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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

(5′ GTAGTCAGTCAGGTCCCG 3′). For the nested PCR, β-actins 7 and 8 (5′ GTTGCTATCCAGGCTGTGC 3′ and 5′ GCCAGCCAGGTCCAGACG 3′, respectively) were used.[18,19]

For the RT-PCR performed by the PTC-200 DNA Engine® Thermal Cycler (Bio-Rad, Belgium), 10 μl RNA was added to 40 μl of a Promega Access-Quick master mix containing Quick master mix buffer (2 ×), 12.5 pmol/μl MV primers (KVW-1 and KVW-2R), 50 pmol/μl β-actin primers (5 + 6), 5 U/μl Avian Myeloblastosis Virus (AMV) Reverse Transcriptase, and nuclease-free H_2O. The steps of the RT-PCR reaction (±3 h) were: the mix was kept at 48 °C for 45 min, followed by 94 °C for 2 min, followed by 40 cycles with each a denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and an elongation step at 68 °C for 30 s. In a final step, the mix was kept at 68 °C for 7 min.

The second amplification step (i.e., in-house developed nested PCR) was performed with a total mix of 50 μl containing 10× Mg^{2+} buffer (Roche), 10 mM dNTPs (Roche), 12.5 pmol/μl MV-1172 and KVW-4R primers, 50 pmol/μl β-actin primers (7 + 8), 5 U/μl Taq DNA polymerase (Roche), nuclease-free H_2O, and 2.5 μl cDNA (from RT-PCR). The nested PCR reaction (± 2 h) involves keeping the mix at 94 °C for 6 min, followed by 40 cycles with each a denaturation step at 94 °C for 30 s, an annealing at 55 °C for 30 s, and an elongation step at 72 °C for 30 s. Finally, the mix was kept at 72 °C for 10 min.

After amplification, the PCR products were visualized on a Bio-Rad UV-transilluminator.

Nasopharyngeal secretions and oral fluid samples were independently and blindly analyzed according to in-house procedures.

A PCR result was considered valid when the positive control for PCR on nasopharyngeal secretions at 1/8000 dilution (2.5 IU/ml) and oral fluid at 1/5000 dilution (4 IU/ml) gave a positive outcome. This ensures that the in-house developed PCR for nasopharyngeal secretions and oral fluid has a sensitivity of >99%.

Samples with an amplicon at 254 bp (presence of measles virus) and 144 bp (β-actin) on gel electrophoresis were considered positive for MV. If no signal was detected at 254 bp, in the presence of the β-actin signal (internal control), the sample was considered negative for MV. In the absence of the β-actin signal, the PCR was considered invalid and the test was repeated from the extraction step.

2.5. Statistical analysis

The kappa coefficient was calculated to determine the level of agreement between assays (serum vs. oral fluid, and nasopharyngeal secretions vs. oral fluid).[20]

3. Results

3.1. Serological assays

All 117 oral fluid and serum samples were tested for anti-measles IgM using the described serological assays. In this study, 73 out of 117 serum samples were IgM-positive in accordance with the clinical measles diagnosis and 44 were IgM-negative (Tables 1 and 2). All positive samples and 28 negative samples were collected in a ghetto in Kinshasa in the Democratic Republic of Congo. Sixteen negative samples came from volunteers working at the IPH, Brussels, Belgium.

Table 1
Results of ELISA assays for the detection of anti-measles IgM in oral fluid and serum

Oral fluid	Serum		
	Positive	Negative	Total
Positive	67	0	67
Negative	6	44	50
Total	73	44	117

ELISA on oral fluid (MicroImmune) versus serum (Siemens = gold standard); sensitivity: 67/73 × 100 = 92% (95% CI 84.7–97.7%); specificity: 44/44 × 100 = 100% (95% CI 92.9–100%).

