Original Article

Molecular Epidemiology Study of Suspected Meningitis Cases in Tehran and Alborz Provinces

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Abstract

Background and Objective: Meningitis is a medical emergency requiring immediate diagnosis and treatment. Bacterial and viral causative agents play a role in meningitis appearance to various degrees. Thus, effective vaccines and antimicrobial and supportive treatments need to be developed and monitoring should be performed in different regions for controlling this disease. Most previous studies have focused on a small number of bacterial agents, and the viral profile of this disease is not precisely monitored in Iran, especially in overpopulated regions. Moreover, limited new applied methods with high precision and sensitivity for the detection of meningitis agents indicate the necessity of determining meningitis agents by rapid molecular methods, which was the aim of the current study.

Materials and Methods: Overall, 148 samples obtained from suspected meningitis patients from different age groups admitted to Tehran and Karaj hospitals were evaluated by new methods involving specific primers for 16s rRNA, PCR, and Real-time PCR tests.

Results: It was found that viral infection, especially infection with human enterovirus, remains the main cause of meningitis in Iran, and Neisseria meningitides is the most common bacterial isolate detected in meningitis cases.

Conclusion: Despite the decreasing trend in meningitis incidence, according to World Health Organization recommendation, implementing an enhanced surveillance system to provide high-quality data on the epidemiological profile of meningitis per each region is necessary.

Keywords: Epidemiology, Meningitis Agents, Molecular Method, Surveillance System

Introduction

Meningitis can occur by infectious and non-infectious causes. Infectious agents include bacteria, viruses, fungus, parasites, and Amoebidae (1-3). The major bacterial causes of meningitis are *Streptococcus pneumoniae, Group B Streptococcus, Neisseria meningitides* (4-6), *Haemophilus influenzae, and Listeria monocytogenes* (7-9). Other bacterial agents like group B *streptococci, Escherichia coli, and Staphylococcus spp. are also among the common bacterial ag*ents of meningitis (10-13). The most common causes of viral meningitis are *Enterovirus* (14- 16), herpesviruses, and Varicella-zoster virus (VZV) (17, 18). Fungi,

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parasites, and Amoebidae are not as prevalent as bacterial and viral causes, except in particular clinical cases or locations (19, 20).

In order to find out the main cause of meningitis, laboratory diagnosis using cerebrospinal fluid (CSF) plays a crucial role. Routine laboratory tests like CSF cell count, glucose and protein concentration, Gram staining, and bacterial culture are commonly used for laboratory meningitis surveillance (21, 22).

By improvement in molecular diagnosis and nucleic acid amplification tests (NAT) like polymerase chain reaction (PCR), a new era in the field of laboratory diagnosis of infectious diseases was opened. HCV infection, enteroviral meningitis, pertussis, HSV encephalitis, and genital infections due to C. *trachomatis* and N. *gonorrhoeae* are some examples of infectious diseases, in which nucleic acid-based tests have become the new golden standard for diagnosis. They are also becoming the method of choice for rapid, sensitive diagnosis of meningitis. these may have some advantages in contrast to microbial culture techniques, as they are not affected by inappropriate transport conditions, improper collection media, or delays in transport. These kinds of tests are highly sensitive and specific (23, 24). The diagnosis of herpes simplex virus, cytomegalovirus, enterovirus, and Varicella-zoster virus infection by NAT has been reported by different studies, which indicated NAT specificity and sensitivity for common meningeal infectious agents (25, 26).

Numerous studies have been conducted worldwide for determining meningitis pathogen profiles, but these studies have largely focused on limited bacterial profiles, especially by applying traditional methods such as cell culture (with lower specificity and sensitivity). However, viral pathogen profile can exceed bacterial agents in meningitis incidence, which should be considered in developing new vaccines and supportive treatments. Thus, the current study was performed to evaluate new molecular methods' efficacy per each pathogen and to identify the proportion of viral and bacterial agents in meningitis incidence.

