Real-time PCR for mumps diagnosis on clinical specimens—Comparison with results of conventional methods of virus detection and nested PCR

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Abstract

Background: Since November 2003, the UK has seen a dramatic rise in the number of mumps cases, resulting in increasing demands on virology laboratories to confirm mumps infection in a timely and efficient manner. Traditional mumps virus detection methods are often insensitive, lengthy, and cumbersome. Some laboratories in the UK now use molecular methods that are based on nested polymerase chain reaction (PCR). Early serological diagnosis often relies on detection of anti-mumps IgM, which may be absent in the first 10 days of illness.

Objectives: We compared a one-step real-time RT-PCR with an established nested PCR (SH-PCR) and virus detection by culture and antigen detection, and assessed the clinical usefulness of mumps real-time PCR for diagnosis from CSF.

Study design: In total, 280 clinical samples were investigated by real-time PCR, nested PCR and a combination of traditional virus detection methods (antigen detection on oral samples, cell culture on all samples). Furthermore, 88 CSF samples submitted for diagnosis of possible viral meningitis were analysed by real-time PCR.

Results: The real-time PCR detected the highest number of positive oral samples (119/180) compared to SH-PCR (92/180) and combined virus culture and antigen detection procedures (90/180). Sensitivity of mumps virus detection in urine was poor for all three methods: 34.0% (traditional detection), 29.8% (real-time PCR) and 2.1% (SH-PCR), respectively. Real-time PCR on 88 CSF samples identified five patients with mumps meningitis, significantly increasing viral diagnosis in this cohort.

Conclusion: Real-time PCR on oral samples is the investigation of choice for mumps infection. Mumps virus detection in urine by any of the PCRs used was clearly less successful. Real-time PCR on CSF samples seems a promising adjunct for diagnosis of mumps meningitis, especially in an age group with high incidence of mumps.

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Keywords: Mumps virus; Real-time PCR; Nested PCR; Virus culture; Virus detection; Meningitis

1. Introduction

Since 2003, there has been a resurgence of mumps in the UK, particularly among adolescents and young adults (Donaghy et al., 2006). The incidence of mumps in susceptible age groups will probably continue to be high in the foreseeable future (Hopkins et al., 2005). Laboratory diagnosis is usually requested on patients with symptoms, necessitating prompt diagnosis on acute samples. Traditionally mumps has been diagnosed by virus isolation in cell culture or complement fixation testing (CFT) of antibody titres on paired sera. The latter has been largely replaced by testing for anti-
mumps-IgM, which is not always detected in the first 10 days of symptoms (Krause, unpublished data). Mumps virus grows quite readily in routine cell culture and can be isolated from CSF (Leinikki, 2004). However, procedures can be lengthy and cumbersome and require highly experienced staff. The sensitivity of mumps virus isolation depends on the quality of samples, but is not expected to exceed 75% (Utz et al., 1958).

The development of molecular diagnostic technology has provided rapid and sensitive tests directly applied to clinical material for many viruses. Comparatively few PCR methods have been described to detect mumps RNA. A nested reverse transcriptase polymerase chain reaction (RT-PCR) amplifying the SH gene has been employed on oral samples for large-scale epidemiological study in the UK by Jin et al. (2004). Uchida et al. (2005) described a real-time PCR amplifying a region located in the fusion (F) gene. That assay had detected mumps virus with high sensitivity in a small number of clinical samples. Its efficiency for different mumps genotypes was not assessed. Here, we describe a study that compared the analytical sensitivity of a real-time PCR with a nested PCR using serial dilutions of known genotypes. We compared the clinical sensitivities and specificities of both PCR assays using clinical specimens that had been analysed using conventional mumps virus detection methods in our laboratory. The usefulness of real-time PCR on unselected CSF samples from patients with meningitis was also assessed.

2. Material and methods

Mumps viruses of known genotype (A, C, D, G1, G2, G5, G6, and J) were obtained from Dr. Li Jin at the Health Protection Agency Colindale and cultured in monkey kidney cells.

In total 280 clinical samples submitted to the Specialist Virology Centre Edinburgh between October 2004 and March 2005 for mumps diagnosis were evaluated. They comprised of 180 oral samples (120 parotid duct swabs and 60 other oral swabs: throat swabs, nose/throat swabs, oral unspecified swabs, and saliva) in virus transport medium (VTM) and 100 urine samples.

