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Molecular Confirmation of the Causative Agents of Diarrhea and Its Antimicrobial Susceptibility Tests

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Objectives: This study aimed to investigate molecular confirmation of the causative agents of diarrhea and its antimicrobial susceptibility tests in the samples collected from patients who have symptoms of diarrhea. There is a relative lack of diagnostic studies on the causative agents of diarrhea in Mongolia, especially on the causative agents of bacterial diarrhea and their antibiotic susceptibility. **Methods:** A rectal swab and stool sample were taken from 187 patients admitted to the NCCD Mongolia with a diagnosis of diarrhea and infection between June 2018 and April 2020 using a hospital-based instantaneous survey model, and bacteriology, molecular biology, and antibiotic susceptibility testing were performed at the laboratory of Bacteriology and Immunology, Institute of Veterinary Medicine. **Results:** Of the 187 samples, N=127 (67.9%), p<0.01) were confirmed by simple and multiplex PCR, and *Salmonella spp., Shigella spp., E.Coli, and S.aureus* were detected in n=20 (15.7%), n=16 (12.6%), n=20 (15.7%), and n=7 samples (5.5%), respectively. Multiplex PCR 3 samples did not detect the *16s rRNA* gene of *Campylobacteraceae* and *C.jejuni* or *cdt C* gene of *C.coli*, all of which were negative. Therefore, multiplex PCR was performed for foursamples to detect pathogenic bacteria, and samples were *E. coli* positive but not *campylobacter, salmonella,* and *shigella.* **Conclusion:** 126 (67.4%) children aged 1-5 years were the most affected by foodborne infection, and most of the diarrhea causative agents detected were *Salmonella spp, Shigella spp, E.Coli*, and *S.aureus*.

Keywords: Foodborne Infection, Bloody Diarrhea, Salmonella, Shigella, Listeria

Introduction

550 million people are infected with foodborne illness each year, and 220 million of whom are children under five. In developing countries, 5-8 million people die each year due to food poisoning [1]. *Enterobacteriaceae Salmonella, Escherichia,* and *Shigella* are the primary bacteria that cause foodborne infections. In the United States, 1.2 million people are infected with salmonella annually, of which 23,000 are hospitalized and about 450 patients die [2]. 80 to 165 million people are infected with shigellosis each year, and 600,000 die. 20–119 million cases of Shigellosis are food-borne, and the death cases were

6,900–30,000 [3].

Hemolytic strains of *E. coli* (STEC toxin, ETEC) cause severe food poisoning. 600 million cases of diarrhea caused by the ETEC strain are reported annually worldwide, including 800,000 deaths among children under five years of age [4]. *S. aureus* enterotoxin-contaminated illness is a significant cause of foodborne infection [5].

In 2016, 1489 pathogens were diagnosed with food poisoning at the NCCD, Mongolia, of which 21.8% were identified as *E. coli*, 62.3% as *Staphylococcus aureus*, 7.8% as *Salmonella enteritis,* and 7.5% as *Shigella flexneri* [6]. According to the 2019 report of the State Inspection Agency, 34 food poisoning outbreaks were registered nationwide in 2016-2018, and 328 of 1028 patients were hospitalized in the NCCD or central hospitals in a province, Mongolia. In 2018, 329 cases of salmonellosis, 6265 cases of dysentery, and 554 cases of bacterial food poisoning were registered, respectively.

Drug-resistant bacterial infections have been increasing year by year due to improper use of antibiotics. 2 million people in the United States become infected with one or more antibiotic-resistant bacteria every year, and death cases are 23,000 [7]. In particular, bacteria such as *Acinetobacter, Pseudomonas, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, Staphylococcus aureus*, and *Streptococcus pneumoniae* have become resistant to broad-spectrum antibiotics [8]. According to the 2018 WHO report, Mongolia has the highest antibiotic use rate at 64.41 units daily per 1,000 population.

