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Frequency of *Listeria monocytogenes* Isolated from Diarrhea Samples of Pediatric Patients at Central Iran

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Abstract

Background: *Listeria monocytogenes* is a primarily foodborne bacterial pathogen that is one of the causative agents of gastroenteritis. However, the prevalence of *L. monocytogenes* infection in pediatric patients with diarrheal disease is not clearly identified in the Iranian population. This study aimed to investigate the frequency of *L. monocytogenes* isolates found in infectious diarrhea samples of pediatric patients in an Iranian population.

Methods: A total of 173 infectious diarrhea samples collected from pediatric patients were used in this cross-sectional study. Samples were collected from patients referred to the Children's Educational-Therapeutic Center affiliated with the Arak University of Medical Sciences in Arak, Iran from May-September 2015. To identify the presence of *L. monocytogenes*, the samples were directly inoculated into the Listeria Enrichment Broth Base through cold enrichment, then plated onto isolated exclusive Listeria Selective Agar Base. As an alternative method for identifying *L, monocytogenes*, Polymerase Chain Reaction (PCR) of the *InlA* gene was used.

Results: Of the 173 infectious diarrhea samples, eight (4.6%) with *L. monocytogenes* were identified using exclusive culture media, while nine (5.2%) were identified using PCR. The majority of *L. monocytogenes* infections (seven cases (77.7%)) were observed in children under the age of five.

Conclusions: Our results show *L. monocytogenes* infections to have a low prevalence for causing diarrhea in children in the central region of Iran. This should be taken into consideration by pediatricians when treating intestinal diseases.

Keywords: Diarrhea, Iran, *Listeria monocytogenes*, Pediatric.

Introduction

According to the World Health Organization (WHO), approximately 525,000 children under the age of five worldwide die as a result of diarrheal disease (1). In addition to morbidity and mortality, diarrheal disease causes significant financial burden on the health care system due to the complications and hospitalizations that it poses (2). Several etiological factors can cause diarrhea, one of which is infection with pathogenic bacteria like *Listeria monocytogenes* (3). Bacteria of the Listeria genus are gram-positive bacillus, non-spore forming, motile, and are catalase-positive bacterium. This genus has seven different strains, of which *L. monocytogenes* is the main bacterial strain that causes listeriosis in both humans and animals (4). Listeriosis causes a variety of symptoms in humans, including spontaneous abortion in pregnant women, granulomatous intrauterine infection, septicemia in infants, endocarditis, myocarditis, encephalitis, meningoencephalitis, hepatic necrosis, skin problems, and gastrointestinal infections (5). Pediatric's stool samples should

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be microbiologically examined for *L. monocytogenes* since diarrhea in pediatric can cause serious complications and sequelae (6). However, screening for *L. monocytogenes* in stool culture is not part of a national routine program in Iran and medical diagnostic laboratories typically do not look for this bacterium. Therefore, the aim of this study was to determine the frequency of *L. monocytogenes* in infectious diarrhea samples obtained from pediatric patients using the standard culture method and through polymerase chain reaction (PCR).

Materials and methods

Sample collection

For this descriptive cross-sectional study, 173 infectious diarrhea samples were collected from pediatric patients referred to the Children's Educational-Therapeutic Center, affiliated with Arak University of Medical Sciences. Samples were obtained from May-September 2015. Questionnaire and consent forms were provided to the parents or guardians of each patient. To be included in the study, pediatric patients with diarrhea required clinical observation of more than five white blood cells per high-power field (HPF) in stool samples and completion of the consent form (7). The patient and guardian described the clinical symptoms which were then recorded. One week prior to the hospital referral, no patients had received antibiotic treatment. This study was approved by the Ethics Committee of the Arak University of Medical Sciences under No. 93-176-30.

Phenotypic investigation

Diarrheal samples were enriched by taking 2 ml of each sample containing either mucus, phlegm, or blood and directly inoculating it into Listeria Enrichment Broth Base (Ibresco, Iran) less than 10 minutes after sample collection. Samples were then maintained at 4 °C for 48 hours. The patient stool samples were then cultured onto isolated Listeria Selective Agar Base (Oxford, UK) with Oxoid Listeria Selective Enrichment Supplement (Thermo Fisher Scientific, Waltham. MA, USA) according to manufacturer's instructions, then incubated at 37°C for 72 hours (8). Gram staining, catalase, camp, hippurate, bile-esculin, motility on Sulfide, Indole, Motility (SIM) medium (motility at 25 °C as a reverse umbrella), and urea agar (Merck, Germany) tests were performed on potential *L. monocytogenes* colonies. Finally, API testing (Biomeriux, France) was used to further identify the presence of *L. monocytogenes* (9).