2.1. Virus isolation methods

All samples had previously been investigated for mumps virus infection in the virus isolation (VI) suite at the SVC Edinburgh, using a combination of three different methods: direct detection of mumps antigen in the sample, shell vial culture, and tube culture. Mumps antigen detection was performed using indirect immunofluorescence testing (IF) with mouse anti-mumps monoclonal antibodies (CHEMICON) and anti-mouse fluorescent antibodies (Light Diagnostics) on oral samples only. Rapid culture with MRC5 shell vials was performed on all sample types. Shell vials were fixed and stained as for IF after 48 h of incubation at 36°C. Cell culture tubes with monkey kidney cells were inoculated and incubated at 36°C, observed for cytopathic effect (CPE) twice weekly, and haemadsorption test with guinea pig erythrocytes was performed. Positive CPE was confirmed by IF.

A positive result in any of these three procedures was reported as significant. Therefore, a VI positive sample was defined by any of the three methods yielding a positive result.

Eighty-eight CSF samples submitted between October 2004 and July 2005 for investigation of viral meningitis from patients aged between 2 and 60 years with a residual volume of at least 200 µl stored at −70°C were investigated.

Of these, 80 samples had been previously tested for viruses in the routine laboratory. In 3 of 88 samples virus isolation had been attempted by cell culture. Other investigations were PCR for HSV1 and HSV2 (43 samples; 1 positive result for HSV2), enterovirus PCR (62 samples; 2 positive results) and VZV PCR (14 samples; all negative).

While 200 µl of oral samples and urine were extracted, 100 µl of VTM was added to 100 µl of CSF initially. CSF samples were re-extracted using up to 200 µl. All samples and controls were extracted using the QIAamp MinElute Virus Spin kit (QUIAGEN) according to manufacturer’s instructions with the following modifications for all clinical samples and controls:

Instead of a spin cycle, a suction device, the QIAGEN vacuum manifold, was used to pull lysate and wash buffers though the membrane of the extraction columns.

An additional washing step with AW1 buffer (QUIAGEN) was included before the standard washing step with AW2 buffer as suggested by the manufacturer to maximise template purification with the vacuum manifold.

The real-time PCR was performed with primer and probe sequences as previously published by Uchida et al. (2005). The TaqMan® probe was synthesized with a reporter dye 6-carboxyfluorescein (FAM) at the 5’-end and a black-hole quencher (BHQ) at the 3’-end by Operon, Germany.

SH-PCR was performed using primers SH1, SH2, SH3, and SH4 as described by Cohen et al. (1999).

The reaction mix for the real-time PCR (final volume of 25 µl) contained 5 µl eluted RNA, 0.8U RNAsin (Promega), 1200 nM of each primer, 50nM probe, and the following components of the QIAGEN one-step RT-PCR kit: 5 µl reaction buffer, 1 µl dNTP mix, 1 µl enzyme mix.

Amplification was performed using an ABI 7500 real-time PCR system (Applied Biosystems) under the following conditions: 50°C for 30 min, 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, and 59°C for 1 min.

The cDNA for the SH-PCR was synthesized in a 20 µl reaction mixture containing 6 µl eluted RNA, 50 U MMLV-RT (Invitrogen) and first strand buffer and 10 mM DTT (supplied with the enzyme), 500 µM of each dNTP, 50 ng random hexamers and 1.6 U RNasin (Promega). The mixture was incubated at 25°C for 10 min, 37°C for 50 min, and 94°C for 5 min. The 50 µl reaction mixture for the first amplification round contained 5 µl of cDNA, 1 U Taq Poly-
merase (Promega), magnesium free reaction buffer supplied with the enzyme, 1.5 mM MgCl$_2$, 200 μM of each dNTP, and 150 nM of each primer SH1 and SH2. Nested amplification was performed with 2 μl first round product in a 50 μl reaction mix as described above. Amplification was carried out as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 15 min. Reverse transcription and amplification were performed on a P2 Thermal Cycler (Thermo Electron Corporation). The amplified product was detected after electrophoresis through 1.5% agarose gels with 6 μl ethidium bromide (10 mg/ml) in 100 ml. The 639 bp product was visualised under UV light.

Ten-fold dilutions series (10$^{-1}$–10$^{-6}$) were prepared from extract of passaged virus of all genotypes available and analysed by both PCR methods. If the highest dilution was still detectable, analysis was repeated including dilutions of up to 10$^{-8}$ (i.e. beyond detection endpoint). Slopes and linear ranges of the standard curves for each genotype were assessed after real-time PCR.

