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Nested PCR for Rapid Detection of Mumps Virus in Cerebrospinal Fluid from Patients with Neurological Diseases

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In this study, we have developed a reverse transcription (RT)-nested polymerase chain reaction (n-PCR) for the detection of mumps virus RNA in cerebrospinal fluid (CSF) from patients with neurological infections. A specific 112-bp fragment was amplified by this method with primers from the nucleoprotein of the mumps virus genome. The mumps virus RT–n-PCR was capable of detecting 0.001 PFU/ml and 0.005 50% tissue culture infective dose/ml. This method was found to be specific, since no PCR product was detected in each of the CSF samples from patients with proven non-mumps virus-related meningitis or encephalitis. Mumps virus RNA was detected in all 18 CSF samples confirmed by culture to be infected with mumps virus. Positive PCR results were obtained for the CSF of 26 of 28 patients that were positive for signs of mumps virus infection (i.e., cultivable virus from urine or oropharyngeal samples or positivity for anti-mumps virus immunoglobulin M) but without cultivable virus in their CSF. Overall, mumps virus RNA was detected in CSF of 96% of the patients with a clinical diagnosis of viral central nervous system (CNS) disease and confirmed mumps virus infection, while mumps virus was isolated in CSF of only 39% of the patients. Furthermore, in a retrospective study, we were able to detect mumps virus RNA in 25 of 55 (46%) CSF samples from patients with a clinical diagnosis of viral CNS disease and negative laboratory evidence of viral infection including mumps virus infection. The 25 patients represent 12% of the 236 patients who had a clinical diagnosis of viral CNS infections and whose CSF was examined at our laboratory for a 2-year period. The findings confirm the importance of mumps virus as a causative agent of CNS infections in countries with low vaccine coverage rates. In summary, our study demonstrates the usefulness of the mumps virus RT–n-PCR for the diagnosis of mumps virus CNS disease and suggests that this assay may soon become the "gold standard" test for the diagnosis of mumps virus CNS infection.

The mumps virus, a member of the *Paramyxovirus* genus, consists of a single-stranded negative-sense genomic RNA with a virion composed of two surface glycoproteins (HN and F), a matrix protein (M), and three core proteins (P, L, and NP) (48).

Mumps is a benign childhood infection, and parotitis is the most common clinical symptom. Cerebrospinal fluid (CSF) pleocytosis has been detected in more than half of all patients with mumps virus infections, showing that dissemination of the virus to the central nervous system (CNS) is a common event in patients with this viral infection (4). The most frequent complication of mumps virus infection is aseptic meningitis (23, 30). Other CNS complications include acute and chronic encephalitis (26, 31), transverse myelitis (35), hydrocephalus (37, 46), and acute cerebellar ataxia (13).

The massive use of live attenuated mumps virus vaccines in developed countries successfully reduced the rate of disease due to mumps virus infection; however, mumps virus outbreaks have not been completely eliminated from these vaccinated populations (3, 6, 12, 16, 29, 44; A. Colville and S. Pugh, Letter, Lancet **340:**786, 1992). Moreover, in the absence of effective vaccination programs, i.e., in developing countries, mumps virus is one of the most common causes of viral meningitis. Although the case fatality rate is only 1 in 10,000, nonfatal complications of mumps virus infection often lead to hospitalization and occasionally to permanent and severe neurological sequelae (20, 28, 36, 42).

Historically, the presence of mumps virus-specific immunoglobulin M (IgM) in a single serum specimen is considered diagnostic of a recent infection. However, the detection of a mumps virus-specific IgM response must be interpreted cautiously since mumps virus may circulate in the community without causing disease. Therefore, the presence of mumps virus-specific IgM may not always establish causality of CNS disease.

Isolation of mumps virus from CSF implies true invasive infection of the CNS and a high likelihood of association with current illness. However, virus isolation from CSF lacks sensitivity, is time-consuming, and represents an expensive, laborious task.

The most promising development in direct detection of virus in CNS has been the application of PCR. This technique has been shown to have high degrees of sensitivity and specificity when applied to the diagnosis of several viral infections of CNS, especially those caused by enteroviruses and herpesviruses (2, 7–9, 15, 17–19, 21, 24, 32, 34, 38–40, 50). However, few procedures have been described for mumps virus RNA detection. Boriskin Yu et al. (5) have developed a PCR method for the detection of mumps virus; however, the virus must be passaged in tissue culture prior to amplification.

