

Review

The role of laboratory confirmations and molecular epidemiology in global eradication of measles

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This review reports on the role of laboratory confirmation and molecular epidemiology in global eradication of measles. The role of laboratory confirmation and molecular epidemiology in defining the origins of measles outbreaks cannot be overemphasized. New serological tests based on recombinant proteins detect only a fraction of the total measles virus (MV) specific antibodies. Several assays based on recombinant MV-haemagglutinin (ELISA and flow cytometry) or MV-fusion protein (flow cytometry) as well as neutralization and haemagglutination test have been evaluated using a large panel of low-titre and negative sera. Isolation of measles virus confirmed the diagnosis. Phylogenetic trees are invaluable tools for monitoring the progress of immunization activities. Recent advances in genomic sequencing technology have lent its support to the monitoring and evaluation of vaccination programmes. More so, indigenous prepared measles antigens has been advocated to be produced, refined further and reproduced massively. This will be highly cost effective especially in field for seromonitoring and surveillance of measles. There is therefore, continual need for simpler diagnostic tests in elimination and eventual eradication of measles.

Key words: Active surveillance, elimination, eradication, haemagglutination inhibiting test, measles, measles antigens, molecular epidemiology, phylogenetic trees.

INTRODUCTION

Measles is also one of the vaccine-preventable diseases (Washington State Department of Health, 2006) and hence one of the World Health Organization (WHO) ex-

panded programme on immunization (EPI)-target diseases for eradication (WHO, 1998, 1999). It accounts for nearly half of the 1.7 million annual deaths due to childhood vaccine-preventable diseases (Baba et al., 2007). The measles virus (MV) is monotypic and essentially without animal reservoir (Norrbj et al., 1975). As a result, measles control and eradication should be within

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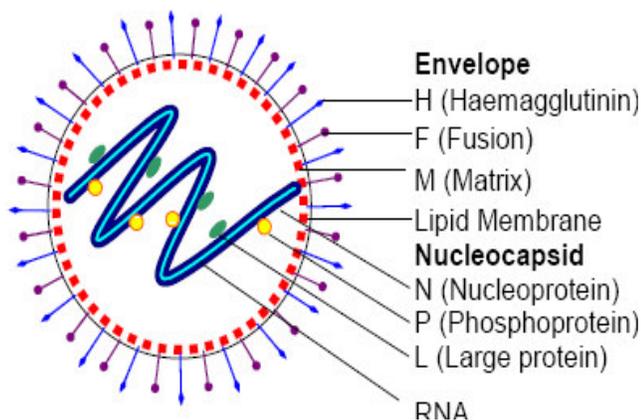


Figure 1. Antigenic Structure of Measles virus (WHO, 2007).

reach of aggressive surveillance and vaccination campaign. Moreover, the virus is antigenically stable making the vaccine derived from the 1954 Edmonston isolate protective against all MVs (Rota et al., 1994a, b) and able to interrupt its transmission from infected to immune individuals. The industrialized nations of the world (for example, the United States) have used the live attenuated MV vaccine to eradicate transmission of indigenous measles strains (Benenson, 1990). Before the introduction of measles vaccines in the 1960s, almost everyone contacted measles usually during childhood with an estimated 2.5 million death [mainly children] out of about 130 million cases every year (Clements et al., 1992).

Measles is endemic worldwide. Although it was declared eliminated from the U.S. in 2000, high rates of vaccination and good communication with persons who refuse vaccination is needed to prevent outbreaks and sustain the elimination of measles in the U.S (Parker et al., 2006). Of the 66 cases of measles reported in the U.S. in 2005, slightly over half were attributable to one unvaccinated individual who acquired measles during a visit to Romania (CDC, 2006a,b). This individual returned to a community with many unvaccinated children. The resulting outbreak infected 34 people, mostly children and virtually all unvaccinated; 9% were hospitalized and the cost of containing the outbreak was estimated at \$167,685. A major epidemic was averted due to high rates of vaccination in the surrounding communities (Parker et al., 2006).

Measles virus (MV) is the agent of acute illness called measles that has been reported as highly contagious and universal in occurrence. Measles produces significant illness, death and disability (Pan American Health Organization, 2005) and infects approximately 40 million people resulting in nearly 1 million deaths annually in developing countries (Oldstone, 1998). It has been reported as a major cause of childhood morbidity and mortality in Nigeria; 212,183 and 168,107 cases were recorded in 2000 and 2001 respectively (WHO, 2001). For instance in 2005, Adamawa State, Nigeria exper-

ience 3,974 cases and 238 measles-deaths (Dubray et al., 2005). WHO, AFRO (2004) reported measles as one of the top five causes of death in children less than five years of age in many African countries. Measles disease is usually characterized by fever of 38°C or more; maculopapular rash of about 3 days or more; with one or a combination of coryza, cough, conjunctivitis and Koplik spots in the oral mucosa of measles' victims (White and Fenner, 1994). It is spread through respiration (contact with fluids from an infected person's nose and mouth, either directly or through aerosol transmission) and is highly contagious, 90% of people without immunity sharing a house with an infected person will catch it.

As measles can pose a significant threat to a person's health, every effort should be made to arrest the indigenous transmission of measles in Nigeria. Measles elimination is defined as the interruption of indigenous measles virus transmission (that is, measles is no longer an endemic disease) in a large geographical area as a result of deliberate efforts. Continuing intervention is required while measles eradication is defined as the interruption of measles transmission worldwide as a result of deliberate efforts; intervention methods may no longer be needed. Eradication represents the sum of successful elimination efforts in all countries (WHO, 2000). There is continual need for simpler diagnostic tests in elimination and eventual eradication of measles. Therefore, this review reports on the role of laboratory confirmation and molecular epidemiology in the global eradication of measles.