Materials & Methods

In this study, presence of 12 common bacterial and viral causes of meningitis were investigated by using molecular methods during two years in Tehran and Karaj provinces. The pathogens included six main viruses, including the human enterovirus, HSV 1, 2, Epstein-Barr virus (EBV), Cytomegalovirus (CMV), and the main bacterial agents were comprised of *Neisseria meningitidis, Mycobacterium tuberculosis, Hemophilus influenzae, Streptococcus pneumonia, and Listeria monocytogenes.* The lab scenario was to identify the causative infectious agents primarily by DNA purification from CSF followed by examining the presence of 16s rRNA by universal primers (27). Positive results indicated the presence of bacterial agents, which were investigated for each individual by specific bacterial species primers, and the bacterial load was assessed by Real-time PCR. Those samples which were negative for 16s rRNA were checked for the six mentioned viral causes by specific primers, followed by Real-time PCR for specific virus quantification (28, 29).

Samples: Overall, 148 clinically suspected meningitis cases (80 men and 68 women) with an extensive age range participated during two years. Patients were admitted to hospitals of Tehran and Karaj, a city about 30 kilometers away from Tehran. Samples were quickly and aseptically transferred to

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the molecular department of six different private laboratories in Tehran (mainly 12th Farvardin Lab and Dr. Izad Dust Lab) and Karaj (mainly Amin Pathobiology Lab and Ghaem Hospital Lab).

Bacterial Genomic DNA and viral nucleic acid purification: Bacterial genomic DNA was purified by 502 MagCore® Bacterial DNA kit, and viral nucleic acid was purified by 202 MagCore® Viral Nucleic Acid Extraction Kit (Low PCR Inhibition).

Agarose gel electrophoresis: All the samples underwent DNA extraction, and the quality of extracted DNA was assessed by 1% agarose gel electrophoresis based on the method proposed by Meyers et al. (30).

16s rRNA PCR setting: The extracted DNA underwent 16s rRNA study by U3 and U8 probes (31). Primers were designed by Oligo6, as shown in Table 1.

Table 1. Primer sequences used for 16s rRNA for studying extracted DNA which was purified from suspected samples

No.	Primer name	Primer sequence	Primer length	Product size
1	U3	5-AACTCCGTGCCAGCAGCCGCGGTAA-3	25 bp	1000 bp
2	U8	5-AAGGAGGTGATCCAGCCGCAGGTTC-3	25 bp	1000 bp

Real-time PCR

Then positive and negative samples were tested for each bacterial and viral agent of meningitis by Real-time PCR and then scrutinized by specific primers for each bacterial and viral agent (the five common bacterial agents of meningitis include: 1- *Mycobacterium tuberculosis* [TB], 2-*Neisseria meningitides* [Nm], 3-*Sterptococcus pneumonia* [Sp], 4-*Haemophilus influenza* [Hi], and 5-*Listeria monocytogenes* [Lm]) and the six common viral agents including HSV1 and 2, EBV, VZV, CMV, HHV-6, and human enterovirus were tested by Real-time PCR (32, 33). Bacterial and viral agent species specific primers are presented in tables 2 and 3, respectively.

Table 2. Bacterial agents' specific primer name, sequence, length and recognized target sequence size. In primer name s column, recorded abbreviation s are described in footnote at first page

No.	Primer name	Primer sequence	Primer length	Target sequence size
1	Lm_f	5′-GGGAAATCTGTCTCAGGTGATGT-3′	24	106
2	$L.m_r$	5′-CGATGATTTGAACTTCATCTTTTGC-3′	25	106
3	$N.m_f$	5′-CCTTATTAGCACTAGCGGTTAG-3′	22	537
4	$N.m_r$	5′-CCGGTCATCTTTTATGCTCCAA-3′	22	537
5	$H.i_f$	5′-CAAGATACCTTTGGTCGTCTGCTA-3′	24	129
6	$H.i_r$	5′-TAGGCTCGAAGAATGAGAAGTTTTG-3′	24	129
7	$S.p_f$	5′-AGCGATAGCTTTCTCCAAGTGG-3′	22	126
8	$S.p_r$	5′-CTTAGCCAACAAATCGTTTACCG-3′	22	126
9	$T.B_f$	5′-TGAGAAGGCAGTAGAAAGCTTAG-3′	22	211
10	$T.B_r$	5′-TGCATGTATGGGTTATCTTCC-3′	18	211

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Table 3. Viral agent species specific primer name, sequence, length and recognized target sequence size