Recently, a PCR for detection of mumps virus RNA directly in clinical samples has been described (14). However, the ef-

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fectiveness of this newly developed mumps virus PCR was evaluated with CSF from only six patients with CNS disease.

In the present study, we describe the development of reverse transcription (RT)-nested PCR (n-PCR) procedure for the direct detection of mumps virus in CSF samples. The method developed in this study was evaluated by testing 101 CSF samples from patients with different CNS illnesses, such as aseptic meningitis, encephalitis, acute cerebellar ataxia, and Guillain-Barré syndrome.

MATERIALS AND METHODS

Patients. The total number of CSF specimens tested by RT–n-PCR was 101. The specimens tested were divided into the groups described below for the purpose of analysis.

(i) Group A. Group A comprised patients ($n = 18$) positive for mumps virus by culture of CSF. The clinical syndromes among the patients were aseptic meningitis ($n = 17$) and encephalitis ($n = 1$).

(ii) Group B. Group B comprised patients ($n = 18$) with mumps virus-specific IgM but with no cultures of CSF, urine, or oropharyngeal swabs positive for virus. The clinical syndromes among the patients were aseptic meningitis ($n = 11$); encephalitis ($n = 5$), acute cerebellar ataxia ($n = 1$), and Guillain-Barré syndrome ($n = 1$).

(iii) Group C. Group C comprised patients ($n = 10$) whose urine or oropharyngeal swabs were culture positive for mumps virus but whose CSF was not positive for mumps virus. The clinical syndromes among the patients were aseptic meningitis ($n = 8$) and encephalitis ($n = 2$).

(iv) Group D. Group D comprised patients ($n = 55$) with otherwise unexplained CSF pleocytosis (negative bacterial culture), with no specimen culture positive for mumps virus, and with no detectable mumps virus-specific IgM. The syndromes among the patients were aseptic meningitis ($n = 34$), encephalitis ($n = 19$), and acute cerebellar ataxia ($n = 2$).

These samples belonged to a panel of 236 CSF samples from patients with a clinical diagnosis of viral CNS infection. In 118 (54%) and 53 (25%) of the CSF samples, enterovirus and herpes simplex virus were detected by PCR methods developed by Severini et al. (43) and Aurelius et al. (2), respectively. The remaining 55 CSF samples were ones retrospectively analyzed in the present study.

(v) Control group. As a control group, CSF samples from 27 patients with meningitis due to the following agents were tested: enterovirus ($n = 17$), herpes simplex virus ($n = 5$), varicella-zoster virus ($n = 1$), *Neisseria meningitidis* ($n = 2$), and *Haemophilus influenzae* type B ($n = 2$). Also, CSF from three patients with meningitis due to trauma were included.

Virus. The Jeryl Lynn strain of the mumps virus vaccine (Mumps Vax; Merck Sharp & Dohme, West Point, Pa.) was used. A viral stock was prepared from virus passaged in LLC-Mk2 cells (ATCC CCL-7; a rhesus monkey kidney cell line) obtained from the American Type Culture Collection.

Virus isolation. Virus isolation in Vero cells (ATCC CCL-81; an African green monkey kidney cell line) and LLC-Mk2 cells was attempted for CSF samples from 101 patients. Tissue culture medium was aspirated from each cell culture tube, 0.1 ml of CSF was added to the cell monolayers, and the cell monolayers were incubated for 1 h at 37°C. After the absorption period, 1.5 ml of minimal essential medium (Gibco BRL, Gaithersburg, Md.) supplemented with antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml), 2 mM L-glutamine (Gibco BRL), and 2% inactivated fetal calf serum was added to each tube. Uninoculated cell culture tubes were used as controls. The tubes were incubated at 37°C for 7 days and were examined daily for the appearance of a cytopathic effect (CPE). The presence of mumps virus in cultures showing typical CPEs was confirmed by indirect immunofluorescence assay (IFA) with a monoclonal antibody (MAb), anti-mumps virus NP (MAb 843; CHEMICON International Inc., Temecula, Calif.). IFA was also carried out for all cultures that lacked a CPE after 7 days. Those cultures negative by IFA were subsequently passaged. Passages were considered negative if no CPE or a lack of staining by IFA was observed. The samples were passaged three times on both cell lines before being considered negative.

Serological test. IgM antibodies to mumps virus were determined by indirect immunofluorescence on slides prepared by using LLC-Mk2-infected cells. Serum samples were depleted of IgG. This was done by incubating equal volumes of samples and sheep antihuman IgG serum (BION, Parke Ridge, N.J.) overnight at 4°C. Negative and positive control sera were included in each assay. Samples with morphologically typical fluorescence at a dilution of 1:10 or greater were considered positive.

