Three cases of mumps virus and enterovirus coinfection in children with enteroviral meningitis

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Abstract
Several viruses are responsible for aseptic meningitis; however, in the region of Southwest Iran, the role played by each virus is still not very well known. The aim of this study is to determine the relative frequencies of mumps virus, herpes viruses, and enteroviruses, as well as coinfections among them, in patients with aseptic meningitis.

In this cross-sectional study, samples of cerebrospinal fluid were collected between December 2012 and December 2013 from patients under 14 years, who were hospitalized in Abuzar Children’s Hospital in Ahvaz, Southwest Iran (the only children’s hospital in Khuzestan province and Southwest Iran).

All 66 cerebrospinal fluid samples and corresponding clinical data were collected from patients with aseptic meningitis by specialists, and with the patients’ consent. The DNA and RNA were extracted from these samples and subjected to polymerase chain reaction as well as reverse transcription polymerase chain reaction (RT-PCR) for detection of mumps virus, herpes viruses, and enteroviruses. Nine of the samples (3 mumps-positive and 6 enterovirus-positive) were sequenced. The mumps virus sequences were investigated for possible mutations in the SH and partial HN regions.

Up to 39 patients (59.09%) were found to be positive for enteroviruses, 3 (4.5%) for mumps virus, and 1 (1.5%) for herpes viruses (specifically, the varicella-zoster virus). Two patients (3.03%) had a mumps virus and enterovirus coinfection. Among the 3 detected mumps virus samples, 1 belonged to genotype B, while the others belonged to genotype N. Six sequenced enteroviruses indicated the highest similarity with Echovirus 30. An amino acid substitution at position 51 (N → T) was detected in the HN region of genotype N mumps virus samples, in comparison to the reference strain.

Abbreviations: cDNA = complementary DNA, CMV = cytomegalovirus, CSF = cerebrospinal fluid, EBV = Epstein–Barr virus, HN = hemagglutinin neuraminidase, MMR = measles, mumps, rubella, PCR = polymerase chain reaction, RT-PCR = reverse transcription polymerase chain reaction, SH = small hydrophobic, UTR = untranslated region, VZV = varicella zoster, WBC = white blood cell.

Keywords: aseptic meningitis, enterovirus, mumps virus, polymerase chain reaction, viral infection

1. Introduction
Aseptic meningitis is an acute but generally self-limited infection caused by a variety of microorganisms, particularly viruses. This disease involves the inflammation of the meninges of the brain and is characterized by a variety of clinical manifestations, such as fever, headache, neck stiffness, vomiting, loss of consciousness, and elevated white blood cell (WBC) count with the predominant lymphocytes.\textsuperscript{[1–3]} There are several viruses that might cause aseptic meningitis, including enteroviruses, mumps virus, herpes viruses, influenza viruses, cytomegalovirus (CMV), and Epstein–Barr virus (EBV). Enteroviruses (HEV) are a large group of viruses responsible for about 90% of aseptic meningitis worldwide, especially in underdeveloped countries with poor sanitation and low standards of hygiene.\textsuperscript{[4–14]} Enteroviruses belong to the picornaviridae family, characterized by a single-
stranded positive-sense RNA and a length of 7.5 kbp. The genome of enteroviruses has 3 different regions, including P1, P2, and P3, which are respectively coded for structural, regulatory, and enzymatic proteins. They have been classified into 4 groups: HEV-A, HEV-B, HEV-C, and HEV-D.[16] The mumps virus is a member of the paramyxoviridae family and genus Rubulavirus, with a single-stranded negative-sense RNA and an approximate length of 15.6 kbp. The mumps virus genome encodes 7 genes, which produce N, P, M, F, SH, HN, and L proteins. It has 1 serotype and 12 genotypes (A to L), which makes it a good target for vaccine production.[19] Mumps virus causes mumps disease, which is characterized by symptoms such as fever, parotitis, pancreatitis, orchitis, deafness, aseptic meningitis, and encephalitis.[10] Mumps vaccine is used as an attenuated vaccine in a triple MMR vaccine, accompanied by measles and rubella viruses. Owing to widespread vaccination, especially in developed countries, a new problem, known as “postvaccination” meningitis, has arisen.[11–17] Urabe Am9, Leningrad, L-Zagreb, Jeryl Lynn, and a variety of mumps strains have been used in vaccine production.[13] However, Urabe Am9 was later rejected because it was seen to cause postvaccination meningitis.[16–19] Several studies revealed that the occurrence of these symptoms, specifically in the HN and SH regions (including in antigenic and immunogenic epitopes), transformed the vaccine strain into its wild type, and may have led to the development of postvaccination meningitis.[20] The other large family of viruses that causes aseptic meningitis is the herpes virus. Herpes viruses are enveloped viruses with large double-stranded DNA with a length of 180 to 220 kbp, which encodes different genes divided into α, β, and γ groups. All members of the herpes virus family, including herpes simplex, varicella-zoster, cytomegalovirus, Epstein–Barr virus, and human herpes virus 6 and 7, are implicated in the cause of aseptic meningitis. However, the roles of herpes simplex 1 and 2 are dominant.[21–23] The aim of this study is to detect enteroviruses, mumps viruses, and herpes viruses in patients with aseptic meningitis, and their molecular characterization in Ahvaz, a city in southwest Iran. Thus, CSF samples of patients with aseptic meningitis were investigated for enteroviruses, mumps viruses, herpes simplex 1 and 2 viruses, and varicella-zoster virus.

