph.D.)