AETIOLOGY OF MEASLES DISEASE

Measles is an acute viral illness that has been reported as highly contagious and universal in occurrence. It is caused by the measles virus (MV) (White and Fenner, 1994). Measles virus (MV) is an enveloped virus belonging to the genus *morbillivirus* of the family-paramyxoviridae (Figure 1). It has a non-segmented, negative sense, single stranded RNA as its genome (White and Fenner, 1994). The RNA genome contains 15,894 nucleotides encoding six structural and two nonstructural proteins. These are the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin (H) and large polymerase (L). The nonstructural proteins V and C are derivatives of the viral P mRNA (Takeuchi et al., 2002).

TRANSMISSION OF MEASLES VIRUS

The highly contagious measles virus is spread by airborne droplets (circulating as a result of coughing and sneezing), close personal contact or direct contact with nasal or throat secretions of infected persons as well as through aerosols. Consequently, measles tends to occur as epidemics which may cause many deaths, especially

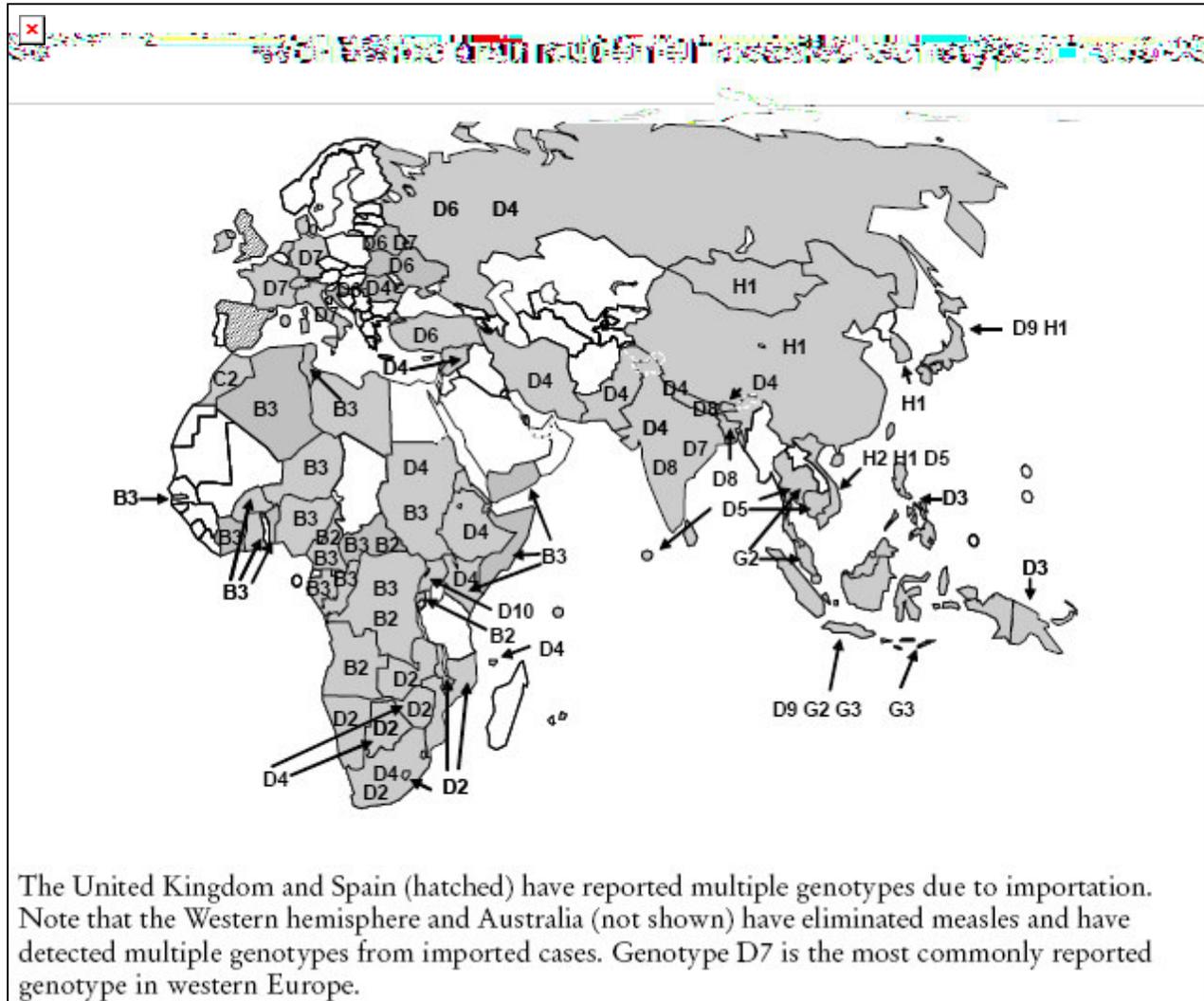


Figure 2. Global Distribution of MV in WHO regions. Distribution of measles genotypes associated with endemic transmission in various areas of the world based on information available in 2002. Genotype designations are shown for each measles-endemic area where virologic surveillance has been conducted. Source: WHO (2007).

among young malnourished children. The virus remains active and contagious in the air or on infected surfaces for up to two hours. It can be transmitted by an infected individual from four days prior to the onset of the rash to four days after the onset. If one person has the disease, a high proportion of their susceptible close contacts will also become infected with the measles virus (WHO, 2007).

In densely populated areas, measles most commonly affect children aged 3 - 4 years old while in less crowded areas, highest incidence was among children aged 5 - 10 years who contracted the disease on entering school (CDC, 2004a). Overcrowding in developing countries facilitates the ease of person-to-person transmission of the virus and increases the likelihood of exposure to high viral loads (Tulchinsky et al., 1993).

In temperate climates, epidemics of measles tended to

occur at 2-5 years intervals and lasted 3-4 months. Generally, larger size of a community leads to a shorter interval between epidemics (WHO, 2007). Although there is only one serotype of MV, genetic variability exists in wild type viruses. WHO currently recognizes 23 genotypes of MV (WHO, 2005) with 16 genotypes identified since 1990 (Figure 2). This variation however does not appear to be biologically significant as the vaccines available protect against them all (WHO, 2007).