The studies on the bacteria that cause diarrhea and their diagnosis have reached, and new methods of prevention and treatment are upgraded. However, the number of foodborne infections has not decreased. There is a relative lack of diagnostic studies on the causative agents of diarrhea in Mongolia, especially on the causative agents of bacterial diarrhea and their antibiotic susceptibility.

To isolate the causative agent from the patients with bacterial diarrhea samples, to study their antimicrobial susceptibility testing, and to confirm resistance genes by molecular analysis.

The main goal of our research is to identify the main bacteria that cause diarrhea in Mongolia, to isolate these bacteria and confirm them by PCR, to determine the antibiotic sensitivity of the pathogens, and to develop treatment tactics.

Materials and Methods

Methods and sampling

We researched 187 samples by selecting purposive Sampling methods and analysis using the experimental research method. A rectal swab and stool sample were taken from 187 patients admitted to the NCCD Mongolia with a diagnosis of diarrhea and infection between June 2018 and April 2020 using a hospital-based instantaneous survey model, and bacteriology, molecular biology, and antibiotic susceptibility testing were performed at the laboratory of Bacteriology and Immunology, Institute of Veterinary Medicine.

Bacterial culture

Enterobacteriaceae were isolated from stool and rectal swab samples to identify bacterial species. BHI, SS, MacConkey, Endo, XM-G, DHL, and chromogenic agar were used to isolate *Enterobacteriaceae*. Isolation of Listeria used special agars, such as Biolab-Fraser broth, Oxford selective agar, and Palcam *Listeria* selective agars. Preston broth and modified charcoal cefoperazone deoxycholate agar were used to isolate *Campylobacteraceae.* The campylobacter was cultured in Preston broth at 42°C for 24 h, then sub-cultured on selective campylobacter agar (mCCDA) and incubated in microaerophilic condition for 48 h at 37°C to isolate a campylobacter-like colony. *Enterobacteriaceae* and others were cultured in aerobic conditions and for 24-48 h at 37°C to isolate a pure colony of the pathogenic bacteria. ATCC standard strains of L. monocytogenes, *C. jejuni*, *S. Typhimurium*, *E. coli*, *Staphylococcus aureus,* and *Shigella spp.* were used to confirm isolation and validation of antibiotic susceptibility tests.

Antibiotic susceptibility tests

Antimicrobial susceptibility test (AST) for amoxicillin (30 µg), chloramphenicol (10 µg), nalidixic acid (30 µg), ciprofloxacin (30 µg), vancomycin (30 µg), tetracycline (30 µg), streptomycin (10 µg), cefotaxime (30 µg), Nitrofurantoin (300 µg), doxycycline (10 µg), gentamicin (50 µg) and erythromycin (30 µg) was performed using disk diffusion method. Experiments were performed, and the results were interpreted as per the Clinical and Laboratory Standards Institute CLSI, 2018, for other antibiotics [16, 17].

Molecular confirmation of causative agents of diarrhea

DNA was isolated from the culture by methods of boiling and phenol-chloroform, and the spectrophotometer checked the purification. Specific primers of *Salmonella spp, Shigella spp, E.Coli, S.aureus, Listeria,* and *Campylobacter* were used to detect by simple and multiplex PCR.

Data analysis

All obtained data were entered into a Microsoft Excel spreadsheet. Descriptive data are given as bar graphs, line graphs, and tables. Chi-squared analysis was performed to determine the trends in the proportion of NTS isolates from periods one and 2. A p-value < 0.05 was considered statistically significant.

Ethical statement

The research study was approved by the Research Ethics Committee of the Mongolian National University of Medical Sciences (2018.06.22 №2018/3-11). All participants gave written informed consent.

Results

1. Sampling

187 patients enrolled in the study, and the patients were n=12 (6.4%) aged 0-1, n=126 (67.4%) aged 1-5, n=24 (12.8%) aged 6-10, n=6 (3.2%) aged 11-15, and n=2 (1.1%) aged 15-19 and n=17 (9.1%) over the age of 20 (see Table 1).