Urine and oral samples were analysed in parallel by both PCRs on the same extract.

CSF samples were analysed by real-time PCR only. Positive CSF results were confirmed by repeating the assay after re-extraction.

Each PCR run also contained a negative control (nuclease free water) and a positive control (extract from cultured mumps virus genotype A diluted 1:20 with CSF).

Respiratory samples positive for respiratory syncytial virus, parainfluenza type 1 and type 3, adenovirus, influenza B, coxsackie A9, untyped enterovirus, rhinovirus and human cytomegalovirus, and CSF positive for enterovirus, herpes simplex type 1, herpes simplex type 2, Neisseria meningitides and varicella-zoster virus were also extracted and subjected to the mumps real-time PCR. There was no internal control.

In an attempt to define true negative (TN) and true positive (TP), results were evaluated using reference standard criteria. All samples positive by tube culture were considered TP. If samples had been negative by tube culture, additional information about other samples submitted at the same time or convalescent serology consistent with recent mumps was sought from the patients’ record. Positive tube culture or serology results on these also defined the sample in question as TP, as did at least two positive results by two methods not hitherto considered (i.e. shell vial, direct antigen detection, SH-PCR or real-time PCR) on the sample itself. In the absence of any of the above, samples were considered TN.

Sensitivity, specificity and accuracy were calculated using 2 × 2 tables. Accuracy was defined as the number of correctly identified (positive and negative) samples per 100 samples analysed. The 95% confidence intervals were produced using the Wilson score method.

### 3. Results

The analytical sensitivity for all analysed genotypes was on average 100-fold greater with real-time PCR than with SH-PCR. Real-time PCR amplified all genotypes with excellent efficiency, documented by slopes from −3.34 cycles/log 10 to −3.75 cycles/log 10 decade with correlation coefficients

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**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Standard curves</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope$^b$</td>
<td>Linear range</td>
</tr>
<tr>
<td>A</td>
<td>−3.58</td>
<td>Neat−10$^{-5}$</td>
</tr>
<tr>
<td>C</td>
<td>−3.40</td>
<td>Neat−10$^{-6}$</td>
</tr>
<tr>
<td>D</td>
<td>−3.60</td>
<td>Neat−10$^{-4}$</td>
</tr>
<tr>
<td>G1</td>
<td>−3.75</td>
<td>Neat−10$^{-6}$</td>
</tr>
<tr>
<td>G2</td>
<td>−3.34</td>
<td>Neat−10$^{-5}$</td>
</tr>
<tr>
<td>G5</td>
<td>−3.45</td>
<td>Neat−10$^{-5}$</td>
</tr>
<tr>
<td>G6</td>
<td>−3.63</td>
<td>Neat−10$^{-5}$</td>
</tr>
<tr>
<td>J</td>
<td>−3.57</td>
<td>Neat−10$^{-5}$</td>
</tr>
</tbody>
</table>

$^a$ The standard curve is the linear graph plotting the Ct value of each sample in a dilution series against its amount of template (on a logarithmic scale).

$^b$ As determined in the linear range of the standard curve and stated in cycles/log 10 decade.

**Table 2**

<table>
<thead>
<tr>
<th>Oral samples</th>
<th>VI positive</th>
<th>VI negative</th>
<th>Total</th>
<th>Urine</th>
<th>VI positive</th>
<th>VI negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time positive</td>
<td>85</td>
<td>34</td>
<td>119</td>
<td>Real-time positive</td>
<td>7</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Real-time negative</td>
<td>5</td>
<td>56</td>
<td>61</td>
<td>Real-time negative</td>
<td>10</td>
<td>74</td>
<td>84</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>90</td>
<td>180</td>
<td>Total</td>
<td>17</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>SH-PCR positive</td>
<td>81</td>
<td>11</td>
<td>92</td>
<td>SH-PCR positive</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SH-PCR negative</td>
<td>9</td>
<td>79</td>
<td>88</td>
<td>SH-PCR negative</td>
<td>17</td>
<td>82</td>
<td>99</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>90</td>
<td>180</td>
<td>Total</td>
<td>17</td>
<td>83</td>
<td>100</td>
</tr>
</tbody>
</table>
The 95% confidence interval is given in parentheses.