PEOPLE MOST AT RISK OF MEASLES DISEASE

Un-immunized persons, especially young children, are at highest risk for measles and its complications, including death. Measles can also affect un-immunized older children, adolescents and young adults. All people who have not

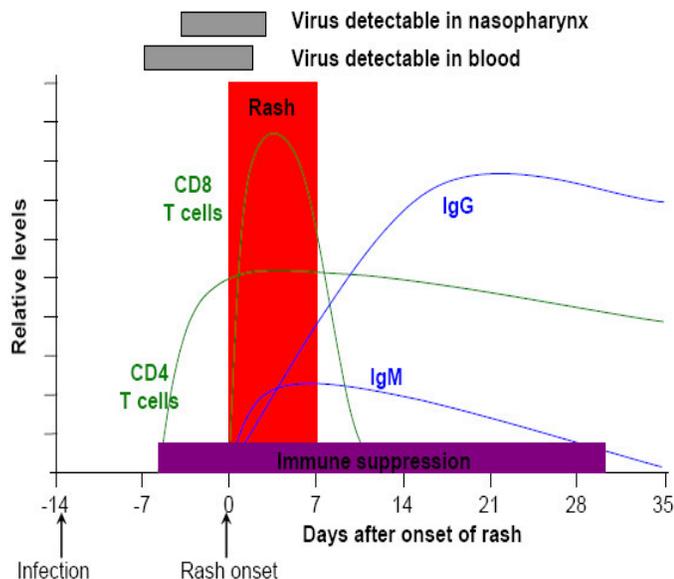


Figure 3. Antibody Response against measles (WHO, 2007).

been immunized with vaccine or who have not acquired immunity through having experienced the disease can become infected. Measles can be particularly deadly in countries experiencing or recovering from war, civil strife or natural disasters. Infection rates soar because damage to infrastructure and health services interrupts routine immunization and overcrowding in camps for refugees and internally displaced persons greatly increase the risk of infection (WHO, 2007).

It causes high mortality in poor populations, particularly among children less than 5 years of age. Case fatality rates in developing countries was estimated to vary between 2 - 6% and as high as 30% during outbreaks (Anon, 1989). Although other primate species may be infected through contact with human, no animal reservoir of measles virus has been identified (Albrecht *et al.*, 1977, 1980). Children and young adults do not usually die directly of measles, but from its complications such as pneumonia and diarrhea as a result of the immunosuppression associated with measles infection.

IMMUNOLOGY OF MEASLES

MV infection induces both humoral and cell mediated immunity (CMI) and provides a life long immunity (Bernstead *et al.*, 1993). Primary infection induces high titres of IgM and IgG which can be detected in serum within few days of rash onset and specific secretory IgA antibodies in nasal specific secretions as illustrated in Figure 3 (Pringle and Heath, 1990; WHO, 2007). With sensitive IgM detection ELISA assays, 90% of measles cases are IgM positive at 3 days past rash onset (Triples *et al.*, 2003). Serum and secretory IgA antibodies levels peak within about 4 weeks and persists long after infec-

tion (Bernstead *et al.*, 1993; WHO, 2007). Figure 2 shows the antibody response against measles.

Re-exposure to MV induces a strong anamnestic immune response with a rapid boosting of IgG antibodies which prevents clinical disease (WHO, 2007), while waning antibodies are found in measles vaccines (Kremer *et al.*, 2006), re-exposure to wild type virus have been found to stabilize specific IgG antibody levels in late convalescents (Kremer *et al.*, 2006). Cytotoxic T cells and natural killer cells (NKC) which constitute cell mediated immunity plays a prominent role in immunity and recovery from acute infection. Patients with defective CMI often suffer severe progressive measles infections and have an increased risk of death (Pringle and Heath, 1990; WHO, 2007). Measles specific immune suppression begins with the onset of clinical disease, before the rash and continues for several weeks after apparent recovery (Redd *et al.*, 1999).

It is known that a one-dose one-time vaccination programme may not be sufficient in conferring immunity against measles. Vaccine-induced protection has been well-documented to be less durable and less robust than naturally-acquired immunity against measles virus (Mossong and Muller, 2003) and high occurrence of symptomatic but mild measles due to secondary vaccine failures has been found among measles patients vaccinated over a decade ago, especially among those who were revaccinated (Paunio *et al.*, 2000). Protection against measles could be incomplete as a result of vaccine failure (Mathias *et al.*, 1989). Also, low vaccinations rates and limited number of related vaccine strains may subject the measles virus to considerable immunological pressure (Rota *et al.*, 1994; Tamin *et al.*, 1994). Neutralization titre of any given serum measured by serological tests like neutralization test and haemagglutination inhibition assays (WHO, 2007) has been shown to correlate well with the degree of protection against MV (Rima *et al.*, 1997). Titres of sera from human vaccines have been demonstrated to be lower against some wild type viruses than vaccine strains while sera from late convalescents neutralized both wild and vaccine strains efficiently (Tamin *et al.*, 1994; Klingele *et al.*, 2000).

LABORATORY ASSAYS AND DIAGNOSIS

Availability of simple quantitative tests for the measurement of measles neutralizing antibodies would be of considerable interest for vaccine related researches and to determine antibody levels of vaccines in the population (Ward *et al.*, 1999). It is known that a one-dose one-time vaccination programme may not be sufficient in conferring immunity against measles. This review summarizes the importance of employing a simple laboratory test (haemagglutination inhibition assay) in determining the antibody levels in vaccines with a view of assessing herd immunity.

Sample collection

Samples needed to be collected in most laboratories include blood, urine, saliva and throat swab, using appropriate materials. Lymphocytes have been isolated from whole blood at collection site. This and all other clinical samples had to be packed in ice-packs and be transported in an insulated vaccine container, to virology surveillance laboratory for further processing and studies.

