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Oral fluid for the serological and molecular diagnosis of measles

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SUMMARY

Objectives: Since measles presents mostly in children, a non-invasive sample collection technique such as oral fluid sampling would be very useful in the early detection of measles RNA and antibodies. The aim of this study was to validate the detection of anti-measles IgM and measles virus RNA in oral fluid and to make a comparison with the gold standard methods of ELISA using serum (Enzygnost® anti-Measles IgM) and in-house nested reverse transcriptase polymerase chain reaction (RT-PCR) using nasopharyngeal secretions.

Methods: Three samples each from 73 measles-positive and 44 measles-negative subjects (serum, oral fluid, and nasopharyngeal secretions) were analyzed.

Results: The anti-measles IgM ELISA (MicroImmune) on oral fluid was validated against the IgM ELISA (Siemens) for serum and this resulted in a sensitivity of 92% and specificity of 100%. A molecular nested RT-PCR using oral fluid was validated against the standard assay on nasopharyngeal secretions and gave a sensitivity of 100% and specificity of 100%.

Conclusions: The results confirm that both serological and molecular oral fluid assays are suitable for routine use. The use of oral fluid samples for the detection of measles virus may encourage patients, general practitioners, and pediatricians to participate in the Belgian measles surveillance system and other epidemiological studies in the framework of the World Health Organization elimination program.

1. Introduction

Measles is a highly contagious viral disease characterized by high fever, coryza, cough, and conjunctivitis, followed by the appearance of a maculopapular rash. Measles virus (MV) is a negative single-stranded RNA virus, belonging to the genus *Morbillivirus*, of the family *Paramyxoviridae*.[1]

Despite the development of an efficient combined vaccine (measles–mumps–rubella), measles remains a major cause of mortality in developing countries and a cause of continuous outbreaks in industrialized countries.

Measles is transmitted orally (coughing, sneezing, and saliva) and has an incubation period of 10–12 days.[2] The measles immunoglobulin M (IgM) antibodies appear in the blood within 1 week of the onset of the rash. The virus itself is detectable during the first 7 days after the appearance of symptoms, in the respiratory epithelium of the nasopharynx and the regional lymph nodes.[3] When oral fluid is sampled at an early stage (day 0–4) after the onset of the rash, more than 80% of patients are positive for the measles virus and approximately 80% are positive for measles IgM antibodies.[3] The measles virus and the highest level of IgM antibodies are detected at day 3 and day 7 after the appearance of the rash, respectively.[2,3] Measles RNA is detectable from the onset of symptoms (i.e., high fever) and declines from day 7 after the rash.[3]

In line with all the other countries in the World Health Organization (WHO) European Region, Belgium is involved in the WHO program for the elimination of measles in Europe by 2010.[4] To achieve this goal, improved measles surveillance in Belgium is necessary. More than 80% of the measles cases should be laboratory confirmed, and vaccination coverage of >95% should be achieved. However, in the case of a clinical measles diagnosis, general practitioners (GPs) can be reluctant to take part in the surveillance system, since sample collection (serum and/or nasopharyngeal secretions) for laboratory confirmation often requires invasive and painful sampling in children.

Currently, in many countries, measles is still confirmed by serological and/or molecular assays on serum and nasopharyngeal secretions, respectively. In Europe, oral fluid sampling and assays have been increasingly introduced as a good alternative, and one of the main WHO recommendations for the European elimination program for measles is that these techniques should be used.[3–5]

Saliva is a mixture of salivary gland secretions and gingival crevicular fluid (GCF). The composition of saliva includes water,

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mucin, enzymes, inorganic salts, and antimicrobial factors.[6] The GCF contains plasma-derived immunoglobulins IgG and IgM and is secreted via the crevicular epithelium, an area of higher permeability than other regions in the oral cavity.[7–9] The levels of IgG and IgM in GCF are approximately 1/800 and 1/400 of those found in serum, respectively.[10]

The detection of IgG and IgM antibodies and viral nucleic acids (MV RNA) in oral fluid enables the laboratory diagnosis of a variety of viral infections. These antibodies transude from capillary beds in the gingival crevice between the teeth and gums into the GCF component of oral fluid.[8] They can be collected using an Oracol collection device (Malvern Medical Development, UK).[11] This device, which was specifically developed for the collection of oral fluid rich in GCF, can yield samples that perform like serum in antibody assays.[8] It has previously been reported that the Oracol collection device yields the highest quality of oral fluid.[12]

The introduction of oral fluid as an alternative medium to serum for the detection of IgM and MV RNA would provide a number of opportunities.[11] Compared to traditional venipuncture, the collection of oral fluid is less invasive, less painful, less expensive (i.e., no trained personnel required), and safer (prevention of needle stick injuries).[10] Since measles presents mostly in children, a non-invasive sample collection method would be very useful for the early detection of MV RNA and antibodies during measles outbreaks. In addition, it could become a useful tool in the control of outbreaks.