The clinical specimen of *L. monocytogenes* available in the microbial collection of the Department of Microbiology, Faculty of Medical Sciences, Arak University of Medical Sciences was used as a positive control.

Genotypic Investigations

DNA extraction was performed directly on the stool sample with the QIAamp DNA Stool Minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The amount and purity of the extracted DNA was measured using a NanoDrop device (Thermo Fisher Scientific, Waltham. MA, USA), then confirmed using universal primers for the bacterial 16S rRNA gene.

As an alternative method for identifying the presence of *L. monocytogenes* in the infectious diarrhea samples and confirming the identity of the isolates, PCR of the *InlA* gene was performed. Table 1 shows the forward and reverse primers used for PCR. The 25 µl final volume of the PCR included 12.5 µl of master mix (1X), 2 µl of DNA template (5 ng), 1 µl each of the forward and reverse primers (10 Pm), 0.5 µl of Taq DNA polymerase (2.5 units), and 8 µl of double-distilled water (all purchased from Yekta Tajhiz Company, Iran). From each positive gene, one sample was used for sequencing through determining the sequence of the PCR product of isolates from the Gene Fanavaran Company and confirmed by basic local alignment search tool (BLAST) analysis.

Table 1. Primers used in this study

Target gene description	Primer	Sequence 5'→3'	Amplicon size (bp)	Annealing Temperature	References
Universal DNA bacterial	*16s-rRNA*-F	5-AGGAGGTGATCCAACCGCA-3	367	55	(22)
	16s-rRNA-R	5-ACCTGGAGGAAGGTGGGGAT-3			
Listeria monocytogenes	*InlA*-F	5- ACGAGTAACGGGACAAATGC -3	800	56	(3)
	InlA-R	5- CCCGACAGTGGTGCTAGATT -3			

Results

Of the 173 diarrhea samples, eight isolates (4.6%) were found to be positive for *L. monocytogenes* using the exclusive culture medium and performing the catalase (+), sodium hippurate (+), camp (+), bile-esculin (+), motility (+), and urea (-) differential tests. Using to the PCR method to identify *L. monocytogenes* from the DNA extracted from diarrhea samples, nine cases (33%) were shown to be positive for *L. monocytogenes* (Table 2).

The average age of the patients with *L. monocytogenes* was three years and six months. The youngest patient was a two-year-old girl, while the oldest was a six-year-old boy. The majority of *L. monocytogenes* infections, seven cases (77.7%), were observed in children younger than five years of age. Within our examined cohort of 173 pediatric patients, five males (55.5%) and four females (44.4%) were determined to be infected with *L. monocytogenes*.

The clinical symptoms in the children with *L. monocytogenes* were fever (88.8%), mucus in stool (77.7%), abdominal pain (44.4%), and blood in stool (11.1%).

Table 2. Classification of age groups and clinical symptoms of pediatric patients infected by *L. monocytogenes*

Age grouping	Gender Male /Female	*L. monocytogenes*	Fever	Mucus in the stool	Abdominal pain	Blood in the stool
≤1–2≥	Male	0	0	0	0	0
	Female	1	1	1	0	0
Total:		1/9, 11.1%	1/9, 11.1%	1/9, 11.1%	0/9, 0%	0/9, 0%
<2-3≥	Male	1	1	1	1	0
	Female	1	1	1	0	1
Total:		2/9, 22.2%	2/9, 22.2%	2/9, 22.2%	1/9, 11.1%	1/9, 11.1%
< 3–4≥	Male	1	1	1	0	0
	Female	2	1	1	1	0
Total:		3/9, 33.3%	2/9, 22.2%	2/9, 22.2%	1/9, 11.1%	0/9, 0%
< 4–5≥	Male	1	1	1	1	0
	Female	0	0	0	0	0
Total:		1/9, 11.1%	1/9, 11.1%	1/9, 11.1%	1/9, 11.1%	0/9, 0%
< 5–6≥	Male	2	2	1	1	0
	Female	0	0	0	0	0
Total:		2/9, 22.2%	2/9, 22.2%	1/9, 11.1%	1/9, 11.1%	0/9, 0%
Final total		9/173, 5.2%	8/9, 88.8%	7/9, 77.7%	4/9, 44.4%	1/9, 11.1%