2. Materials and methods

This cross-sectional study was performed on children with aseptic meningitis who were hospitalized in Ahvaz Children’s Hospital (the only children’s hospital in the Khuzestan province and Southwest Iran) in Ahvaz, the capital city of Khuzestan, between December 2012 and December 2013. This study was registered with the ethics committee, under registration number 122, and patient information was collected with permission from the hospital’s questionnaires. Among the 66 CSF samples collected from patients with aseptic meningitis, 48 cases (72.73%) were male and 18 cases (27.27%) were female, with an average age of 2.5 ± 3.1 years. All patients had received the polio vaccine and 37 cases (56%) had received the measles, mumps, rubella (MMR) vaccine. Most patients were observed during summer and fall (Fig. 1A). Inclusion criteria for this study consisted of clinical symptoms such as fever, headache, seizure, neck stiffness, vomiting, diarrhea, or nausea at the time of admission (Fig. 1B), and the presence of pleocytosis in the CSF (WBC count above 5 per mm³) with lymphocyte predominance. Exclusion criteria included positive bacterial cultures, samples with very high neutrophil counts, and turbid or bloody appearance. All clinical data were collected from questionnaires completed by a specialist physician and with the consent of the patients and/or their parents. Laboratory data of each patient included measurements of glucose concentration, protein, and WBC count (lymphocyte and neutrophil) from the patient’s history, which were documented in the hospital’s laboratory, as illustrated in Table 1. All tests were conducted in the hospital by an expert technician.

3. Statistical analysis

Statistical analysis was conducted with the χ² and Fisher exact tests for qualitative variables, and Student t tests and analysis of variance (ANOVA) for quantitative analysis of 2 or more groups (SPSS, version 20, IBM) with CI = 95%.[24,25] P < 0.05 was considered a significant difference. Missing data were considered in every analysis by the SPSS.

4. Genome extraction and cDNA synthesis

The viral genome was extracted from each CSF sample using the High Pure Viral Nucleic Acid Kit (Roche, Indianapolis, IN) as per manufacturer’s instructions, and stored at −70°C for further processing. cDNA was synthesized for the viral RNA using a commercial kit (Thermo Scientific, Vilnius, Lithuania) as follows: 10 μL RNA was used as a template, 1 μL random primer, and 1 μL water was used to prepare the first master mix. After incubating the master mix at 65°C for 5 minutes and chilling it on ice, the second master mix was prepared in accordance with the manufacturer’s instructions. The master mixes were combined and incubated at 42°C for an hour. The reaction was terminated at 70°C for 5 minutes.