Mumps RT–n-PCR. A 100-μl aliquot of each clinical sample was mixed with 500 μl of extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate [pH 7.0], 1% sarcosyl, 2.5 M β-mercaptoethanol, 50 μl of 2 M sodium acetate [pH 4.0], and 500 μl of phenol saturated with diethyl pyrocarbonate [DEPC]-treated water). The mixture was then vortexed and left at room temperature for 15 min. A total of 100 μl of a chilled chloroform-isoamyl alcohol mixture (49:1) was added, and the sample was vortexed and placed on ice. After 10 min, the mixture was centrifuged at 15,000 × *g* for 5 min at 4°C, and the aqueous phase was transferred to a tube with 600 μl of chilled isopropanol and 20 μl of dextran T500 (20 μg/μl) and was then left at −20°C overnight. On the following day, the sample was centrifuged at 15,000 × *g* for 30 min at 4°C, and the final pellet was washed once with 70% ethanol. The RNA pellet was briefly dried and was resuspended in 10 μl of DEPC-treated water (11).

The synthesis of cDNA was performed in a 20-μl reaction volume containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 40 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.), 20 pmol of primer NP7, and 10 U of RNasin (Promega). The mixture was incubated at 37°C for 90 min. After the elongation, the tubes were inactivated at 95°C for 5 min.

The primer used for the cDNA synthesis was NP7 (5′-CATCTTGTTGAGA ATCACCA-3′; virion sense primer), and the first amplification was done with this same primer and the NP8 primer (5′-CTTCAGAGTACAGCCACTCA-3′; mRNA sense primer), which flank the region between nucleotides 1490 and 1680 of the NP gene of the mumps virus Miyahara strain (45). These primers are the same as those described by Boriskin Yu et al. (5).

The primers used for the second amplification were Mumps-3 (5′-CAGGAT CCAATTCAAGCACA-3′; virion sense primer) and Mumps-4 (5′-AATCTTG GTGTTCCATCCCC-3′; virion antisense primer). These two primers were designed on the basis of the known sequence of the initial products and were designed by using Primer 3 software (S. Rozen and H. Skaletsky, Whitehead Institute for Biomedical Research, Massachusetts of Technology Center, http://www.genome.wi.mit.edu/genome_software/other/primer3.html).

The first round of PCR amplification was carried out in a 50-μl reaction volume containing 20 μl of the cDNA product, 5 μl of the PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl [Promega]), 6 μl of 25 mM $MgCl_2$ (Promega), 20 pmol of each primer (NP7 and NP8), and 1.5 U of *Taq* DNA polymerase (Promega). This reaction mixture was amplified for 35 cycles in a programmable thermal cycler (model 2400; Perkin-Elmer Cetus) by using the following conditions: 94°C for 60 s, 47°C for 60 s, and 72°C for 60 s, plus a final extension step at 72°C for 5 min.

One microliter from the first PCR was further amplified with the inner pair of primers in a 50-μl reaction mixture containing 10 mM deoxynucleoside triphosphate mixture, 20 pmol of each inner primer, 4 μl of 25 mM $MgCl_2$ (Promega), 5 μl of the PCR buffer described above, and 1 U of *Taq* DNA polymerase (Promega). The second round of amplification was performed as follows: 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s, plus an additional final extension step at 72°C for 5 min.

To monitor the PCR, negative controls (CSF from patients with proven non-mumps virus-related CNS infections) and positive controls (Jeryl Lynn vaccine strain diluted with CSF from healthy subjects to obtain a final dilution 0.1 PFU/ml) were included in each run. The controls were extracted at the same time as the test samples.

The 112-bp PCR products were analyzed by electrophoresis on a 2% agarose gel and were visualized by staining the gel in an ethidium bromide (0.5 μg/ml) solution.

The specificity of the mumps virus amplicon was validated by restriction enzyme analysis with the *Hin*fI enzyme (Promega). The *Hin*fI restriction enzyme was predicted to cleave within a GANTC motif present in all published mumps virus nucleoprotein sequences. This would result in two fragments of 70 and 42 bp.

Prevention of contamination. To carry out the complete assay procedure and due to the extreme sensitivity of the n-PCR, four distinct areas were established, including a reagent preparation area (area 1), a specimen preparation area (area 2), a nested area (area 3), and a general work area which includes the area where the amplified product was detected (area 4). In areas 1, 2, and 3, laminar-flow biosecurity cabins were used for the processes performed in those areas. A separate set of micropipets with plugged tips was used in each cabin in the different areas. We included a control sample (sterile water) every two samples to detect possible cross-contamination between the specimens.