Discussion

Our findings reveal the frequency of *L. monocytogenes* in infectious diarrhea samples of Iranian pediatric patients to be rather rare. Of the 173 patient samples tested, eight cases (4.6%) identified through the culture method and nine cases (5.2%) identified through PCR were determined to be positive for *L. monocytogenes*. Similar research exploring the prevalence of *L. monocytogenes* in human diarrheal samples found the presence of this bacterium to be relatively low. The separate studies found 0% of patients in Korea and China and 0.2% Canada (10-12) to be positive for *L. monocytogenes* in their stool. The increase in *L. monocytogenes* isolates found in the diarrheal samples of the patients in our study and the differences among previous reports may be a result of variances in the sensitivity of the diagnostic, enrichment, and culture methods used to determine the presence of *L. monocytogenes*. Additionally, among the different geographic regions there may be variances in diet, degree of immunity to *L. monocytogenes* infection and the level of exposure to natural reservoirs of *L. monocytogenes* bacteria in the environment such as contact with animals and drinking water conditions (6, 13).

PCR-based molecular methods provide powerful tools that significantly improve the diagnosis of intestinal pathogens (14). The presence of intestinal bacterial infection requires rapid diagnosis and identification. However, traditional methods for identifying *L. monocytogenes* uses standard selective and differential culture media followed by a sequence of isolation and phenotypic tests which can be time-consuming. Delays in diagnosis leaves patients at risk for untreated infection, which can enhance the spread of infection to other individuals. The use of PCR provides a more rapid and sensitive method for the identification of intestinal pathogens (15). Using PCR leads to faster diagnosis times, has a higher sensitivity than standard culture methods, can better identify bacterial species, and produces less laboratory waste. Additionally, the use of antibiotics and the presence of low dose bacterium in culture can encourage the growth of bacteria, and potentially antibiotic resistant bacteria which is a possible biohazard (14, 16). Rapid and accurate identification of *L. monocytogenes* holds a critical role in the management of acute illness by ensuring the use of correct antibiotic therapy and containing the spread of infection (17).

In this study, the average age of patients with *L. monocytogenes* was three years and 6 months. Previous reports show the average age of patients with *L. monocytogenes* to be 15.5 years in Los Angeles and 52 years in Sweden (18, 19). In our study, five cases (55.5%) of the patients with *L. monocytogenes* infection were male and four cases (44.4%) were female. In Los Angeles, half of the patients were male and in Sweden, 25 cases (52%) were female and 23 cases (47.9%) were male (18, 19). This difference in symptoms can likely be attributed to bacterial virulence, the dose of the bacteria that entered the body and the strength of the patients' cellular immune systems(20, 21). In our study, the most common clinical symptom displayed in children with *L. monocytogenes* was fever (88.8%), followed by the presence of mucus in the stool samples (77.7%), abdominal pain (44.4%) and blood in stool (11.1%). Previous research conducted in Italy observed the most frequently reported clinical symptoms of patients with gastroenteritis due to *L. monocytogenes* infection to be fever (60-100%), diarrhea (88-88%), erythromelalgia (20-100%), headache (80%), and over 70% of patients had at least one digestive problem such as diarrhea, vomiting, nausea, or abdominal pain (6).

Our study reveals *L. monocytogenes* to be a causative agent of gastroenteritis with low prevalence in pediatric patients in Arak, Iran. The *L. monocytogenes* bacteria is a foodborne pathogen that is present throughout the environment in a variety of food sources (12). Additionally, microbiological tests are required to isolate *L. monocytogenes* in medical diagnostic laboratories and hospitals. Healthcare measures must be taken to control this infection. Furthermore, paediatricians should consider that *L. monocytogenes* can be causative agent of diarrhea with low

frequency in this geographic region particularly during the course of an empirical therapy. In addition, although the frequency is low we would like to suggest that identification of this bacteria in diarrhea patients must be added to the routine stool culture program.

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