The oral fluid of 67 out of the 73 anti-measles seropositive subjects tested positively. The oral fluid of all 44 anti-measles seronegative subjects tested negatively. These results show that the measles IgM capture EIA on oral fluid has a sensitivity of 92% (95% CI 84.7–97.7) and specificity of 100% (95% CI 92.9–100). The strength of agreement between the assays was very good (κ = 0.90). Two technicians on two different Etimax 3000 machines analyzed all oral fluid and serum samples blindly at different times.

The Measles IgM Capture EIA was evaluated and compared to Enzygnost Anti-Measles Virus IgM ELISA. In this study, no false-positive or false-negative results were obtained with the Measles IgM Capture EIA on serum, so the specificity was even higher than that reported on the manufacturer's datasheet (sensitivity 100%, specificity 96.1%). However, the Enzygnost Anti-Measles Virus IgM ELISA, which had a sensitivity of >99.9% and a specificity of >99.9%, remained the gold standard in this study.

The results for the 117 samples obtained by the different serological and molecular assays are shown in Table 2.

3.2. Molecular assays

The optimized and validated PCR assays on oral fluid and nasopharyngeal secretions were performed for their validation on clinical samples. Two technicians evaluated the in-house developed nested RT-PCR at different times and on different thermocyclers. Logarithmic and semi-logarithmic dilutions (from 10^{-1} up to $10^{-6.5}$) of a standard reference strain (Schwarz vaccine strain containing 20 000 IU/ml) were performed for both sample types. Each dilution was analyzed 24 times. The results were subjected to a probit analysis (by Xlstat 4.0).

In each run of the in-house developed nested RT-PCR on nasopharyngeal secretions, three positive controls (1/1000, 1/5000, and 1/8000 dilution of reference Schwarz strain in negative control containing MRC5 cells) were included (Figure 1). After validation, the 99% end-point was calculated and it corresponded to the 1/8000 dilution containing 2 IU/ml. This dilution was chosen as a positive validation control for each run using nasopharyngeal secretions.

The in-house nested RT-PCR on oral fluid also included three positive controls (1/100, 1/1000, and 1/5000 dilution of the reference Schwarz strain in negative saliva) in each run (Figure 2). The 1/5000 dilution (i.e., 4 IU/ml) corresponded to the 99% end-point and was chosen as a positive validation control for each run using oral fluid.

The optimized in-house nested RT-PCR on nasopharyngeal secretions and oral fluid was also tested on positive MV RNA samples of different genotypes, such as genotype A, B3, D4, D5, D6 and D9.

For the validation on clinical samples, all 117 oral fluid samples and nasopharyngeal secretions were tested for MV RNA using the above described PCRs. Our results show that 73 out of 117 nasopharyngeal secretions were MV RNA-positive (Table 2), confirming the clinical measles diagnosis. All 73 MV RNA-positive nasopharyngeal secretions were also positive for oral fluid. Similarly, the 44 MV RNA-negative nasopharyngeal secretions were also negative for oral fluid. Using nasopharyngeal secretions as the gold standard the results show that the nested RT-PCR on oral fluid has a sensitivity of 100% (95% CI 95.1–100) and specificity of 100% (95% CI 92.9–100). The positive and negative predictive values were 100% (95% CI 95.1–100) and 100% (95% CI 92.9–100), respectively. The strength of agreement between the assays was very good (κ = 1).

Table 2
ELISA and PCR results obtained for the detection of anti-measles IgM (oral fluid and serum) and measles virus RNA (oral fluid and nasopharyngeal secretions), respectively