5. Seminested and nested polymerase chain reaction for virus detection

The nested polymerase chain reaction was used for the detection of enteroviruses, herpes viruses, mumps virus, and varicella-zoster virus in the CSF samples. For each PCR reaction, specific primers were used, including primers for the 5’ untranslated and viral protein 1 regions of enteroviruses, the HN region of the mumps virus, Glycoprotein D of the herpes simplex virus, and Glycoprotein A of the varicella-zoster virus (Table 1).[26–29] A master mix of the PCR reactions, containing 2.5 μL 10× PCR buffer mixed with 0.5 μL dNTP mix (0.2 mM), 0.75 μL (1.5 mM) MgCl₂, 10 pM of each primer, 0.2 μL (1 unit) Taq polymerase, and double distilled water up to 25 μL was prepared for the first round of 35 cycles. About 2 μL of the first round PCR product was used as a template for the second round. The reaction mixture in the second round of PCR was the same as the mixture used in the first round; however, PCR was done for 30 cycles in the second round. The PCR products of each sample were run by electrophoresis on 2% agarose gel, supplemented with safe stain. The predicted PCR products of 155, 640, 282, and 200 bp length bands on the agarose gel were analyzed and recorded under UV light for enteroviruses, mumps virus, herpes simplex 1 and 2 virus, and varicella-zoster virus respectively. The PCR products for Enterovirus VP1 were 640 and 440 bp. Some of the positive samples were sequenced by an ABI 3500 sequencer (Applied Biosystems, Foster City, California, USA). Negative control reaction consisting of all PCR components, except the template, was conducted at each stage of testing, including extraction,
cDNA synthesis, and PCR. Every stage was done in a separate room and laminar hood.

6. Results

In this study, 72.73% of the subjects were male and 27.27% were female. The average ages of patients were respectively 2.5±3.2 and 2.6±3 years for positive and negative cases. There was no significant relationship between the distribution of viral infections across different age groups and gender (P=0.65 and P=0.29, CI: 95%, Table 3). Among the 66 CSF samples, 43 patients (65.1%) were positive and 23 patients (34.8%) were negative for viral infection, 39 patients (59.1%) were positive for enteroviruses, and 3 patients (4.5%) were positive for mumps virus. The ages of the mumps-positive cases were 12 months (2 patients), and 13.5 months (1 patient). There was a 14-day-old patient (1.5%) who was positive for the varicella-zoster virus. All patients had been vaccinated for polio virus as well as given the oral polio vaccine, and 37.65 patients (57%) (the data of 1 patient was missing) had been vaccinated against mumps virus. The 3 children with mumps infection in this study had been vaccinated against mumps virus. No herpes simplex virus infections were detected in the patient samples. Table 3 illustrates the frequency of viral infections in different age groups. Table 1 shows laboratory data such as leukocyte (lymphocyte and neutrophil) count, as well as protein and glucose concentrations among the patients. While no significant relationship was found between glucose concentration and viral infection (P=0.27), a significant relationship was observed between protein concentration and viral infection (P=0.03), as well as between 3 groups of patients with different viral infections (P=0.03). The most common symptoms observed among patients were fever, vomiting, lethargy, seizure, and headache (Fig. 1B). Among the 3 patients with mumps infection, 2 of them (3%) exhibited simultaneous infection with enteroviruses. Both were males, respectively aged 13 months and 12 months. One of these patients displayed symptoms of fever, TCG seizure with a duration of several minutes, lethargy, malaise, and developmental delay, while his brain CT scan indicated hydrocephalus. VZV infection was detected in a 14-day-old female, with clinical
manifestations of fever, generalized maculopapular rash 3 days after birth, and normal brain sonography. Her mother also had had chickenpox at the time of childbirth.