Laboratory procedures

Other laboratory procedures needed to be carried out during any study include:

1. Sample preparation
2. Isolation of measles virus
3. Storage of measles virus isolates and other clinical samples
4. Detection of measles virus N and H genes
5. Measles' cDNA sequencing
6. Phylogenetic analysis

Sample Preparation

Lymphocyte isolation from whole blood

In 2001, Amersham (the manufacturer of Ficoll-Paque™) described isolation of lymphocyte from whole blood. At room temperature, freshly drawn blood collected in EDTA-treated tube has to be mixed with minimum essential medium (MEM) in a clean plastic centrifuge tube while Ficoll-paque™ has to be added to an empty centrifuge tube, then the diluted blood could be carefully layered without mixing. This tube has to be screw-capped and centrifuged at room temperature. The upper layer of centrifuged fluid has to be removed and the lymphocyte layer has to be also removed and dispensed into MEM. All samples should always be properly labeled to identify each patient, location and date.

Urine specimen

Most of the time, all urine specimen collected during any study has to be centrifuged 4°C to pellet the sediment (Papania et al., 2002). The supernatant has to be decanted and the pellet re-suspended in virus transport medium (VTM) (that is, 2% MEM plus antibiotics).

The throat swabs

WHO (AFRO) (2004) recommended that throat samples be placed in labeled virus transport swab tube ensuring

that the swab is immersed in the sponge containing the viral transport medium. One of the tubes has to be kept frozen and the other processed to isolate measles virus.

The blood and saliva samples on filter paper

There was enthusiasm for salivary antibody assays and other assays that did not require blood sampling. Viral RNA has been extracted from the isolated virus, as well as, filter-paper blood and saliva samples. It was also recommended that these samples be properly labeled and placed in separate plastic tubes. If further processing could not be done on arrival at laboratory, all samples should be stored at -20 °C until use.

Measles isolation

Collection of viral samples for isolation and genotyping of measles virus is recommended to establish the genotype responsible for the case or outbreak. MV RNA could be extracted from isolated virus and filter-paper samples. The N and H genes could be subjected to RT-PCR. Products of the latter can be subsequently subjected to sequencing and phylogenetic analyses (Hanses et al., 1999). However, clinical samples that test negative for measles virus isolation could be further tested for rubella and human B-19 parvovirus isolation. The clinical signs/symptoms of the diseases caused by these two viruses were similar to measles'.

MV isolation from lymphocytes

Lymphocytes (fresh or freeze-thawed) have to be co-cultured by inoculating 0.4 ml cell suspension, under biosafety cabinet (BSC) using semi-confluent monolayer of B95a cells and incubated at 37°C for 1 - 14 days. Presence of measles virus-specific cytopathic effects (CPE), that is, syncytium/giant cells indicates the presence of the virus. When the CPE has become advanced, the complete culture has to be harvested, aliquoted and stored in liquid nitrogen or at -20°C until it is used for RNA extraction (Hanses et al., 1999). Peripheral lymphocytes of individuals with SSPE have been shown to contain MV (Fournier et al., 1985). Alternatively, the source of MV sequences may have been contaminated during autopsy (Simmonds et al., 2006).

MV isolation from urine

Urine sediment (fresh or freeze-thawed) has to be mixed with MEM and antibiotics. This have to be inoculated on semi-confluent monolayer of B95a cells (in 25 cm² tissue culture flask) (Papania et al., 2002). Maintenance medium

containing antibiotics has to be added after an hour of virus adsorption. If the CPE becomes advanced, the whole culture has to be harvested, pelleted by centrifugation and the pelleted cells should then be re-suspended in MEM and kept frozen (-70°C).

MV isolation from throat swab

WHO (AFRO) (2004) also described that under BSC, swab will be broken and put in a vial containing 2 ml viral transport medium (VTM). The remaining fluid from the sponge in the transport tube will be pipetted and added to the vial. This will be screw-capped and kept at -20°C if not further processed immediately. The virus isolation will be done as described above.

Serological detection of MV

Haemagglutination inhibition test

The Haemagglutinin (H) protein is an immunogenic trans-membrane glycoprotein spike found on viral envelopes which is responsible for binding of virus to cells thereby playing important role in viral pathogenesis and immunity (Griffin, 2007). Antibody to H protein is measured by haemagglutination inhibition (HI) test which is relatively easy and inexpensive and has been of proven use in seroepidemiologic assays for viruses with H peplomer such as measles, influenza and rubella. They correlate strongly with level of protection against wild type viruses (Obi et al., 1996) and correspond to virus neutralization potential (Norrbj et al., 1975).

The HI test is the most widely acceptable test in most developing countries like Nigeria. Haemagglutination inhibition test has been used for a long time as a gold standard for laboratory diagnosis of measles infection (WHO, 2007). This is because, it is very sensitive and specific and easily performed in any laboratory. HI antibody test due to its easy and simple nature, has been an assay of choice in seromonitoring of protective level of immunity to measles virus in developing countries and the sole problem of non-availability of an highly potent HA antigen being faced in employing this test. Furthermore, recent studies have shown a considerable high level of protection against measles among youths. It was however reported in recent studies, that cases of unprotected young individuals against measles are still present in Nigerian population, which is an indication that our vaccination campaigns in this country needs to be intensified and properly evaluated to improve on the successes and correct the faults. It was also further indicated in a study by Motayo, (2007) and Ogundiji, (2008) that subtle genetic changes in the RNA genome of measles virus is translating into increase in virulence thereby posing a threat to antibody mediated protection.

Enzyme-linked immunoassay (ELISA) test

The ELISA is another sensitive test which is also used for diagnosis of measles virus infection but it is more expensive and requires the use of special equipment not found in many laboratories in developing countries like Nigeria (WHO, 2007). To standardize testing and control costs, PAHO elected to use a single ELISA test kit in national laboratories throughout the region and to rely on several specialized reference laboratories to conduct further testing if indicated (Dietz et al., 2004).