Sample number	ELISA Micro ser[a]	OD Micro ser[b]	CO Micro ser	OD/CO Micro ser[c]	ELISA Micro sal[d]	OD Micro sal[e]	CO Micro sal	OD/CO Micro sal[f]	PCR naso[g]	PCR sal[h]
Mea/01	POS	3.248	0.372	8.731	POS	1.949	0.243	8.021	POS	POS
Mea/02	POS	3.254	0.372	8.747	POS	3.635	0.243	14.959	POS	POS
Mea/03	POS	0.869	0.372	2.336	NEG	0.184	0.243	0.757	POS	POS
Mea/04	POS	1.658	0.372	4.457	POS	3.699	0.243	15.222	POS	POS
Mea/05	POS	4.435	0.372	11.922	POS	1.231	0.243	5.066	POS	POS
Mea/06	POS	2.036	0.372	5.473	wPOS	0.285	0.243	1.173	POS	POS
Mea/07	POS	1.657	0.372	4.454	POS	0.971	0.243	3.996	POS	POS
Mea/08	POS	1.294	0.372	3.478	NEG	0.143	0.243	0.588	POS	POS
Mea/09	POS	1.674	0.372	4.500	POS	3.299	0.243	13.576	POS	POS
Mea/10	POS	4.365	0.372	11.734	POS	1.948	0.243	8.016	POS	POS
Mea/11	POS	3.91	0.372	10.511	POS	4.442	0.243	18.280	POS	POS
Mea/12	POS	1.671	0.372	4.492	POS	0.639	0.243	2.630	POS	POS
Mea/13	POS	2.797	0.372	7.519	wPOS	0.460	0.243	1.893	POS	POS
Mea/14	POS	1.445	0.372	3.884	NEG	0.157	0.243	0.646	POS	POS
Mea/15	POS	0.771	0.372	2.073	POS	1.844	0.243	7.588	POS	POS
Mea/16	POS	4.189	0.372	11.261	POS	2.193	0.243	9.025	POS	POS
Mea/17	POS	3.482	0.372	9.360	POS	2.284	0.243	9.399	POS	POS
Mea/18	POS	2.212	0.372	5.946	NEG	0.154	0.243	0.634	POS	POS
Mea/19	POS	1.45	0.372	3.898	wPOS	0.244	0.243	1.004	POS	POS
Mea/20	POS	2.819	0.372	7.578	wPOS	0.413	0.243	1.700	POS	POS
Mea/21	POS	1.556	0.372	4.183	wPOS	0.485	0.243	1.996	POS	POS
Mea/22	POS	3.568	0.372	9.591	POS	2.934	0.243	12.074	POS	POS
Mea/23	POS	3.398	0.372	9.134	POS	3.571	0.243	14.695	POS	POS
Mea/24	POS	3.517	0.372	9.454	POS	0.558	0.243	2.296	POS	POS
Mea/25	POS	1.205	0.372	3.239	wPOS	0.327	0.243	1.346	POS	POS
Mea/26	POS	0.777	0.372	2.089	NEG	0.137	0.243	0.564	POS	POS
Mea/27	POS	3.565	0.372	9.583	POS	3.075	0.243	12.654	POS	POS
Mea/28	POS	3.242	0.389	8.334	POS	3.382	0.224	15.098	POS	POS
Mea/29	POS	1.487	0.389	3.823	wPOS	0.341	0.224	1.522	POS	POS
Mea/30	POS	4.064	0.389	10.447	POS	2.803	0.224	12.513	POS	POS
Mea/31	POS	3.878	0.389	9.969	POS	4.410	0.224	19.688	POS	POS
Mea/32	POS	1.349	0.389	3.468	POS	0.531	0.224	2.371	POS	POS
Mea/33	POS	4.586	0.389	11.789	POS	3.856	0.224	17.214	POS	POS
Mea/34	POS	2.87	0.389	7.378	POS	0.923	0.224	4.121	POS	POS
Mea/35	POS	4.086	0.389	10.504	POS	2.883	0.224	12.871	POS	POS
Mea/36	POS	3.011	0.389	7.740	POS	4.144	0.224	18.500	POS	POS