VIa

SH-PCR

Real-time PCR

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral samples</td>
<td>98.06 (93.19–99.17)</td>
<td>100.00 (85.13–100.00)</td>
<td>98.40 (94.35–99.56)</td>
</tr>
<tr>
<td>Urine</td>
<td>29.79 (18.65–43.98)</td>
<td>97.14 (85.47–99.49)</td>
<td>58.54 (47.73–68.58)</td>
</tr>
</tbody>
</table>

SH-PCR

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral samples</td>
<td>89.32 (81.88–93.93)</td>
<td>100.00 (85.13–100.00)</td>
<td>91.20 (84.93–95.02)</td>
</tr>
<tr>
<td>Urine</td>
<td>2.13 (0.38–11.11)</td>
<td>100.00 (90.11–100.00)</td>
<td>43.90 (33.67–54.68)</td>
</tr>
</tbody>
</table>

VIa

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral samples</td>
<td>83.50 (75.15–89.43)</td>
<td>100.00 (85.13–100.00)</td>
<td>86.40 (79.30–91.33)</td>
</tr>
<tr>
<td>Urine</td>
<td>34.04 (22.17–48.33)</td>
<td>100.00 (90.11–100.00)</td>
<td>62.20 (51.38–71.92)</td>
</tr>
</tbody>
</table>

The 95% confidence interval is given in parentheses.

a VI: virus isolation methods.

above 0.99. For each genotype, endpoint dilutions of SH-PCR and real-time PCR and details of the standard curves are listed in Table 1.

Results of PCR and VI methods on clinical samples are detailed in Table 2. Real-time PCR yielded the highest number of positive results for all oral samples: 119 out of 180 samples. SH-PCR yielded only slightly more positive results than VI methods (92 samples versus 90 samples). VI methods and real-time PCR were near equivalent in detecting mumps in urine. However, sensitivity was low. Mumps RNA was detected in only 1 urine sample out of 100 by SH-PCR.

Details of sensitivity and specificity of each test method for the different specimen types are listed in Table 3. Fifty-five of 180 oral samples could not be included in the reference standard because neither serology nor results from an alternative sample were available to refute or confirm results ofshell vial, antigen detection or PCR on the sample in question. In contrast, 82 of 100 urine samples could be classified as TP or TN, mainly because of results from oral samples submitted at the same time.

Real-time PCR performed with the highest sensitivity of all three methods on all oral samples. Specificity of SH-PCR always reached 100%, as SH-PCR positive samples were all confirmed by real-time PCR.

Mumps virus RNA was initially detected by real-time PCR in six CSF samples with logarithmic amplification of fluorescence (Ct: 35.81–43.72). After re-extraction all but the sample with the highest Ct value remained positive. The two patients concerned had all presented with sterile CSF and CSF lymphocytes. Parotitis had been diagnosed in two patients. In two further cases, mandibular or submandibular “swelling” was noted after onset of meningitis. Virus culture had been attempted (unsuccessfully) in three of these five CSF samples. Infection with mumps virus was confirmed in two patients with parotitis (by serology or virus isolation from an oral sample). The Ct values in their CSF were 35.81 and 42.15, respectively. Evidence of mumps infection was not found in the laboratory reporting system for any of the 82 patients where samples were negative by real-time PCR.