Table 1. Age group of respondents

Age group	Male	Female	P-value
0-5	2.57±1.40	3.06±1.24	0.0815
6-14	8.29±2.17	7.78±1.92	0.5551
15-20	28.60±17.77	22.11±1.90	0.2922

In terms of clinical symptoms, there were n=117 (62.5%) cases of fever, n=42 cases of vomiting (22.5%), n=30 cases of diarrhea (16.1%), and n=57 cases of stomach cramps (30.5%) (see Table 2).

Table 2. Clinical symptoms

Clinical symptoms	Male	Female	Total	P-value
Fever				
Yes	62 (53%)	55 (47%)	n=117 (62.5%)	0.9278
No	32 (45.7%)	38 (54.3%)	n=70 (37.5%)	
Vomiting				
Yes	19 (45%)	23 (55%)	n=42 (22.5%)	0.6709
No	76 (52.4%)	69 (47.6%)	n=145 (77.5%)	
Tenesmus				
Yes	19 (63.3%)	11 (36.7%)	n=30 (16.1%)	0.0483
No	68 (43.3%)	89 (56.7%)	n=157 (83.9%)	
Belly cramps				
Yes	31 (54.4%)	26 (45.6%)	n=57 (30.5%)	0.5258
No	63 (48.5%)	67 (51.5%)	n=130 (69.5%)	

There were n=19 (10.2%) cases of diarrhea 5 times, and n=2 (1.07%) cases of diarrhea 25 times. 61%, 42%, and 24% of patients had symptoms of bloody stool, mucus in stool, and floating stool, respectively.

2. The bacteriological analysis and molecular confirmation

Stool and rectal swab samples were collected from 187 patients to identify causative agents of diarrhea. 70 of 187 samples were isolated pathogenic bacteria by the identification of bacteriological analysis. Of the 187 specimens, n=70 (37.4%) were culture-positive samples, of which n=39 (55.7%) were detected *Salmonella, Shigella, E.Coli, and S. aureus*, n=24 (34, 3%) were cultured with mixed bacteria. 5 2 of 70 culture-positive samples were detected, listeria-like cultures and campylobacter-like cultures were isolated, respectively.

Of the 187 samples, n=127 (67.9%) ($p<0.01$) were confirmed by simple and multiplex PCR, and *Salmonella spp., Shigella spp., E.Coli, and S. aureus* were detected in n=20 (15.7%), n=16 (12.6%), n=20 (15.7%), and n=7 samples (5.5%), respectively (see table 3).

Table 3. Frequency of diarrhea

Organism	Times per day		Total N=70	P-value
	<5	>10		
Salmonella spp				
+	15(75%)	5(25%)	n=20 (28.6%)	0
-	5 (10%)	45 (90%)	n=50 (71.4%)	
Shigella spp				
+	12(75%)	4(25%)	n=16 (22.8%)	0
-	4 (7.4%)	50 (92.6%)	n=54 (77.2%)	
E.coli spp				
+	16(80%)	4(20%)	n=20 (28.6%)	0
-	12 (24%)	38 (76%)	n=50 (71.4%)	
S.aureus spp				
+	7(100%)	0 (0%)	n=7 (10%)	0
-	0(0%)	63 (100%)	n=63 (90%)	
Listeria				
+	5(100%)	0 (0%)	n=5 (7.1%)	0
-	0(0%)	65 (100%)	n=65 (92.9%)	
Camphylo bacteria				
+	2(100%)	0 (0%)	n=2 (2.9%)	0
-	0(0%)	68 (100%)	n=68 (97.1%)	

The PCR was selected from listeria and campylobacter-like colonies grown on mCCDA agar. DNA was isolated by phenol-chloroform and purified and concentrated using nano drops to check purification and concentration (see Table 4).