Mea/37	POS	5.133	0.389	13.195	POS	1.185	0.224	5.290	POS	POS
Mea/38	POS	4.858	0.389	12.488	POS	5.200	0.224	23.214	POS	POS
Mea/39	POS	3.715	0.389	9.550	POS	2.730	0.224	12.188	POS	POS
Mea/40	POS	0.825	0.389	2.121	NEG	0.197	0.224	0.879	POS	POS
Mea/41	POS	2.826	0.389	7.265	POS	0.900	0.224	4.018	POS	POS
Mea/42	POS	3.92	0.389	10.077	POS	1.426	0.224	6.366	POS	POS
Mea/43	POS	1.625	0.389	4.177	wPOS	0.270	0.224	1.205	POS	POS
Mea/44	POS	1.276	0.389	3.280	wPOS	0.326	0.224	1.455	POS	POS
Mea/45	POS	2.768	0.389	7.116	wPOS	0.340	0.224	1.518	POS	POS
Mea/46	POS	2.427	0.389	6.239	POS	0.514	0.224	2.295	POS	POS
Mea/47	POS	3.539	0.389	9.098	POS	4.653	0.224	20.772	POS	POS
Mea/48	POS	2.883	0.389	7.411	POS	0.462	0.224	2.063	POS	POS
Mea/49	POS	4.656	0.389	11.969	POS	4.938	0.224	22.045	POS	POS
Mea/50	POS	2.157	0.389	5.545	POS	3.226	0.224	14.402	POS	POS
Mea/51	POS	3.331	0.389	8.563	POS	1.530	0.224	6.830	POS	POS
Mea/52	POS	5.088	0.389	13.080	POS	5.091	0.224	22.728	POS	POS
Mea/53	POS	4.118	0.389	10.586	POS	3.555	0.224	15.871	POS	POS
Mea/54	POS	4.07	0.389	10.463	POS	4.618	0.224	20.616	POS	POS
Mea/55	POS	4.506	0.389	11.584	POS	3.347	0.224	14.942	POS	POS
Mea/56	POS	2.928	0.389	7.527	POS	3.178	0.224	14.188	POS	POS
Mea/57	POS	2.086	0.389	5.362	POS	0.879	0.224	3.924	POS	POS
Mea/58	POS	1.912	0.389	4.915	POS	1.002	0.224	4.473	POS	POS
Mea/59	POS	2.002	0.389	5.147	POS	0.639	0.224	2.853	POS	POS
Mea/60	POS	4.314	0.389	11.090	POS	3.382	0.224	15.098	POS	POS
Mea/61	POS	4.096	0.389	10.530	POS	4.618	0.224	20.616	POS	POS
Mea/62	POS	5.04	0.389	12.956	POS	5.062	0.224	22.598	POS	POS
Mea/63	POS	3.306	0.389	8.499	POS	0.768	0.224	3.429	POS	POS
Mea/64	POS	3.91	0.389	10.051	POS	4.115	0.224	18.371	POS	POS
Mea/65	POS	2.336	0.389	6.005	POS	0.643	0.224	2.871	POS	POS
Mea/66	POS	3.062	0.389	7.871	POS	0.812	0.224	3.625	POS	POS
Mea/67	POS	1.611	0.389	4.141	POS	0.660	0.224	2.946	POS	POS
Mea/68	POS	3.59	0.389	9.229	POS	3.731	0.224	16.656	POS	POS
Mea/69	POS	4.582	0.389	11.779	POS	4.797	0.224	21.415	POS	POS
Mea/70	POS	4.253	0.389	10.933	POS	4.710	0.224	21.027	POS	POS
Mea/71	POS	1.48	0.389	3.805	POS	2.101	0.224	9.379	POS	POS
Mea/72	POS	1.862	0.389	4.787	POS	2.189	0.224	9.772	POS	POS
Mea/73	POS	4.147	0.389	10.661	POS	4.736	0.224	21.143	POS	POS
Mea/74	NEG	0.071	0.372	0.191	NEG	0.055	0.243	0.226	NEG	NEG
Mea/75	NEG	0.184	0.372	0.495	NEG	0.037	0.243	0.152	NEG	NEG