Immunohistochemistry

Formalin fixed, paraffin embedded tissues should be analysed by immunohistochemistry. The method used by McQuaid et al. (1990) should be modified as the case may be. Simmonds et al. (2006) modified McQuaid et al. (1990) method as follows: Formalin fixed, paraffin embedded $7\ \mu\text{m}$ thick tissue sections on silane-coated slides were hydrated in xylene and alcohol. Endogenous peroxidase activity was blocked by 10 min incubation in 3% H_2O_2 in methanol. Tissues were washed in water and blocked in blocking solution (1:20 dilution of normal rabbit serum in PBS-T (0.1% Tween-20 in PBS)) for 10 min. Sections were incubated with a monoclonal mouse antibodies against the N protein (clone 7C11, (Samuel et al., 2003) at a dilution of 1:2000 in blocking solution overnight at 4°C , washed in PBS-T and incubated with a biotin conjugated secondary antibody (Dako, UK) at 1:400 in blocking solution for 1 h. After further washing, streptavidin coupled HRP (Dako, UK) was added for 30 min, tissues washed and incubated with DAB, followed by haematoxylin counterstaining.

Immunoblotting

Tissues should be homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100. Protease inhibitors should also be added) and protein concentrations of whole lysates, without removal of cell debris, should be determined by spectrophotometry. Equal amounts of total protein of each sample should be analyzed by SDS-PAGE and immunoblotting. The 7C11 MV N specific antibody has been used to probe blots, which should be followed by detection with an anti-mouse-HRP antibody (Dako, UK) and ECL plus reagents (Amersham Bioscience, UK, 2001) as described by Simmonds et al. (2006).

Genotypic study and phylogenetic analyses

For detection, the measles virus nucleoprotein and haemagglutinin genes were reverse transcribed by RT-PCR.

Tissues should be homogenised and total RNA extracted with RNeasy Mini kit (Qiagen, UK) or any other standard kit. RNA should be treated with DNase to avoid amplification of genomic DNA. The concentration of extracted RNA should be determined by spectrophotometry and the desired quantity of RNA should be reverse transcribed using random primers. PCR primers should be designed using Primer3 software (Rozen and Skaletsky, 2000) to yield a desired base pair amplification fragments such as 192, 223, 206, 238, 234 and 218 base pair amplification fragment of MV N, M or H gene, human GAPDH, beta-actin and 18S rRNA, respectively and where possible, it should be read across intron/exon junctions to avoid amplification of genomic DNA (Simmonds et al., 2006). The cDNA product obtained from the latter should be subjected to comparative sequence and phylogenetic analyses in order to assign clades and genotypes to the virus isolates. The genetic diversity of all isolates and influence of measles vaccination on measles virus genetic evolution, among others, have been reviewed. To investigate a possible link between virus integrity and viral spread, MV loads detected should be compared with the extent of MV M gene and protein mutation reported previously (Kuhne et al., 2005). Increased MV RNA levels per cell may indicate a more actively replicating virus, which may cause faster progressing disease. Alternatively, infection of a greater number of cells may lead to destruction of more tissue, which may also lead to accelerated disease. In summary, the amount of MV protein and RNA differs greatly in different cases of SSPE, which may influence progression of the disease. Further studies of greater numbers of cases are required to confirm any findings (Simmonds et al., 2006).

MV nucleic acid sequencing

De Swart et al. (2001) described the extraction of viral RNA from the blood and saliva on filter paper using high pure viral nucleic acid isolation kit. Total viral RNA could also be extracted from stored MV using guanidinium-acid phenol method (Chomczynski and Sacchi, 1987). Briefly, the virus has to be incubated with Trizol and viral RNA extracted with chloroform and precipitated with isopropanol. The extracted MV N and H mRNA has to be used to synthesize the required and respective cDNAs using RT-PCR with appropriate primers (Hanes et al., 1999). The RT-PCR products have to be purified in 1% agarose gel electrophoresis. The amplicons should then be directly sequenced with the dye terminator cycle sequencing kit using appropriate primers. As MV can usually be detected in brain tissue from biopsy or autopsy by RT-PCR and/or immunohistochemistry, it is thought to persist and spread within the CNS. Although the brain appears to be the main location of persistent MV in SSPE, it is not entirely clear if this is also the site of persistence and the only target during disease. Viral antigen or RNA has been

detected outside the CNS in SSPE cases (Fournier et al., 1986; Jin et al., 2002). To address the question of MV persistence outside the CNS in SSPE, Simmonds et al. (2006) included available peripheral tissues of two SSPE patients in their analysis on measles viral load which may reflect SSPE disease progression.

Phylogenetic analyses

The sequence data are usually analyzed using the CLUSTAL X software (Thompson et al., 1994) for multiple sequence alignments. A PHYLIP package has also been used for the phylogenetic analysis in order to assign the MV isolates clades and genotypes (Felsenstein, 1993). The dendrogram showing the genetic relationship of the isolates with the reference sequence has been provided. All the isolates, genotypes and clades could be designated using WHO recommended nomenclature (WHO, 1998).

Viral load

Viral load has been correlated to disease development, progression and outcome in several infections, including HIV, SIV, cytomegalovirus (Bowen et al., 1996; Gor et al., 1998) and Epstein-Barr virus (Kimura et al., 2002). Occurrence of cognitive impairment in HIV infected patients and development of SIV encephalitis in macaques was linked to the level of viral RNA in the cerebrospinal fluid (CSF). In contrast, CNS disease did not correlate with plasma viral load in either HIV or SIV (Ellis et al., 1997; McArthur et al., 1997; Zink et al., 1999). With the exception of the study by Simmonds et al. (2006), no studies have been carried out so far trying to relate the progression of SSPE with viral load. Due to the rarity of the condition and the invasiveness of removing biopsy samples, obtaining information on viral load during the course of disease is problematic. However, the use of autopsy samples may provide some insight into this relationship (Simmonds et al., 2006).