Table 4. Association between symptoms and organism

Organism	Bloody stool N=114 (59.4%)	Mucus in stool N=78 (40.6%)	Total N=192	p-value
Salmonella spp				
+	32 (28%)	24 (30.8%)	n=56(29.2%)	0.7472
-	82 (72%)	54 (69.2%)	n=136(70.8%)	
Shigella spp				
+	48 (42%)	40 (51.3%)	n=88(45.8%)	0.2393
-	66 (58%)	38 (48.7%)	n=104(54.2%)	
E.coli spp				
+	30 (26.3%)	30 (38.5%)	n=60(31.3%)	0.0829
-	84 (73.7%)	48 (61.5%)	n=132(68.7%)	
S.aureus spp				
+	5 (4.3%)	4 (20.5%)	n=9(4.7%)	1
-	109 (93.7%)	74 (79.5%)	n=183(95.3%)	

Multiplex PCR 3 samples did not detect the *16s rRNA* gene of *Campylobacteraceae* and *C. jejuni* or *cdtC* gene of *C. coli*, all of which were negative. Therefore, multiplex PCR was performed in four samples to detect pathogenic bacteria, and samples were E. coli positive but not *campylobacter, salmonella,* and *shigella* (see Table 5 and Figure 1)

Table 5. Results of PCR for detection of *Salmonella spp, Shigella spp, E.coli spp, S.aureus spp*

Organism	Number of sample	Positive in PCR	Total (%)
Salmonella spp	187	20	15.7 %
Shigella spp	187	16	12.6 %
E.coli spp	187	20	15.7 %
S.aureus spp	187	7	5.5 %

3. Antimicrobial susceptibility testing

To determine the antibiotic susceptibility of the bacterium, the colony-forming unit of the culture was adjusted to McFarnald's 0.5 concentration and placed in the Mueller Hinton agar. *Salmonella spp* was 100% resistant to vancomycin nalidixic acid but sensitive to chloramphenicol, tetracycline, streptomycin, cefotaxime, and colistin. *Shigella. spp* was 100% resistant to erythromycin but sensitive to chloramphenicol, cefotaxime, nitrofurantoin, ciprofloxacin, doxycycline, and colistin. 80% of E. coli were resistant to amoxicillin; however, 100% were susceptible to chloramphenicol, tetracycline, streptomycin, cefotaxime, colistin, and nitrofurantoin.

All isolated bacteria were 86-100% sensitive to chloramphenicol, streptomycin, cefotaxime, ciprofloxacin, colistin, nitrofurantoin, gentamicin, doxycycline, and tetracycline. However, 33 to 61% of isolates were resistant to ampicillin, amoxicillin, vancomycin, nalidixic acid, and erythromycin.

Listeria-like cultures were resistant to cefotaxime, ciprofloxacin, and nalidixic acid and sensitive to amoxicillin, streptomycin, and tetracycline. The antibiotic susceptibility of two Campylobacter-like cultures was determined by resistance to fluoroquinolone antibiotics, such as ciprofloxacin and ofloxacin.

Discussion

Our research was extensive and multidisciplinary. We studied from samples N=187 isolated main bacteria that cause diarrhea in Mongolia to isolate these bacteria and identified pure culture, confirmed them by PCR, determined the antibiotic sensitivity of the pathogens, and developed treatment tactics.

Food poisoning is considered a significant issue in today's world. So far, nearly 250 types of food poisoning diseases have occurred in the world, and more than two-thirds of them have a bacterial origin. Among the bacteria causing food poisoning,

L. monocytogenes, *C. jejuni,* and *Salmonella* are particularly important. Simultaneous amplification of greater than one particular locus is essential for rapidly diagnosing multiple microbes. It is just a technique termed *Multiplex PCR,* through which several distinct primer packages tend to be blended into one PCR assay. We also used this method in our study.

Rapid tests are widely used to diagnose infectious diseases worldwide, but traditional bacteriological methods are used to identify and confirm the pathogen by molecular biological analysis. In the United States, it is performed by bacteriological culture and antibiotic susceptibility testing after positive results of rapid tests to diagnose infectious agents [3].