Table 2 (*Continued*)

Sample number	ELISA Micro ser[a]	OD Micro ser[b]	CO Micro ser	OD/CO Micro ser[c]	ELISA Micro sal[d]	OD Micro sal[e]	CO Micro sal	OD/CO Micro sal[f]	PCR naso[g]	PCR sal[h]
Mea/76	NEG	0.062	0.372	0.167	NEG	0.031	0.243	0.128	NEG	NEG
Mea/77	NEG	0.094	0.372	0.253	NEG	0.035	0.243	0.144	NEG	NEG
Mea/78	NEG	0.062	0.372	0.167	NEG	0.038	0.243	0.156	NEG	NEG
Mea/79	POS	0.643	0.372	1.728	NEG	0.051	0.243	0.210	NEG	NEG
Mea/80	NEG	0.115	0.372	0.309	NEG	0.057	0.243	0.235	NEG	NEG
Mea/81	NEG	0.063	0.372	0.169	NEG	0.055	0.243	0.226	NEG	NEG
Mea/82	NEG	0.153	0.372	0.411	NEG	0.067	0.243	0.276	NEG	NEG
Mea/83	NEG	0.052	0.372	0.140	NEG	0.049	0.243	0.202	NEG	NEG
Mea/84	NEG	0.153	0.372	0.411	NEG	0.072	0.243	0.296	NEG	NEG
Mea/85	NEG	0.131	0.372	0.352	NEG	0.036	0.243	0.148	NEG	NEG
Mea/86	NEG	0.18	0.372	0.484	NEG	0.052	0.243	0.214	NEG	NEG
Mea/87	NEG	0.177	0.372	0.476	NEG	0.058	0.243	0.239	NEG	NEG
Mea/88	NEG	0.173	0.372	0.465	NEG	0.032	0.243	0.132	NEG	NEG
Mea/89	NEG	0.063	0.372	0.169	NEG	0.045	0.243	0.185	NEG	NEG
Mea/90	NEG	0.06	0.372	0.161	NEG	0.046	0.243	0.189	NEG	NEG
Mea/91	NEG	0.227	0.372	0.610	NEG	0.060	0.243	0.247	NEG	NEG
Mea/92	NEG	0.075	0.372	0.202	NEG	0.042	0.243	0.173	NEG	NEG
Mea/93	NEG	0.096	0.372	0.258	NEG	0.065	0.243	0.267	NEG	NEG
Mea/94	NEG	0.118	0.372	0.317	NEG	0.065	0.243	0.267	NEG	NEG
Mea/95	NEG	0.15	0.372	0.403	NEG	0.159	0.243	0.654	NEG	NEG
Mea/96	NEG	0.075	0.372	0.202	NEG	0.066	0.243	0.272	NEG	NEG
Mea/97	NEG	0.179	0.372	0.481	NEG	0.067	0.243	0.276	NEG	NEG
Mea/98	NEG	0.064	0.389	0.165	NEG	0.027	0.224	0.121	NEG	NEG
Mea/99	NEG	0.171	0.389	0.440	NEG	0.040	0.224	0.179	NEG	NEG
Mea/100	NEG	0.083	0.389	0.213	NEG	0.041	0.224	0.183	NEG	NEG
Mea/101	NEG	0.06	0.389	0.154	NEG	0.038	0.224	0.170	NEG	NEG
Mea/102	NEG	0.184	0.389	0.473	NEG	0.150	0.224	0.670	NEG	NEG
Mea/103	NEG	0.324	0.389	0.833	NEG	0.044	0.224	0.196	NEG	NEG
Mea/104	NEG	0.085	0.389	0.219	NEG	0.042	0.224	0.188	NEG	NEG
Mea/105	NEG	0.077	0.389	0.198	NEG	0.046	0.224	0.205	NEG	NEG
Mea/106	NEG	0.046	0.389	0.118	NEG	0.060	0.224	0.268	NEG	NEG
Mea/107	NEG	0.203	0.389	0.522	NEG	0.058	0.224	0.259	NEG	NEG
Mea/108	NEG	0.101	0.389	0.260	NEG	0.042	0.224	0.188	NEG	NEG
Mea/109	NEG	0.053	0.389	0.136	NEG	0.041	0.224	0.183	NEG	NEG
Mea/110	NEG	0.097	0.389	0.249	NEG	0.062	0.224	0.277	NEG	NEG
Mea/111	NEG	0.125	0.389	0.321	NEG	0.063	0.224	0.281	NEG	NEG
Mea/112	NEG	0.075	0.389	0.193	NEG	0.057	0.224	0.254	NEG	NEG
Mea/113	NEG	0.092	0.389	0.237	NEG	0.049	0.224	0.219	NEG	NEG
Mea/114	NEG	0.104	0.389	0.267	NEG	0.070	0.224	0.313	NEG	NEG
Mea/115	NEG	0.203	0.389	0.522	NEG	0.066	0.224	0.295	NEG	NEG
Mea/116	NEG	0.075	0.389	0.193	NEG	0.036	0.224	0.161	NEG	NEG
Mea/117	NEG	0.066	0.389	0.170	NEG	0.057	0.224	0.254	NEG	NEG