RESULTS

Mumps virus RT–n-PCR sensitivity and specificity. The sensitivity of the RT–n-PCR was determined by using serial dilutions of the Jeryl Lynn mumps viral stock diluted with CSF from healthy subjects. The 10-fold dilutions were subjected to the same procedure described above for the extraction of CSF. The RT–n-PCR detected 0.001 PFU/ml and 0.005 50% tissue culture infective doses per ml. The two fragments obtained after enzyme digestion of the nested products were of the expected sizes of 70 and 42 bp (Fig. 1).

The specificity of the RT–n-PCR was determined by analyzing the 30 CSF specimens from patients with meningitis due to bacteria and viral agents other than mumps virus. All these specimens were found to be negative for mumps virus RNA.

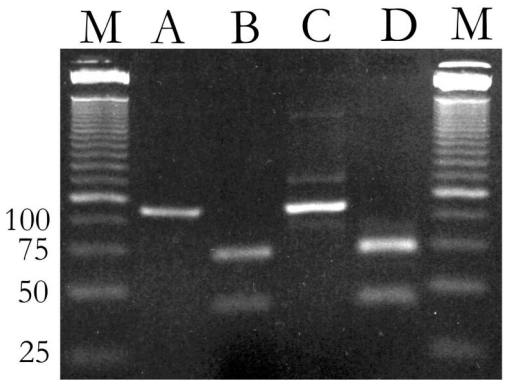

FIG. 1. Electrophoretic analysis of the RT–n-PCR products of the Jeryl Lynn strain and a mumps virus field strain before and after being digested with the restriction enzyme *Hin*fI. DNA fragments from the undigested (lane A) and digested (lane B) Jeryl Lynn strain and the undigested (lane C) and digested (lane D) field strain are shown. The gel shown is a 4% agarose gel. Lanes M, DNA molecular size marker (25-bp ladder). The sizes of the bands are indicated to the left (in base pairs).

Mumps virus RT–n-PCR with CSF from patients with CNS disease and confirmed diagnosis of mumps virus infection. Mumps virus RNA was detected in 18 (100%) of the samples from the 18 patients from whose CSF mumps virus was isolated. A positive mumps virus RT–n-PCR result was detected for 16 of 18 (89%) patients negative for virus isolation from CSF and positive for mumps virus-specific IgM. For the two patients whose CSF was negative by RT–n-PCR, the samples were available 16 and 21 days after the onset of symptoms. Mumps virus RNA was detected in CSF of 10 of 10 (100%) patients with CNS disease and positive for mumps virus isolation from urine or oropharyngeal swab specimens but negative by culture of CSF for mumps virus and mumps virus-specific IgM.

Overall, mumps virus RNA was detected in 44 of 46 (96%) CSF samples from patients with a clinical diagnosis of viral CNS disease and confirmed mumps virus infection, while mumps virus was isolated from only 18 of 46 (39%) CSF samples.

Mumps virus RNA was detected in 97% (35 of 36) and 88% (7 of 8) of the patients with meningitis and encephalitis, respectively. The rates of isolation were 48% (17 of 36) and 13% (1 of 8), respectively.

Mumps virus RT–n-PCR with CSF from patients with suspected viral CNS disease and without laboratory evidences of mumps virus infection. Fifty-five CSF samples from patients with a clinical diagnosis of viral CNS infection were also analyzed. All samples were negative for enterovirus by RT–n-PCR (43), for herpesvirus by n-PCR (2), mumps virus specific IgM, and mumps virus by culture of CSF. Mumps virus RNA was detected in CSF from 25 (46%) of the patients.

Mumps virus RNA was detected in 38% (13 of 34) and 53% (10 of 19) of the patients with meningitis and encephalitis, respectively, while no virus was isolated from CSF from patients with either of these clinical entities.

DISCUSSION

In this paper we report on the development of a sensitive and specific PCR assay for the detection of mumps virus RNA directly in the CSF from patients with mumps virus infections of the CNS.

We have shown that our nested RT–n-PCR is sensitive and is capable of detecting 0.001 PFU/ml. It has been estimated that the particle/infectivity ratio for RNA viruses ranges between 100 and 1,000 (27); therefore, we concluded that RT–n-PCR allowed the detection of 10 to 100 copies of mumps virus RNA per 100 μl of biological sample. This detection limit for mumps virus is 1,000-fold higher than those of the mumps virus PCRs reported by other investigators (5) and is comparable to those of the n-PCRs developed for enteroviruses (9, 41).