THE ROLE OF LABORATORY CONFIRMATION IN MEASLES ERADICATION

In 1994, countries in the WHO region of the Americas set themselves the goal of interrupting the transmission of endemic measles by the end of 2000 using strategies developed by the Pan American Health Organization (PAHO, 1995). These strategies included recommendations for vaccination activities intended to achieve high population immunity together with sensitive surveillance for suspected measles cases and effective virological and serological surveillance (de Quadros et al., 1996). In the Americas, a suspected measles case is defined as any

individual with a febrile rash illness (de Quadros et al., 1996). Since 21 November 2002, no endemic measles transmission has been reported in Latin America (PAHO, 2003a, b).

Most strategies for reducing global measles morbidity and mortality and eliminating measles are based on the ability to enhance immune responses to measles virus (Moss and Polack, 2001). During the WHO campaign on the eradication of measles, accurate discrimination between immune and non-immune individuals became increasingly important. Due to waning immunity in vaccinated population, the performance of a measles IgG assay depends mainly on its ability to detect reliably seronegative individuals among many vaccinees with low antibody levels. New serological tests based on recombinant proteins detect only a fraction of the total measles virus (MV) specific antibodies. Therefore, several assays based on recombinant MV-haemagglutinin (ELISA and flow cytometry) or MV-fusion protein (flow cytometry) as well as neutralization and haemagglutination test have been evaluated using a large panel of low-titre and negative sera (Harterter et al., 2000). Isolation of measles virus confirms the diagnosis. A viral specimen can be evaluated for the presence of measles virus by PCR in a specialized network laboratory if culture attempts fail. Moreover, the molecular analysis of the virus may be essential to confirm its source (Dietz et al., 2004).

A key component of the programme has been a laboratory network, established in 1995, comprising 22 national laboratories, 10 of which function as reference centres, and three as specialized reference laboratories (Venczel et al., 2003). National laboratories were responsible for the testing of blood samples for immunoglobulin M (IgM) antibodies. A case is confirmed serologically, virologically or by epidemiological linkage to another confirmed case. Commercially available enzyme immunoassay (EIA) kits for IgM-class antibodies are currently in use throughout the region and all network laboratories use the same test kit (Ozanne and d'Halewyn, 1992). PAHO recommends that blood sample be taken at the first contact with a suspected case and within 30 days of onset of rash. The test kits in use have been shown to have high sensitivity and specificity. However, cross-reactions with other viral diseases, e.g. rubella and *Parvovirus*, may occur (Thomas et al., 1999; Ratnam et al., 1995).

As seen in Figure 3, if the first serum sample is found to have IgG antibodies, but IgG titres in the second sample show no change when compared to the first sample, the case would not be considered measles and could be discarded. The IgM-positive test result would be considered a false-positive. However, if the second sample shows a fourfold higher IgG antibody titre than the first sample, the case should be considered an acute measles infection and confirmed (Dietz et al., 2004). If the second sample shows an increase in IgG titres, but is

less than fourfold higher than in the first sample, it would not be possible to determine whether or not it was an acute infection. Thus, no conclusion could be reached as to the actual status of the suspected case. In this situation, for programmatic considerations, the suspect case should be confirmed solely on the basis of the positive IgM test result. For the purposes of elimination programmes, it is better to incorrectly confirm a non-case than to discard a true measles infection (Dietz et al., 2004). If both the first and second samples are negative for IgG antibodies, the case would not be considered to be measles and should be discarded. If, however, the second sample is IgG-positive for measles, the case should be confirmed as acute measles infection (Dietz et al., 2004). Sometimes, there may be an insufficient volume from the first sample for IgG antibody testing, but a second sample would still be required. If the second sample is negative for IgG, the case should be discarded. If, however, the second sample is IgG-positive, it would not be possible either to confirm or discard the case, because the positive result could represent an acute or past infection. In such a situation, the case must be confirmed based on the IgM test result. Regardless of the scenario or testing sequence results, when in doubt, the case should be confirmed on the basis of the positive IgM test result (Dietz et al., 2004).

No laboratory test is 100% sensitive or specific and false-positive results of laboratory tests do occur (Dietz et al., 2004). The positive predictive value of a laboratory test decreases as the prevalence of the disease decreases resulting in an increase in the number of false-positive results (Mausner and Kramer, 1985). Therefore, as progress towards elimination is made, that is, as the prevalence dramatically decreases, some false-positive laboratory results should be expected. In countries that maintain high levels of vaccination activity and of surveillance for rash and fever, the notification of febrile rash illness in recently vaccinated persons should be anticipated. This is an important consideration because at least 5% of primary vaccinations can result in a febrile rash illness (CDC, 2002). Thus, epidemiologists and managers of measles elimination programmes must be prepared to interpret a positive IgM laboratory test in the setting of greatly reduced disease, or when the clinical and epidemiological data indicate that the case in question is not measles (Dietz et al., 2004). In such situations, the dilemma is to determine whether a measles IgM-positive result occurs because: 1) the subject has an acute measles infection; 2) the test result is a false-positive, or 3) the subject was recently vaccinated against measles.

Despite active surveillance, there has been no evidence of an extended chain of measles transmission among individuals infected in most country, for instance, United States where measles is no longer endemic (MMWR, 2002). However, the elimination of endemic measles is not equivalent to an absence of measles

cases. As long as measles circulates in other countries, measles will be imported into the United States (CDC, 2004b). The possibility of transmission to susceptible contacts mandates a commitment to high rates of immunization, active surveillance with rapid diagnosis to prevent the reestablishment of endemic measles (Meissner et al., 2004).

Laboratory findings comprise only part of the process used to determine whether a suspected measles case is a true case. Test results are affected by the quality of samples received, inherent limitations of the test because of the low prevalence of measles and the technical expertise of the laboratory staff conducting the tests (Dietz et al., 2004). In addition, when a sample is taken within 3 days of onset of rash, up to 30% of true measles infections may be IgM-negative (Helfand et al., 1997). The proficiency of the laboratory performance can be assured through site visits, exchange of samples for re-testing and participation in annual proficiency testing (Venczel et al., 2003).