OD, optical density; CO, cut-off; PCR, polymerase chain reaction; POS, positive result; wPOS, weak positive result; NEG, negative result.
[a] ELISA Micro ser: qualitative result for each serum sample obtained by the Measles IgM Capture EIA (MicroImmune, UK).
[b] OD Micro ser: OD of each serum sample of the Measles IgM Capture EIA (MicroImmune, UK).
[c] OD/CO Micro ser: ratio of the OD/CO of the Measles IgM Capture EIA (MicroImmune, UK) for each serum sample.
[d] ELISA Micro sal: qualitative result for each oral fluid sample obtained by the Measles IgM Capture EIA (MicroImmune, UK).
[e] OD Micro sal: optical density of each oral fluid sample of the Measles IgM Capture EIA (MicroImmune, UK).
[f] OD/CO Micro sal: ratio of the OD/CO the Measles IgM Capture EIA (MicroImmune, UK) for each oral fluid sample.
[g] PCR naso: qualitative results of the in-house nested reverse transcriptase (RT)-PCR on nasopharyngeal secretions.
[h] PCR sal: qualitative results of the in-house nested RT-PCR on oral fluid.

The absence of cross-reactivity of the PCRs was evaluated to make sure that no other exanthematous virus could be detected by these PCRs. Different dilutions of the rubella virus (80–800 IU/ml), parvovirus B19 (10^4–10^5 IU/ml), and mumps virus (800–8000 IU/ml) were tested. The measles PCRs did not give a positive result for any of these viruses.

The accurate detection of a panel consisting of 12 dilutions positive for MV RNA (i.e., 2000 IU/ml), alternating with 12 negative oral fluid samples, proved the absence of contamination during the test, since the negative samples remained negative for MV RNA after PCR.

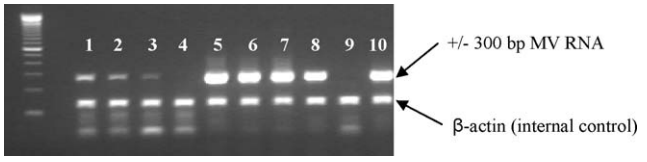

Figure 1. Nested RT-PCR on nasopharyngeal secretions showing positive and negative controls and measles positive and negative results. Lane 1: positive nasopharyngeal control (PC naso) (1/1000 dilution = 20 IU/ml); lane 2: PC naso (1/5000 dilution = 4 IU/ml); lane 3: PC naso (1/8000 dilution = 2.5 IU/ml); lane 4: negative nasopharyngeal control (NC = MRC5 cells); lanes 5, 6, 7, 8 and 10: nasopharyngeal sample positive for measles virus RNA; lane 9: nasopharyngeal sample negative for measles virus RNA.