Mumps virus RNA was not detected in control samples of patients with meningitis or encephalitis from other causes. Most of these CSF samples were from patients with bacterial, enteroviral, or herpetic CNS infections. Therefore, the lack of detection of mumps virus RNA in these CSF samples indicates that our RT–n-PCR is able to achieve the most common differential diagnoses for most patients with mumps virus CNS disease.

Our method detected virus in all CSF samples with culture-confirmed mumps virus infection as well as nearly all patients whose CSF was negative by culture but with other evidence of mumps virus infection (i.e., positive urine or oropharyngeal swab cultures or positive reactions for anti-mumps virus IgM). These results suggest that the mumps virus PCR is more sensitive than viral culture and may detect mumps virus in CSF when viral culture does not. Different PCRs for the detection of the mumps virus genome have been developed by other investigators; however, there is scarce or no information regarding a comparison between PCR and tissue culture assays for the diagnosis of mumps virus CNS infections (1, 5, 14, 22).

The PCR assay with CSF yielded negative results for two patients with mumps virus infection confirmed by a mumps virus-specific IgM test. Mumps virus-specific IgM antibody reaches a peak titer within 10 to 14 days of the onset of the infection and can be detectable for 2 to 4 months in most patients (25). Remarkably, the CSF from these patients had been sampled 16 and 21 days after the onset of the neurological symptoms, respectively, while CSF from the PCR-positive patients had been obtained after a median of 4 days (range, 1 to 12 days) from the time of the first appearance of symptoms (data not shown). These results indicate that the sensitivity of PCR is influenced by the time of CSF sampling in the course of disease. More longitudinal data are required to establish the sensitivity of our PCR of CSF at different points in the time course of mumps virus CNS infections.

The difference in sensitivity between PCR and cell culture might be explained in different ways. First, demonstration of the presence of the mumps virus genome by PCR, in contrast to virus culture, does not require maintenance of the replication competence of the virus. Second, different factors, such as loss of viability by specimen handling or the presence of a small number of infectious virus particles and/or replication-defective or antibody-complexed virus in CSF, may notably lower the sensitivity of virus isolation. In contrast, the rate of detection of mumps virus by PCR is scarcely or not at all affected by the factors mentioned above.

Mumps virus RNA was also detected in 25 of the 55 CSF samples from patients who had negative laboratory evidence of viral infection including mumps virus infection. These findings confirm the great sensitivity and usefulness of our RT–n-PCR. A high degree of sensitivity is associated with a risk of false-positive results. However, we can exclude the possibility of false-positive PCR results in this study since inclusion of different controls was required to validate each run. Moreover, the PCR results were highly reproducible, showing that amplification was not due to technical error or sporadic contamination.

The 25 patients who had positive mumps virus PCR results represent 12% of the 236 patients with a diagnosis of a viral CNS disease. This finding, together with those from others investigators (10, 49) show the importance of mumps virus as a causative agent of meningitis and encephalitis in countries with low rates of vaccine coverage. It is interesting that in some PCR-positive patients for whom the etiology of their infection had not been established by conventional virological methods, CNS infection occurred without recognized parotitis (data not shown). This observation underlines the importance of performing a mumps virus-specific PCR with CSF from all patients with presumed viral CNS diseases, especially in countries where the mumps virus vaccine is not included in national immunization program.

It has been shown that a specific diagnosis of encephalitis due to any virus is much more problematic than a diagnosis of viral meningitis, because CSF is much less likely to yield cultivable virus from patients with encephalitis (33, 41, 47). Consistent with these observations is the fact that, in our study, the rates of isolation of mumps virus from CSF from patients with meningitis were significantly higher than those from patients with encephalitis (48 and 13%, respectively). In contrast, the sensitivities of RT–n-PCRs for the diagnosis of mumps virus-confirmed meningitis and encephalitis were similar (97 and 88%, respectively). Our results emphasize the diagnostic power of analyzing CSF by RT–n-PCR, which is more evident in the diagnosis of mumps virus encephalitis.

We conclude that our RT–n-PCR assay is a reliable, specific, and sensitive tool for the diagnosis of mumps virus CNS infections. However, more clinical samples will need to be tested before the sensitivity and specificity can be firmly established. Also, the validity of this assay for detection of the mumps virus genome in serum, urine, and oropharyngeal samples should be determined.

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