Vaccination of susceptible individuals through the full implementation of the strategy recommended by PAHO in all countries remains the foundation of the regional measles elimination initiative (de Quadros et al., 2003). Until programmes for measles elimination are implemented worldwide, importations will continue to occur in the Americas. Sensitive measles surveillance and high population immunity must be maintained to prevent the resumption of endemic transmission. Laboratory surveillance remains a central activity within the elimination programme (Cutts and Brown, 1995). Standardized approaches to laboratory testing and interpretation of results are critical to ensure the continued success of the programme (Dietz et al., 2004).

THE ROLE OF MOLECULAR EPIDEMIOLOGY IN MEASLES ERADICATION

Molecular epidemiology of MV entails genetic characterization of the wild-type (wt) virus and it is an important component of measles surveillance (WHO, 2003). Measles surveillance and epidemiological investigation is able to identify the source and transmission pathways of the virus. This is most beneficial when the change in viral genotypes over time in a particular region can be observed, because the information from the genetic analyses can document the interruption of transmission of endemic measles. Consequently, molecular characterization of wt MVs has become a valuable tool for the evaluation of the effectiveness of measles control and elimination programmes. WHO therefore recommended that viral surveillance is conducted during all phases of measles control and that virological activities be expanded to provide an accurate description of the global distribution of measles genotypes.

In line with the above, WHO published guidelines for a

uniform nomenclature for designating wt MVs and describing genotypes (WHO, 2003). The guidelines include the laboratory methods used for genetic characterization. It also stated that the minimum amount of data required for determining the genotype of a MV is the sequence of the hypervariable 450 nucleotides (nt) that code for the carboxyl terminal 150 amino acids of the viral nucleoprotein (N). These sequence data can be obtained from a viral isolate or by amplification of MV sequence directly from RNA extracted from a clinical specimen. When a new genotype is suspected, in addition to N sequence, complete H gene (1854 nts) sequence should always be obtained. WHO established standard reference sequence with which the nt sequence of MV isolates should be compared. New genotypes that were identified as a result of expanded virological surveillance made WHO (2001) update the virus nomenclature to cater for new MV genotypes. New genotypes are suspected and designated if the nt sequence differs from the closest reference sequence by more than 2.5% in N and 2.0% in H. Proposed new genotypes should be assigned lower case letter (e.g. g3).

MV genetic characterization essentially includes clade-typing, genotyping and phylogenetic analysis. The terms clade and genotype are used to describe the genetic characteristics of wt MVs. For the purpose of molecular epidemiology, the genotype designation is the operational taxonomic unit, while the clades indicate the genetic relationship between the various genotypes. Currently, there are eight clades (A-H) which have been divided into 22 genotypes and one proposed genotype (i.e. d10) making 23 genotypes recognized by WHO. Clades B, C, D, G, and H each contain multiple genotypes (B1-3; C1-2; D1-10; G1-3; H1-2) while clades A, E and F each contain a single genotype (WHO, 2001; 2003). The nt sequences of the MV vaccine strains indicated that the wt viruses from which they were derived were all members of genotype A.

Since molecular epidemiology has permitted different MV isolate to be grouped into clades and genotypes, the major group which has been identified indigenous to Africa, is clade B. In Nigeria, a measles endemic nation, the first genotyping and phylogenetic characterization of circulating MV revealed that the viruses were in clade B genotype 3 (B3) (Hanses et al., 1999). However, the MVs studied then were from Ghana and Nigeria of which the Nigerian isolates were exclusively from south-western Nigeria, precisely from Lagos and Ibadan. Another 2002 report identified two MVs belonging to B3.1 genotype circulating from Sudan to Nigeria and Ghana and to Cameroon, whereas the B3.2 genotype was said to be found in West Africa (Kouomou et al., 2002).

However, recent molecular epidemiologic studies have demonstrated interruption of circulation of genotype D6 viruses that were responsible for the large measles outbreak in Saõ Paulo in 1997 and subsequent outbreaks in Rio de Janeiro, Argentina, Chile, Bolivia, Haiti, and the

Dominican Republic (Canepa et al., 2000; Oliveira et al., 2002). The recorded low number of cases and the identification of genotypes other than D6 in association with measles cases imported into South and Central America are consistent with regional elimination (PAHO, 2001; Hersh et al., 2000; CDC, 2000a, b).

In 1998, WHO published guidelines for a uniform nomenclature for designating wild type measles viruses and describing genotypes thereby providing guidelines for laboratory methods used for genetic characterization (WHO, 2007b). In 2001, 2003 and 2005, World Health Organization (WHO) recommendations were updated to take into account the identification of new genotypes resulting from expanded virological surveillance (WHO, 1998, 1999, 2001, 2002, 2003, 2005, 2007b). Molecular epidemiology of MVs is an important component of measles surveillance as it provides a method for identifying the geographical origin and tracing transmission pathways of a virus (WHO, 2007b). Two measles strain banks, the MV section of the Center for Disease Control and Prevention (CDC) USA and the Health Protection Agency (HPA) UK, have been designated by WHO to acquire, analyze, store and dispense representative strains (WHO, 2007b). The terms clade and genotype are used to describe genetic characteristics of wild type MVs. For molecular epidemiological purposes, the genotype designations are the operational taxonomic unit while clades are used to indicate genetic relationship between the various genotypes. Altogether, eight Clades are recognized designated A-H, within the Clades 23 genotypes. With the exception of genotype F, all of the genotypes have a corresponding reference strain (WHO, 2007b). Many laboratory networks have used various molecular approaches and epidemiology to:

1. Determine the source of imported viruses
2. Follow the pathway of virus circulation
3. Monitor the progress or lack of progress of the vaccination programme
4. Identify reservoirs sustaining virus circulation
5. Detect gaps in surveillance; and
6. Show geographical distribution of the virus.