7. Sequencing, phylogenetic, and mutation analysis

There were 3 mumps-positive samples and 6 enterovirus-positive samples, which had sufficient concentrations of PCR product to be sequenced. These samples were identified and sequenced using an ABI 3500 sequencer. All 6 of the enterovirus sequences showed high similarity with human Echovirus 30, strains 06.065.4448 and 1-MRS2013, from Australia and France respectively. Among the 3 mumps-positive samples, 2 (3%) belonged to the L-Zagreb vaccine strain (genotype N) and 1 showed the highest similarity to the Hoshino vaccine strain (genotype B). These results were demonstrated by drawing a phylogenetic tree based on the SH region using the neighbour-joining method (MEGA6), as shown in Fig. 2. The entire SH region and the first 53 amino acids of the HN region of the mumps-virus genome were investigated for the analysis of possible mutations (MEGA6). There were no mutations in the SH region of the 3 mumps-positive samples, as compared with reference strains of genotype N and B (Fig. 3). An amino acid substitution was identified in position 51 (N→T) in the HN region of genotype N mumps virus (KT346350 and KT346351), as compared with the reference strains (L-Zagreb and L3). No amino acid substitutions were found in the SH and partial HN regions of genotype B, mumps virus (KT346332) as compared with the Hoshino vaccine strain (Fig. 3).

8. Discussion

Meningitis is a disease caused by inflammation of the protective layers covering the brain and spinal cord, called the meninges, which consist of the dura mater, arachnoid mater, and pia mater. The condition can result in encephalitis or meningoencephalitis.[30–32] Various agents, including bacteria, viruses, fungi, protozoa, chemical agents, and drugs, can cause meningitis and encephalitis.[32,33] Several viruses have been reported among children with aseptic meningitis or encephalitis, and postvaccination meningitis in children who have received live attenuated vaccines is also a great global concern.[17,18,32,33] Thus, this study was conducted to evaluate molecular detection of certain viral infections, including enteroviruses, herpes simplex viruses 1 and 2.

### Table 1

| Virus detected | Protein (mg/dL) | Glucose (mg/dL) | Total cell count (cell/mm³) | WBC (cell/mm³) | Lymphocyte (cell/mm³) | Neutrophil (cell/mm³) |
|---------------|----------------|----------------|-----------------------------|----------------|----------------------|----------------------|
| Yes Mean      | 37.13          | 69.21          | 393.46                      | 290.31         | 156.90               | 71.64                |
| N Std. Deviation | 22.508        | 26.625         | 533.056                     | 485.243        | 355.087              | 127.956              |
| No Mean       | 25.75          | 62.54          | 443.54                      | 265.25         | 73.42                | 40.79                |
| N Std. Deviation | 14.022        | 20.619         | 635.518                     | 504.468        | 99.661               | 115.433              |
| Total Mean    | 32.79          | 66.67          | 413.83                      | 280.28         | 125.10 (0–1947)      | 59.89 (0–572)        |
| N Std. Deviation | 20.358        | 25.533         | 572.142                     | 526.874        | 257.461              | 123.314              |

WBC = white blood cell.

### Table 2

| Genome region | Sequence Product size |
|---------------|----------------------|
| Enterovirus   | Outer Antisense: | 440 and 155 bp |
| 5′ UTR        | Outer Antisense: | |
|               | Inner Sense:      | |
|               | Inner Antisense:  | |
| Enterovirus   | (008) Outer Antisense: | 640 bp for 008 and 013 set |
| VP1           | (011) Outer Antisense: | 440 bp for 011 and 012 set |
|               | (012) Inner Antisense: | |
| Mumps virus   | Outer Antisense: | 676 and 639 bp |
| SH and partial HN | Outer Antisense: | |
|               | Inner Antisense:  | |
| HSV 1 and 2   | Outer Antisense: | 383 and 282 bp |
| Glycoprotein D| Outer Antisense: | |
|               | Inner Antisense:  | |
| VZV           | Outer Antisense: | 272 and 200 bp |
| Gene 29       | Outer Antisense: | |
|               | Inner Antisense:  | |