No.	Primer name	Primer sequence	Primer length	Target sequence size
1	CMVf	5′-CCAGTGCCCGCAGTTTTTATT-3′	21	86
2	CMVr	5′-ACCGGAGAAGAGCCCATGTC-3′	20	86
3	HSV1,2f	5′-CGGAATTCCGTCATCTCACGGGGACAC-3′	27	323
4	HSV1,2r	5′-CGGGATCCCGACGGTATCGTCGTAAA-3′	26	323
5	EBVf	5′-GCAGCCGCCCAGTCTCT-3′	17	83
6	EBVr	5′-ACAGACAGTGCACAGGAGCCT-3′	21	83
7	VZVf	5′-CCCGCGGTGGAGACGACTT-3′	19	257
8	VZVr	5′-GCGGTGGAGACGACTTCAATAGCA-3′	24	257
9	HEVf	5′-CPXGCCZGCGTGGC-3′	14	206
10	HEVr	5′-GAAACACGGACACCCAAAGTA-3′	21	206
11	HHV6f	5′-CAAAGCCAAATTATCCAGAGCG-3	22	133
12	HHV6r	5′-CGCTAGGTTGAGGATGATCGA-3′	21	133

DNA extraction from bacterial and viral agents

Ultimately, the extracted DNA was run on 1% agarose gel electrophoresis, and 1000-bp bands were apparent in the samples.

Results

The results showed that 39 (26%) samples were without infection, and among the 109 remaining infected samples, 38 (35%) samples were positive for 1000-bp band by specific primer for 16s rRNA (bacterial infection) and 71 (65%) samples were found positive for viral agents. Among the 71 viral positive samples, human enterovirus was isolated from 29 (41%) samples, Herpes simplex virus1, 2 (HSV 1,2) from 21 (30%) samples, EBV from 8 (11%) samples, CMV from 7 (10%) samples, and Varicella zoster virus (VZV) from 6 (8%) samples.

Among the 38 positives bacterial samples, the main bacterial isolates were *Neisseria meningitidis* isolated from 10 (26%) samples, *Mycobacterium tuberculosis* from 8 (21%) samples, *Hemophilus influenzae* from 7 (18%) samples, *Streptococcus pneumonia* from 5 (13%) samples, and *Listeria monocytogenes* from 4 (11%) samples (Table 4, Figure1). There were 4 (11%) cases in the bacterial group that reacted with 16s rRNA, but were negative for the above bacteria using specific primers. It seems that viral infection remains the leading cause of meningitis infection in Iran, and human enterovirus is the main causative agent of viral infection and *Neisseria meningitides* is the most common bacterial agent isolated from meningitis cases. Fungi, parasites, amoebidae, and bacteria play a smaller role as meningitidis causes in Iran.

Table.4. Based on current findings, 26% of samples (38 samples) were non- infected and 74% (109 samples) were infected samples. At below table , bacterial and viral agent proportions in infected samples are recorded as a clarified path for detecting predominant cases

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Sample Result	Frequency	Percentage	Causative Agent	Frequency	Percentage	Causative Agent	Frequency	Percentage
Not infected	39	26%	NA	NA	NA	NA	NA	NA
Infected	109	74%	Bacterial	38	35%	Neisseria meningitides	10	26%
						Mycobacterium tuberculosis	8	21%
						Hemophilus influenzae	7	18%
						Streptococcus pneumoniae	5	13%
						Listeria monocytogenes	4	11%
						Others*	4	11%
			Viral	71	65%	Human enterovirus	29	41%
						HSV 1,2	21	30%
						EBV	8	11%
						CMV	7	10%
						VZV	6	8%
						HHV	0	0%

- NA not applicable.
- *Not reacted by species specific primers used in this study but still has positive 16s rRNA result.