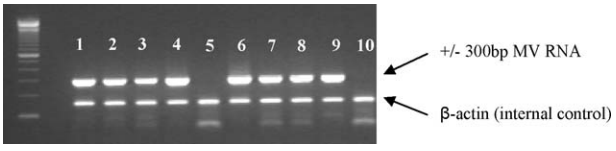

Figure 2. Nested RT-PCR on oral fluid showing positive and negative controls and measles positive and negative results. Lane 1: positive oral fluid control (PC OF) (1/100 dilution = 200 IU/ml); lane 2: PC OF (1/1000 dilution = 20 IU/ml); lane 3: PC OF (1/5000 dilution = 4 IU/ml); lane 4: oral fluid sample positive for measles virus RNA; lane 5: negative oral fluid control; lanes 6, 7, 8 and 9: oral fluid sample positive for measles virus RNA; lane 10: oral fluid sample negative for measles virus RNA.

4. Discussion

Our results show that oral fluid assays are suitable for routine laboratory confirmation in the diagnosis of measles cases. The oral fluid assay should make it easier for patients, GPs, and pediatricians to participate in the Belgian measles surveillance system and epidemiological studies (e.g., seroprevalence surveys).

The collection of oral fluid, unlike blood collection, is non-invasive, less painful, safe, and less expensive (i.e., no trained personnel required). Furthermore, only one sample (i.e., oral fluid) needs to be collected for serological, as well as molecular analysis.

This study shows that serological and molecular assays performed on oral fluid samples have a very high sensitivity and specificity compared with the gold standard tests on serum and nasopharyngeal secretion samples. The level of agreement between the commercially available EIA and ELISA for the detection of anti-measles IgM in oral fluid and serum was very good ($\kappa = 0.90$). The in-house nested RT-PCR assays for the detection of MV RNA in oral fluid and nasopharyngeal secretions also resulted in a very good level of agreement ($\kappa = 1$).

The excellent concordance of the PCR on oral fluid and nasopharyngeal secretions shows that nasopharyngeal samples can be replaced by oral fluid for the detection of MV RNA. In our study, samples were taken at an early stage of the disease, after the onset of the rash, when the viremia was still very high. This was probably the major reason for the high level of concordance between the two PCRs.

Previous studies have demonstrated that nasopharyngeal secretions can be replaced by oral fluid for measles virus detection, without loss of reliability.[21,22]

Six oral fluid samples out of 73 were IgM-negative, although the corresponding sera were positive. Since all samples were subjected to an IgG quantification assay, which showed that they all contained sufficient total IgG, an inadequate sample collection procedure could be excluded. These negative results could be explained by the timing of sample collection, since samples were collected at different times within 3 days after the onset of the rash. This is in line with the previously described study of Perry et al. who used a monoclonal IgM capture EIA[23] and proved that the sensitivity of a measles IgM assay depends on the timing of sample collection. An alternative explanation is that these negative results came from cases with a lower IgM concentration, which was detectable in serum (weakly positive) using the Enzygnost Anti-Measles Virus IgM ELISA (Table 2), since IgM is at a higher concentration in serum than in oral fluid.[10]

The high efficiency of the Oracol collection device (targeting GCF) has also been confirmed in oral fluid samples used for detecting anti-measles IgM and MV.[12] The use of this device reduces the presence of interfering factors, such as water, protein inorganic salts, mucin, and enzymes.[6] Although the salivary gland secretion contains most of the IgA, the GCF contains the highest concentrations of IgG and IgM.[12]

The Oracol device specifically collects oral fluid from areas of the mouth rich in crevicular fluid. There is good correlation between the performance of these oral fluid samples and sera in antibody assays of immunoglobulin concentrations.[12] In addition, it has been shown that participants favor the Oracol collection device over other sample collection methods.[12]

Previous studies have demonstrated that oral fluid can be used as a good alternative to serum for the detection of different viruses such as hepatitis A, B and C viruses, human immunodeficiency virus, measles and rubella.[7,8,11,21–41]

No other study groups have used identical assays and procedures, therefore it is not possible to extrapolate the sensitivities achieved in the present study on oral fluid (92% for the serological assay and 100% for the molecular assay) with those obtained by other study groups.[21–24,29,31,34,38,39]

Helfand et al. used their own antibody capture EIA for the detection of measles IgM in oral fluid using the OraSure collection device.[29] They compared the oral fluid assay with the traditional serum assay and obtained a sensitivity of 91% and specificity of 95%.