VZV = varicella zoster.
2, mumps virus, and varicella-zoster virus in children with aseptic meningitis. In this study, 39 patients (59.1%) with aseptic meningitis were detected to have enterovirus infection. This result is similar to previous reports (52% and 57%) in Ahvaz, which indicates the predominance of enterovirus infections in patients with aseptic meningitis. Hosseininasab et al. reported a 43% prevalence of enterovirus infections in patients with aseptic meningitis in Shiraz, Iran. Another study, conducted by Mamishi et al. in Tehran, Iran, found that 10.16% of patients with aseptic meningitis have enterovirus infections. Six sequenced samples among the 39 enterovirus-positive samples were recognized as Echovirus 30. Mamishi et al. reported 1 case of Echovirus 30 in 12 children with enteroviral aseptic meningitis in Tehran, Iran. Several factors may be involved in the spread of enterovirus infection across the city, including climate, environmental pollution, low levels of hygiene, and inadequate sanitation. Furthermore, among the 10 patients who were surveyed about their environment in a questionnaire, 50% lived in poor regions of the city with inadequate sanitation and poor levels of hygiene. Different studies reported the presence of echovirus 30 and other HEV-B in patients with aseptic meningitis. No herpes simplex viruses were found in this study. Only one 14-day-old boy, who was born of a mother infected with the varicella-zoster virus at the time of labor, tested positive for VZV infection. Kim et al. reported a 14-year-old boy with VZV aseptic meningitis, and Hosseininasab et al. reported 2 cases (ages 1–5) of aseptic meningitis with VZV infection. Three cases of mumps virus infection were detected despite the vaccination programme against the mumps virus in Iran. Currently, the Hoshino strain is used as mumps vaccine in Iran. Based on this vaccination programme started in March 2004, every child should receive the first dose of the vaccine (measles, mumps, rubella vaccine, otherwise known as the MMR vaccine) at 12 months, followed by the second dose between the ages of 4 and 6. Noorbakhsh et al. reported 62.7% IgM positivity against the mumps virus among children between 9 months and 14 years of age. This is similar to the report of Modares et al., prior to the installation of the vaccination programme in Iran, which introduced the country as an endemic region for mumps virus infection. According to the age of the patients as well as their vaccination status, any of the following reasons can be considered to explain the occurrence of infection:

1. Vaccination failure, that is, some scientists report low antibody protection after administration of the first dose of vaccine;

Avijgan et al have reported low seroconversion (86.1%, 77.7%, and 75%) respectively at 3, 12, and 24 months after the first dose of vaccine;

2. The presence of any amino acid substitutions resulting from mutation, which can lead to changes in virus neuro-virulence.

As mentioned previously, the only strain of mumps virus used in vaccines in Iran is the Hoshino strain, which was detected in 1 patient in our study. Certain strains of mumps virus, such as Urabe AM9 (genotype B) and Odate-1 (genotype I), showed high neuro-virulence and were responsible for the high incidence of meningitis in Japan. Accordingly, the Urabe AM9's use as a

| Age group | Viral infection |
|-----------|----------------|
| Less than 6 mo | None | Mumps | VZV | Enterovirus |
| 6–24 mo | 8 | 0 | 1 | 14 | 23 (34.8%) |
| 24 mo to 6 y | 7 | 3 | 0 | 10 | 20 (30.3%) |
| More than 6 y | 6 | 0 | 0 | 5 | 8 (12.1%) |
| Total | 24 | 3 | 1 | 37 | 65 |

**Table 3**

Frequencies of different viruses in different age groups (1 sample was unknown).

VZV = varicella-zoster. 