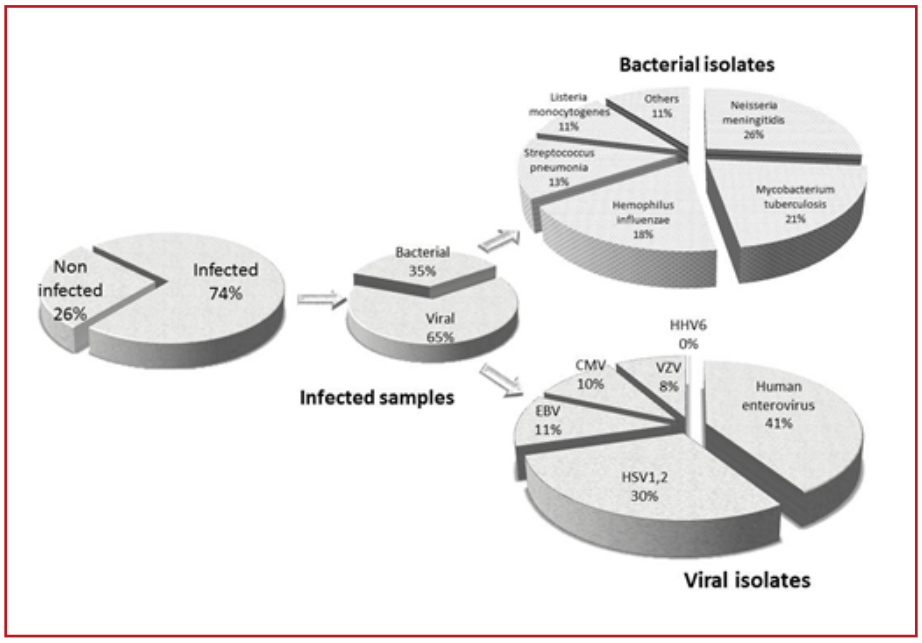


Figure1. Representing bacterial and viral agents' proportion in meningitis incidence. Viral causes have allocated highest rank to themselves. Between bacterial causes, Neisseria meningitides has gained first place

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Discussion

Acute purulent meningitis is the most common infection of the central nervous system, with a yearly incidence of about 3 cases in 100,000 inhabitants in industrialized countries. This disease is caused by different viral and bacterial pathogens, the epidemiology of which has changed as a result of the widespread use of conjugate vaccines and preventive antimicrobial treatments. Thus, accurate information regarding the critical etiological agents is necessary to ascertain public health measures and ensure appropriate management, which need continuous monitoring in different regions. Accordingly, by describing the shifting epidemiology of viral and bacterial meningitis throughout Tehran and Alborz provinces (with overpopulation), we exhibit a clear path for detecting empirical antimicrobial and adjunctive treatments for clinical subgroups, which would help centers for their preventive schedules.

Due to the significance of the age factor in determining meningitis agents' profile, designing studies based on age factor can provide more accurate and valuable results. Thus, this factor should be addressed in future studies.

Rapid and precise detection of bacterial and viral meningitis agents by appropriate methods could help develop more effective treatments (34). The identification of pathogens from CSF through culture usually takes 1-2 days. Moreover, culture frequently remains negative, especially if the CSF is taken after the initiation of antimicrobial therapy. Since the outcome of infection highly depends on the early initiation of adequate therapy, new rapid diagnostic methods are urgently needed. The disease diagnosis by molecular methods like PCR and Real-time PCR has opened new era in laboratory diagnosis, and specific tools have been developed for different bacterial causes of meningitis such as N. meningitidis, S. pneumoniae, and S. agalactiae. It has been shown that the use of 16s rRNA for the diagnosis of bacterial meningitis yields promising results in terms of sensitivity (100%) and negative predictive value (100%), but the specificity (40.6%) and positive predictive value (48.6%) of the assay are low. Thus, selection of an appropriate primer pair is an essential factor in the PCR technique. This limitation can be solved by using broad-range bacterial primers designed based on the conserved region of 16S rDNA of bacteria. This might be followed by applying appropriate restriction enzymes to accomplish digestion patterns of universal PCR products to identify the bacterial species, which needs future studies. The above-mentioned points were considered in the present study, which is one of the strengths of the current study.

Most studies have focused on the detection of bacterial meningitis causes rather than viral causes, which could be due to the availability of simple methods for the detection of bacterial agents. Accordingly, the proportion of viral and bacterial agents' role in meningitis incidence has not been specified in epidemiological studies, which leads to an unclear path for determining dominant agents specified per region. In addition, local studies performed in Iran are fewer than needed for continuous monitoring, and in the following dedicated molecular methods per each pathogen should be studied as well.

In sum, the obtained bacterial profiles were consistent with the results of other studies performed in Iran, but this is not true for viral agents' profile. Results of the current research showed that human enterovirus had

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the highest prevalence (41%), followed by Herpes simplex virus1, 2 (HSV 1,2) (30%), and EBV (11%). Meanwhile, in summarizing the results of former studies, the following distribution rated were obtained: Enterovirus (41.7%), Herpes simplex virus-1 (HSV-1) (33.3%), and Mumps' virus (25%). These differences necessitate further studies on the viral meningitis agents' profile (34-37).

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Conflict of Interest

Authors declare no conflict of interest.

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