Perry et al. developed an in-house M-antibody capture radioimmunoassay (MACRIA) for oral fluid. They collected oral fluid by dribbling into a sterile plastic container.[34] In their study, a distinction was made in the sensitivity obtained at the different time points of sample collection, i.e. first 7 days following the rash, or within 1–5 weeks after the appearance of the rash. For these time points, they measured a sensitivity of 92% and 100%, respectively.

Although both research groups used other serological assays, we can assume that the obtained sensitivity of 92% with the EIA from MicroImmune is an excellent result. Van Binnendijk et al. used another molecular assay–real-time PCR, which is generally known as a more sensitive assay in comparison with the traditional nested RT-PCR.[22] Although, they mentioned a detection limit of 0.01–0.001 IU/ml, it was not clear in their paper to which end-point it corresponded.

In 2007, Thomas et al. also described the evaluation of a real-time PCR for the rapid detection of MV RNA in oral fluid.[38] The major difference between the real-time PCR of Thomas et al. and Van Binnendijk et al. was the choice of the targeted gene: H-gene and N-gene of the measles genome, respectively. Thomas et al. routinely performed the real-time PCR (H-gene) and nested RT-PCR (N-gene) in parallel for the purposes of genotyping. However, they found the real-time PCR to be more sensitive and rapid.

In our nested RT- PCR, 4 IU/ml corresponds to the 99% end-point for the detection of MV in oral fluid. In every run, a positive control containing 4 IU/ml is introduced and should be positive. In some cases we were able to detect 0.02 IU/ml, however no 95% or 99% end-point could be guaranteed.

Our results show that our in-house developed nested RT-PCR on oral fluid has a very good sensitivity (100%). However, it should be mentioned that the MV RNA positivity depends on the time-point of sampling, which, of course, has an influence on the calculation of the sensitivity of the assay. MV RNA can be detected very well until 7 days after the onset of the rash.[3,22] Afterwards the measles viremia declines rapidly, but remains detectable until 2 weeks after the appearance of the rash.

Different European countries have already implemented oral fluid assays in their measles surveillance systems. Oral fluid sampling is recommended by the WHO as a suitable surveillance tool for measles. Measles IgM and MV RNA have been proven to be detectable very early after the onset of symptoms.[3,4] A comparative study has demonstrated that results obtained on oral fluid and dried blood spots are comparable to those obtained on serum. A very good result was achieved for the detection of measles IgM and MV RNA in oral fluid, which was not the case for the dried blood spots.[3]

In the UK, oral fluid assays for the detection of measles have been carried out since the beginning of the 1990s.[5,23] Between 1995 and 2001 in England and Wales, the program to eliminate measles resulted in a surveillance campaign in some schools. This surveillance relied upon oral fluid sampling, and interesting and reliable results were produced.[40] The Department of Health in England even recommended that all suspected cases of measles should be tested for the presence of salivary measles IgM. In Ireland, they used oral fluid samples to evaluate the molecular epidemiology of circulating measles virus from 2002 to 2007.[41] In Belgium, oral fluid sampling has been shown to be a useful tool in the surveillance and control of the recent measles outbreak in

Antwerp.[42] These studies prove that oral fluid is already a standard approach for measles detection in different European countries.

The implementation of oral fluid sample collection has significant advantages over the sampling and analysis of serum and/or nasopharyngeal secretions in measles virus surveillance.

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