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Figure 2. Phylogenetic tree based on the entire SH region (316 nucleotides) of mumps virus using the neighbor-joining method with a 1000 bootstrap test. Three accession numbers, marked by black squares, represent the patient samples. An accession number marked with a black circle is the first prevalent mumps virus strain reported from Iran. Other strains included are reference strains from different genotypes, derived from the NCBI.
vaccine strain was discontinued in Japan, Canada, and the United Kingdom.\(^{[19]}\) Based on these facts, it seems that the mumps vaccine strain may need an alteration in Iran to achieve better immunization after vaccination. Previous studies have indicated that amino acid substitutions at positions 335, 354, 356, 360, 464, and 466 in the HN region, and in positions 29 and 48 in the SH region, resulted in neuro-virulence of the virus.\(^{[19,42,43]}\) The HN region is the main immunogenic part of the mumps genome and contains glycosylation sites in 3 epitope domains (aa265-288, aa329-340, aa352-360)\. The SH region is the most variable part of the genome and is used for genotyping as well as for phylogenetic analysis of the virus.\(^{[44,45]}\) In this study, about 316 nucleotides of the SH and the first 162 nucleotides of the intravirion and transmembrane domains of the HN gene were sequenced. Their amino acid sequences were aligned and compared with wild type and vaccine strains in the gene bank. The mumps virus strains detected in this study were identified as genotypes N (KT346350, KT346351) and B (KT346352) (Fig. 1). This is the first report of a mumps genotype in Southwest Iran and is different from the RS 12 strain (genotype H) which was reported in 1986, in Tehran.\(^{[45]}\) In this study, the SH region of genotype N (KT346350 and KT346351) showed amino acid substitutions at positions 9 (Y→H), 29 (I→T), 42 (A→T), 44 (H→Y), and 56 (S→P) compared with the RS 12 (genotype H) strain. The amino acids at positions 29 and 48 of the SH region are important due to their effect on virus attenuation, especially when substitutions occur simultaneously.\(^{[19]}\) Amino acid substitutions at positions 10 (L→P), 19 (Y→S), 30 (L→S), and 42 (A→T) were seen in the KT346352 (genotype B) strain in the SH region, when they were compared with the RS 12 strain. No amino acid substitutions were found in the SH region of our strains (KT346350, KT346351, and KT346352) compared with the L-Zagreb, L3, and Hoshino reference strains. Amino acid substitutions were seen at position 23 (V→A) in the KT346350 and KT346351 strains, when compared with the HN region of the RS 12 strain. The KT346352 strain showed amino acid substitutions at positions 9 (I→M) and 21 (I→V) in the HN region as compared with strain RS 12. Additionally, there was a substitution in position 51 (N→T) in the HN region of the KT346350 and KT346351 strains when compared with reference strains from genotype N (L-Zagreb and L3), but there were no amino acid substitutions in genotype B (KT346352) compared with the HN region of the Hoshino reference strain (Figs. 2 and 3). Several reports have suggested the presence of different substitutions that result in virus attenuation. Based on the report of Cui et al, it seems that multiple mutations in a region or a combination of several mutations in various regions of the genome are responsible for virus attenuation or virus reversion from vaccine strain to wild type.\(^{[19]}\) The presence of mumps virus infection among children who received an MMR vaccine shows the probability of the vaccine reversion or vaccine failure as cause of mutation. It is logical to evaluate the potency of the mumps vaccine strain used in Iran in comparison to other strains, such as Jeryl Lynn strain that is used in the United States and has 95% effectiveness,\(^{[18]}\) or other strains that decrease any chances of vaccination failure and virus reversion. Finally, the sampling period (sampling through an extended period of time) could indicate better results. Missing data about the patient’s economic situation, locations they have lived in, etc. (some patients do not fill in the questionnaires completely) could be considered limitations of the study. We also could not do whole genome sequencing for better analysis of mutations in the mumps virus genome, because of low sample concentration and budget limitations. It seems that this study cannot be applied to other populations in the Middle East because their mumps vaccination programme and coverage as well as the mumps virus strains that they use in their vaccines are unknown to us. The low number of mumps meningitis cases is due to the rarity of mumps virus infection in vaccinated patients, given that there is a vaccination programme against the mumps virus in Iran for several years, which has decreased mumps virus infection significantly. Therefore, further studies are required to sequence other regions of the 3 mumps genome, which were detected in this study.

9. Conclusion
This study shows the high rate (59.09%) of enterovirus infection among children with aseptic meningitis observed during spring and fall. Several factors could be responsible for the spread of enteroviruses: poor quality of drinking water, low levels of
hygiene, and inadequate sanitation. Therefore, improvement of sanitation is of highest priority. The presence of mumps virus infections among children who receive vaccines indicates the importance of evaluating the mumps vaccine potency and safety in Iran, as well as making necessary alterations in the mumps vaccine strain to achieve higher immunization levels.

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