CURRENT TRENDS

In 1991, a measles outbreak occurred in Fukuoka, Japan, in which Hidaka et al. (1994) observed 15 cases of measles vaccine failure (MVf). However, the introduction of enhanced diagnostic tests for IgM detection such as IgM-capture EIA, with results which may be positive for patients with measles reinfection due to secondary vaccine failure, has highlighted the difficulty in differentiating between primary infection or reinfection due to primary and secondary vaccine failure (Erdman et al., 1993; Hidaka et al., 1994; Helfand et al., 1997; Paunio et al., 2000; Pannuti et al., 2004). Measles reinfection due to

secondary vaccine failure is probably more common than suggested by studies relying on specific IgM (Paunio et al., 2000), because measles-specific IgM is also inducible by reinfection (Erdman et al., 1993). The estimation of IgG antibody avidity is useful for identifying primary and secondary immune responses, but there have been few reports of its use during measles outbreaks (Pannuti et al., 2004). The results of the study by Hamkar et al. (2006), which showed that 18.4% of 365 measles cases confirmed by a positive IgM test mounted a secondary immune response, provide further evidence that the presence of IgM cannot be used as a reliable indicator of a primary immune response (Pannuti et al., 2004).

However, while many developed countries have successfully controlled measles and are currently in measles elimination and eradication phase (CDC, 2005); Nigeria, in tune with global goal, is just on measles control goal (that is, morbidity and mortality reduction). As a result, it recently concluded a nationwide accelerated measles vaccination campaign in June /July, 2006. Measles as one of the leading cause of childhood morbidity and mortality and the need to effectively monitor through active surveillance, control and eventually eradicate measles cannot be overemphasized.

Where relatively extensive virologic surveillance has occurred, two general patterns of measles genotype distribution have been observed. Figure 3 shows the global distribution of MV in WHO regions. In countries that still have endemic transmission of measles, most cases are caused by relatively few endemic genotypes. In countries that have eliminated measles, the small numbers of cases are caused by a number of different genotypes that reflect various imported sources of virus and suggest the lack of sustained transmission of an endemic genotype (Rota and Bellini, 2003).

Recent advances in genomic sequencing technology have lent its support to the monitoring and evaluation of vaccination programmes. Phylogenetic trees are invaluable tools for monitoring the progress of immunization activities. Viruses of the same genetic lineages cluster geographically together in a phylogenetic tree (Adu, 2008). In a study, Nigerian measles isolates was classified into Cluster I and II of a newly discovered B3 genotype of Clade B (Hanses et al., 1999). According to Adu (2008) before 1999, no field isolate of the measles virus from Nigeria had ever been studied and that study put Ibadan and Nigeria in general, in the measles epidemiological map of the world for the first time. According to Adu (2008), these efforts are with ultimate aim of detecting the viruses and the un-immunized children and of reaching every Nigerian child with the vaccine in order to stop the unnecessary suffering and untimely death resulting from these vaccine preventable diseases.

Maintenance of high routine vaccination coverage and community-based surveillance (that is, case identification, reporting and investigation) require adequately trained

and equipped primary health-care personnel. Strengthening the primary health-care system and EPI in developing countries, although perhaps not essential for interruption of measles virus transmission, greatly facilitates achieving and maintaining measles elimination in a country or region.

SUMMARY

Often, identification of vaccine virus from a sample collected from an individual presented with rash after vaccination has provided the evidence necessary for classification of the case as vaccine-associated. Although facilities for both virus isolation and direct detection of measles virus by reverse transcription followed by the polymerase chain reaction (RT-PCR) are available in certain regional reference and specialized laboratories, only serological confirmation is performed in all national laboratories. Viral samples have limited utility in the network for case confirmation because negative results may result from poor quality of the sample and therefore cannot be used to rule out a suspected case. Thus, RT-PCR has not been utilized at the country level throughout the region (Dietz et al., 2004).

The HA/HI test is not only relevant in the diagnosis of measles infection but is also very important for seromonitoring of measles infection. A readily available measles HA antigen will be a very valuable and useful tool for monitoring the progress of measles seroconversion following immunization in Nigeria. To perform the HA/HI test, a potent measles antigen is therefore needed. Up till now, this measles antigen is still being imported at very expensive amount into Nigeria. Also to import the foreign brands of this measles antigen into Nigeria takes a very long time and often a time, they arrive having lost their potency or very close to their expiry date. There have been previous attempts to produce indigenous available measles antigen in Nigeria laboratory by sonication which had not been too successful because of the very low titre of the antigen (Adu Personal Communication, 2007; Motayo, 2007). The indigenous prepared measles antigen has also been advocated to be produced, refined further and then massively produced. This will be highly cost effective especially in field for seromonitoring and surveillance of measles.

Critical therefore to measles surveillance is the molecular characterization of all MV isolates through genetic study. This is because genetic study can identify source and transmission pathway of wild type (wt) MVs; thereby elucidating local virus transmission and or importation (Lo et al., 2001). Identification of wt MV in any country had necessitated epidemiological investigation and measles vaccination if wt virus was identified. A wide genetic diversity between indigenous wt MVs indicates whether or not measles vaccination was able to interrupt local transmission of the virus. Importantly, most study provides genetic characteristics and distribution of wild-type

measles virus circulating in a country. In addition, the genetic study may reveal presence of some genotypes reportedly inactive. These include the B1, D1, E, F and G1 (Riddell et al., 2005). The mutation rate amongst field isolates of measles virus has been reported to be low and appear to be random rather than driven by vaccine pressure or immune responses (Rima et al., 1997). It has been considered significant that studies show the effect of mass vaccination on evolution of the virus in the vaccination era in developing countries like Nigeria. It is important to genetically differentiate between wt and vaccine viruses associated with measles cases or outbreaks. Recent studies have allowed observation of changes in MV genotypes over time in Nigeria to be made. Clarification of epidemiological links during measles outbreaks and separation of indigenous strains from newly imported strains is also needful.

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