The aim of this study was to validate the detection of anti-measles IgM and MV RNA in oral fluid and to make a comparison with the assays that are considered to be the gold standard: ELISA on serum (Enzygnost® anti-Measles IgM) and in-house nested reverse transcriptase polymerase chain reaction (RT-PCR) on nasopharyngeal secretions.

2. Materials and methods

2.1. Study population and sample collection

The study population consisted of 65 males and 52 females ($N = 117$) with an age range of 3 months to 58 years from whom three samples each were collected. The number of samples was calculated to reach significance for the validation of both assays.[13]

Most samples were collected in the Democratic Republic of Congo by the National Measles Laboratory (Kinshasa) and came from children living in a ghetto of the Congolese Police, situated in the region of the city of Kinshasa. The positive samples came from children with clinical measles symptoms, such as high fever, Koplik spots, and rash and were from the same measles outbreak. Healthy children with no rash-illness, no fever or other disease symptoms donated the control samples. All samples were collected within the same time period.

Some control samples were obtained from volunteers at the Scientific Institute of Public Health (IPH), Brussels, Belgium. The volunteers were healthy adults, with no rash-illness and no fever at the time of sampling.

Three samples (serum, oral fluid, and nasopharyngeal secretion) were collected simultaneously from each participant.

2.2. Sample treatment

Each blood sample was collected into a sterile tube, centrifuged at 2000 rpm (approximately $700 \times g$) for 10 min and stored at $-20\,^{\circ}C$.

Oral fluid samples were collected using the Oracol collection device following the manufacturer's instructions. Afterwards (within 7 days), 1 ml of transport medium was added to each collection device. The medium was phosphate-buffered saline (PBS) pH 7.2 with 10% fetal calf serum, 0.2% Tween20, 0.5% gentamicin, and 0.2% fungizone (treatment for oral candidiasis).[14] The addition of this transport medium has several purposes, firstly it minimizes the effects of degradation of the oral fluid and secondly it facilitates pipetting. After adding this medium, the vials were centrifuged at 2000 rpm (approximately $700 \times g$) for 5 min and the oral fluid was removed and stored at $-20\,^{\circ}C$.

The collection of nasopharyngeal secretions was performed using a throat swab preserved in a transport medium containing PBS (pH 7.2) with gentamicin and amphotericin.

Each set of three samples was collected within 3 days after the onset of the exanthematous rash. All samples were centrifuged and stored at $-20\,^{\circ}C$ on the day of sampling at the National Measles Laboratory in Kinshasa. The sets of three samples were shipped on dry ice, by courier, from Kinshasa to the National Laboratory for Measles and Rubella (NLMR) at the IPH in Brussels, Belgium. All oral fluid samples were visually checked for blood contamination. Contaminated samples were rejected to avoid false-positive results.

To verify the quality of the oral fluid samples, a total IgG quantification test was performed. To be acceptable for use in the assays, the sample needed to contain at least a level of 0.625 μg/ml IgG (i.e., detection limit of the IgG quantification assay). This test was first described for anti-HIV in urine[15] and has been modified for oral fluid and eluates of dried blood by the Virus Reference Laboratory, Health Protection Agency, London (2000). The assay was performed at the NLMR according to the protocol of the Virus Reference Laboratory.

2.3. Serological assays—detection of anti-measles IgM in serum and oral fluid

The detection of anti-measles IgM was performed using Enzygnost Anti-Measles Virus IgM ELISA (Siemens, Germany) for serum and a Measles IgM Capture EIA (MicroImmune, UK) for oral fluid on an automatic bench-top microplate analyzer (Etimax 3000, DiaSorin, Italy).

Serum and oral fluid samples were independently and blindly analyzed according to the manufacturer protocols.

2.4. Molecular assays—detection of measles virus RNA in nasopharyngeal secretions and oral fluid

The extraction of RNA from samples was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

An in-house nested RT-PCR was developed, optimized, and validated. Three dilutions of the reference Schwarz vaccine strain containing 20 000 international units (IU)/ml and negative controls were designed for this purpose.

For the PCR on nasopharyngeal secretions, 1/1000 (20 IU/ml), 1/5000 (4 IU/ml), and 1/8000 (2.5 IU/ml) dilutions of the Schwarz vaccine strain into MRC5 cells were used as positive controls and MRC5 cells were used as negative control.

A 1/100 (200 IU/ml), 1/1000 (20 IU/ml), and 1/5000 (4 IU/ml) dilution series of the Schwarz vaccine strain in negative oral fluid was used as positive controls in a PCR performed on saliva samples. For this latter PCR, negative oral fluid was used as the negative control.

RNA was reverse-transcribed and cDNA amplified using a one-step RT-PCR kit, followed by a nested PCR with specific primers for the measles virus N-gene region: KVW-1 (external forward), KVW-2R (external reverse), MV-1172 (internal forward), and KVW-4R (internal reverse).[16,17] A human β-actin fragment was amplified with primers developed in-house as internal control for the integrity of RNA and the efficiency of the reactions for both PCRs. The specific primers for the targeted region in the RT-PCR were β-actin 5 (5′ AACACCCCAGCCATGTAC 3′) and β-actin 6