4. Discussion

Cell culture has retained its value as a “catch all” method to diagnose unexpected or unidentified viruses (Ogilvie, 2001). In the SVC Edinburgh it enabled the laboratory staff to confirm large numbers of mumps infections after over a decade of mumps being exceptionally rare. However, PCR techniques have shown superior sensitivity on many occasions (Guney et al., 2003; Slomka et al., 1998). Large variations in sensitivity between different PCRs should be addressed before an assay to replace conventional virus detection is chosen. The SH-PCR performed less well than anticipated. We have shown a 100-fold difference in the lower level of detection of the two PCR assays, and this is mirrored in their comparative sensitivity when used on all specimen types. A previous report also commented on the surprisingly low sensitivity of an SH-PCR protocol (Jin et al., 2004). Degradation of RNA during transport was named as a possible reason, but this appears highly unlikely here. Differences in sensitivity between SH-PCR and real-time PCR might be due to primer location and product length. The SH-PCR product covers the entire length of the SH gene, known to be the most variable region of the mumps genome. The F gene, the target of the real-time PCR, appears to be the most conserved (Uchida et al., 2003). While mumps virus is considered genetically relatively stable, a PCR targeting the most conserved region is less likely to loose sensitivity due to strain variation. Primers and probes for the real-time PCR were designed from the sequence of a genotype G strain (Uchida et al., 2005). Alignment of representative sequences of mumps viruses from all genotypes showed very few primer/probe mismatches (data not shown). We showed near-ideal efficiency for all genotypes assessed in the linear range of the standard curve with slopes between −3.34 cycles/log 10 and −3.75 cycles/log 10 decade. We, therefore, expect this real-time PCR to have an excellent sensitivity for all genotypes. The 100-fold lower analytical sensitivity of the SH-PCR made it unsuitable to serve as “gold standard” in this evaluation. We thus devised a “reference standard” where TP and TN are defined. Using such a “reference standard” tends to overestimate both sen-
sitivity and specificity, but might be useful when assessing several tests in parallel. We aimed to evaluate the performance of the molecular methods: criteria were weighted so that these methods would be assessed more critically. Almost one-third of all oral samples, could not be classified as TP or TN due to the stringent definitions used.

Specificity was very high for all test methods. Real-time PCR was the most sensitive test for mumps virus on oral samples, even without taking into account 19 oral samples that were positive by real-time PCR alone, as they could not be considered in the reference standard.

In the detection of mumps RNA in urine, however, both PCR methods failed to show any advantage compared to virus isolation. This was despite the fact that virus isolation in this study was considerably less successful than one might expect from the literature (Utz et al., 1958). In contrast to oral specimens, a considerable number of urines (nine samples) from which virus had been cultured remained negative by PCR.

The reasons for this remained unclear, but could lie in the presence of variable amounts of PCR inhibitors in the eluate. Effective extraction of nucleic acid from cerebrospinal fluids and nasopharyngeal swabs using our chosen method has been described (Seferis et al., 2005) but similar data on urine were not found. Inhibition of PCR by urinary substances has been well described for Chlamydia detection (Mahony et al., 1998), but few studies exist on extraction of RNA virus from urine. Real-time RT-PCR on cat urine spiked with feline calicivirus showed superior results after extraction with silica gel-based columns compared to other methods according to Scansen et al. (2005). However, results might not be directly transferable to human urine conditions. Repeat extraction of some select urine samples with two alternative methods (phenol chloroform and using the QIAGEN QIAamp Viral RNA Mini Kit) yielded comparable Ct values to using the QIAbamp MinElute Virus Spin kit. A commercial kit for urine preparation for chlamydia PCR yielded negative results only. Detection of mumps virus RNA in urine by PCR has been reported, but success rates appear low compared to oral samples (Afzal et al., 1997). This contrasts with historical data presented for virus culture, where both urine and oral samples appear equally suitable. Further studies are needed to elucidate the reasons, with optimal sample preparation an obvious starting point, before PCR can be recommended for mumps detection in urine.

Our results suggest real-time PCR to be a sensitive and specific test for mumps meningitis. Real-time mumps PCR remained negative in all samples with known other pathogens, but suspected mumps meningitis was confirmed in cases where no virus had been isolated. Mumps RNA was also detected in the CSF of several cases, where mumps virus had not been specifically queried. One patient presented with submandibular swelling 1 week after recovery from meningitis, one patient suffered from sore throat only. Delayed onset or absence of typical mumps symptoms is well described (Leinikki, 2004). Good amplification was observed in all positive samples and CSF samples from patients with confirmed mumps yielded similar Ct values to those without additional confirmation. Diagnosis of mumps might have been improved, had virological investigations been incomplete: virus culture had been attempted from three of six CSFs and throat swabs in VTM had been received in two of the six cases with initially positive mumps real-time PCR result. Presence of mumps RNA could not be confirmed in one CSF sample from a 19-year-old patient with a 1-week history of headache. Nonspecific amplification seems unlikely, as the amplification followed a sigmoidal pattern, and no sample containing other known pathogens had yielded a false positive result using this assay. A false positive result due to contamination is possible. Alternatively, the very low quantity of viral RNA in this sample, suggested by the high Ct value in the initial PCR, could have been lost during repeated freezing and thawing.

Real-time PCR for mumps in CSF increased identification of a viral pathogen significantly in our sample cohort and could be a valuable adjunct for diagnosis of aseptic meningitis in young adults.

Acknowledgments

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