Bacteriological identification of the pathogen using a variety of selective media based on the biochemical characteristics of the pathogen [9].

This study had significant limitations. First, bacteriological methods were used to identify pathogenic bacteria. Second, the isolated agent was confirmed by PCR. Thirdly, antibiotic sensitivity of the pathogens was determined.

Our study used bacteriological methods to identify listeria and campylobacter from various samples. We used biochemical techniques to isolate the pathogen using enriched and selective media based on the biochemical characteristics of the pathogen, carbohydrate, and sugar degradation activity. The specimens were cultured in selective nutrient media such as Fraser broth, Palkam agar, Oxford, Brain heart infusion broth, agar, campylobacter mCCDA agar, and Priston broth, and similar pathogens were identified in terms of culture, shape, color, and size.

Recently, chromogenic media have been widely used to differentiate between serum types of *Salmonella* and *E. coli*, and researcher L.M.F. Kuijper compared 4 types of chromogenic media with SS agar and isolated *Salmonella* ParatyphiA serum from 20 samples [10]. In this study, *Salmonella* and *E. coli* were isolated using two types of chromogenic media.

Wences Arvelo et al. reported that 103 (19%) of the 554 patients with diarrhea tested positive for *E. coli* 94 (17%), *Shigella spp* 31 (6%), and *Salmonella spp.* 4 (1%) cases [11].

Iranian researcher Roya Nicfarin isolated the pathogen using MacConkey and XLD agar in a bacteriological test for *Shigella* in children under 12 with diarrhea [12]. We also used this agar and medium to identify Enterobacteriaceae from the samples.

Abbasi, et al found that *L. monocytogenes* in only 4.6% of the 173 stool samples collected from the pediatric hospital [13]. Irving et al. identified 13 cultures (5.8%) of *Campylobacter spp* from 225 stool samples collected from a hospital in Philadelphia, USA [14]. These studies show that the low detection of Listeria and Campylobacter pathogens makes it challenging to detect pathogens by bacteriological methods, and the infection rate is low in the public—Indian researchers Elnaz. The results of these studies show that the low detection rate of *Listeria* and *Campylobacter* pathogens makes it challenging to detect pathogens by bacteriological methods. Therefore, in this study, 5 (7.1%) cases of listeria-like and 2 (2.9%) cases of campylobacter-like cultures were isolated from 70 samples collected from patients who have symptoms of diarrhea. However, molecular analysis of listeria and campylobacter was not confirmed in these 7 cases or previous studies.

According to the "Antibiotic Use and Bacterial Resistance" survey conducted in 20 health facilities in Mongolia in 2019, the usage of antibiotics in most hospitals was high, about 70-95%. In this study, the urinary pathogen *E. coli* had the highest resistance to ampicillin at 85% and cefazolin at 35.9% and was sensitive to imipenem and meropenem [7]. *E. coli* isolated from the stool and rectal swab samples in this study is 80% resistant to amoxicillin, a group of penicillin, proving that *E.coli* which causes severe infections, is resistant to ampicillin and its group.

Many drug-resistant *Shigella*, including azithromycin and ciprofloxacin-resistant pathogens, have been reported in Australia, Europe, Taiwan, Canada, and the United States [3].

57.5% of ampicillin-resistant *Shigella* strains were isolated by Iranian researchers, as well as our study.

Romania et al. 120 strains isolated from the samples to detect *Salmonella* infection, and 60.53%, 51.2%, 37.25%, 25.1%, and 12.65% of isolated strains were tetracycline, streptomycin, sulfamethoxazole, nalidixic acid, and ciprofloxacin-resistant, respectively, performed by antibiotic susceptibility testing [15]. In our study, S*almonella* was 100% resistant to vancomycin and nalidixic acid and 100% sensitive to chloramphenicol, tetracycline, streptomycin, cefotaxime, and colistin.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has risen worldwide over the past decade, and much research is being done. Researchers Sergelidis et al. identified enterotoxin genes from MRSA in a study of different geographical regions in 2019. Japanese scientists recently identified the toxic factor *Staphylococcus argenteus* from the species of *Staphylococcus aureus* [5, 16]. When we studied the antibiotic resistance of the samples, the following results were obtained. *S.aureus* was 100% resistant to amoxicillin erythromycin and sensitive to

chloramphenicol, tetracycline, streptomycin, cefotaxime, nalidixic acid, nitrofurantoin, and doxycycline; it was detected by antibiotic susceptibility testing in our study. We have yet to do toxic factors of Staphylococcus aureus research.

When we confirmed the bacterial isolation by PCR, not all of the specific genes of listeria and campylobacter were detected; on the other hand, there were no cases of listeria and campylobacter in the samples of 187 patients. These results suggest that foodborne pathogens are similar in biochemical properties, culture, and shape and that it is important to differentiate them at the genetic level by molecular biology [17, 18, 19, 20, 21].

Listeria monocytogens, Campylobacter jejuni, and *Bacillus cereus* are toxin-releasing bacteria, and many studies have been conducted worldwide to find a straightforward and rapid way to identify these microorganisms. Iranian scientists have detected multiple bacteria by PCR based on their toxicity [22, 23]. They successfully used a PCR primer to detect the hly A gene of *Listeria monocytogens* and the cdtC gene of *Campylobacter jejuni* and tested it on 30 milk products. We also experimented with four cultures isolated from 64 human specimens using the Multiplex PCR primer to simultaneously detect several food poisoning bacteria developed by Startup Primer LLC. No specific genes for *Shigella* and *Campylobacter* were detected in 7 cultures. These results suggest that it is possible to use this in-house Multiplex PCR primer to detect foodborne infection in food products. Overall, 126 (67.4%) children aged 1-5 years were the most affected by foodborne infection, and most of the diarrhea causative agents detected were *Salmonella spp, Shigella spp, E.Coli*, and *S.aureus*.

In Tanzania, diarrhea-causing pathogens were determined the effect of co-infection on clinical symptoms. Total nucleic acid was extracted from archived stool samples (N = 146) collected from children (0-59 months) admitted with diarrhea in health facilities in Moshi, Kilimanjaro. Pathogen detection was performed using the quantitative polymerase chain reaction with custom TaqMan Array cards. The Poisson model was used to determine the effect of co-infection on clinical presentation during admission. Of all the participants, 56.85% were from rural Moshi with a median age of 11.74 months (IQR: 7.41-19.09). Vomiting (88.36%) and fever (60.27%) were the most frequent clinical manifestations. At least one diarrhea-associated pathogen was detected in 80.14% (n=117) of the study population. The most prevalent pathogens were rotavirus 38.36% (n=56), adenovirus 40/41 19.86% (n=29), *Shigella* /EIEC 12.33% (n=18), norovirus GII 11.44% (n=17) and *Cryptosporidium* 9.59% (n=14). Co-infections were detected in 26.03% of the study population (n=38). Multiple pathogens in the stool samples of children with diarrhea indicate poor sanitation and may have significant implications for disease management and patient outcomes [24].

In our study, n=70 (37.4%) cultures from N=187 specimens were confirmed by 2 types of PCR, *E.Coli*, *Salmonella spp*, and *S.aureus* were 28.6%, 28.6%, and 10%, respectively. The incidence of *Escherichia coli* is close to that of the results of other researchers, and the incidence of different bacteria is higher than others n=126 (67.4%) children aged 1-5 years were the most affected by foodborne infection, and most of the diarrhea causative agents detected were *Salmonella spp, Shigella spp, E.Coli*, and *S.aureus*.

The findings have implications for clinical treatment, and based on this basic research, it is essential to investigate further the serotypes, virulence, and antibiotic resistance mechanisms of pathogens.

Conflict of Interest

There are no conflicts of interest to declare.

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