Identification Viable but Not Culturable Bacteria in Water Sources "PCR and Culture" Kermanshsh, Iran

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ABSTRACT

Background and Aim: As the world population grows, it is predicted that freshwater supplies will be rare in the 21st century. Despite abundant advances in water and wastewater treatment, waterborne diseases still threaten the health of the people of the world. *Listeria monocytogenes* bacterium is a pathogen that causes listeriosis. This pathogen can also cause meningitis, poisonous sepsis, and abortion in humans. One way of transmitting this microorganism is water and foodstuffs. Quick and accurate identification plays an important role in preventing infections. Also, due to the importance of *Campylobacter jejuni* in water and food industries and causing infection, toxication, and digestive problems in humans, the identification of this bacterium can be an effective step in preventing water contamination with *Campylobacter jejuni*. The aim of the present study was to identify *Listeria monocytogenes* and *Campylobacter jejuni* through culture and PCR and compare them in the water supply of Kermanshah city. **Materials and Methods**: 18 samples were collected from different water supplies of Kermanshah. DNA was extracted from standard *Campylobacter jejuni* and *Listeria monocytogenes* using a DNG-Plus kit. PCR reaction was optimized using specific primers. After determining the specificity and PCR detection limit, the collected water samples were examined and at the same time, the samples were cultured and examined. **Results**: From 18 samples of water supply sources in Kermanshah by PCR, *Campylobacter jejuni* was isolated from all samples, and *Listeria monocytogenes* was isolated from 17 samples, and also 4 cases of *Campylobacter jejuni* and 2 cases of *Listeria monocytogenes* were isolated by culture method. **Conclusion**: The results showed that PCR has a better performance than culture for detecting *Listeria monocytogenes* and *Campylobacter jejuni*.

Key Words: *Listeria monocytogenes, Campylobacter jejuni, PCR, drinking water*

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INTRODUCTION

Today, new studies have shown that adherence to old standards can no longer prevent the spread of contamination and pathogenic microorganisms in the environment [1]. Listeria is one of the bacteria that is widely distributed in the natural environment and its main sources are dust, soil, vegetables, animal excrement, water, sewage, and sludge [2, 3]. Listeria is in the group of aerobic bacteria, gram-positive, bacillus, without spores, motile, catalase-positive and has 6 species, the main species that causes listeriosis in humans and animals is Listeria monocytogenes. Listeriosis is a foodborne disease and is manifested in humans in the form of meningoencephalitis, sepsis, and abortion. Transmission of the disease in humans is through the consumption of milk, meat, vegetables, and generally contaminated food and water, inhalation of airborne bacteria, insects, direct contact with vector human feces and infected animal products [4, 5]. Campylobacter is the leading cause of gastroenteritis in many countries of the world and in addition to causing dysentery, it also causes secondary diseases such as

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meningitis and cholecystitis. Jejuni and coli species play an important role in the development of campylobacteriosis. The disease is mainly common in spring and summer. Water contamination with this bacterium has been reported from different parts of the country [6, 7] . The microorganism will be killed within 5 minutes at 60 °C. It is sensitive to drought but may survive for a relatively long time in the excrement and soil because of covering by organic materials. The bacterium grows better in alkaline environments. The bacterium is unable to grow at 25 °C. It grows better at 42 °C rather than 35 °C. In humans, *Campylobacter jejuni* and coli cause acute intestinal inflammation. Campylobacter can be isolated in cases of intestinal outbreaks [7].

Campylobacter jejuni:

Morphology: *Campylobacter jejuni* is a gram-negative bacillus, curved, motile, rhabdoid, non-sporogenic, without spores, non-fermenting of positive oxidase, thermophilic and microaerophile of the Campylobacteriaceae family and Campylobacteriosis is one of the leading factors of enteritidis. The main source of this bacterium is the gastrointestinal tract of animals, especially chickens and turkeys. Consumption of raw meat, chicken, milk, and non-chlorinated water are the main causes of the transmission of this bacterium to humans and the occurrence of campylobacteriosis. Symptoms of campylobacteriosis include fever, stomachache, and diarrhea will be manifested from two to five days after eating contaminated food, that diarrhea may result in dysentery. There is usually no vomiting in this foodborne infection. Meat foods should be thoroughly cooked, raw milk, and non-chlorinated water should be avoided to control this infection. Campylobacter with different species and hosts is one of the most important and common bacteria between humans and animals. One of the most common diseases of this genus has 18 species and dosage infection is low for animals and humans. Campylobacter family has two important species called *Campylobacter jejuni* and *Campylobacter coli*, which are responsible for most cases of Campylobacter infections in humans [8].

Table1: Characteristics of major species of Campylobacter [9]

Campylobacter Upsaliensis	*Campylobacter Lari*	*Campylobacter Coli*	*Campylobacter jejuni*	Species / Characteristic
Negative or weak	Positive	Positive	Positive	Catalase
Sensitive	Sensitive/Resistant	Usually sensitive	Usually sensitive	Nalidixic acid
Sensitive	Resistant	Resistant	Resistant	Cefalotin

Campylobacter jejuni (table 1) can also cause bacteremia, meningitis, and abortion. Although the disease is usually self-limiting after a few days, it can last for several weeks [10, 11]. The most important cases after infection with some strains of Campylobacter are noninfectious reactive arthritis, which is more common in people with HLA-B27, and an autoimmune disease called Guillain-Barree syndrome, which accounts for about %1 and %/1 of post-infection cases, respectively. In Guillain-Barree syndrome, including Miller-Fisher and Acute Motor Axonal Neuropathy, antibodies against the bacterial O19 antigen have cross-reactivity with peripheral nerve surface gangliosides, leading to the destruction of the myelin sheath [10, 12]. Although Campylobacter infection is largely non-fatal, there are reports, showing that annually, about 100 people die from Campylobacter infection in the United States. Statistics show that the prevalence of this bacterium is high, especially in children under five years in developed and developing countries, and even show a higher prevalence of Shigellosis [11, 13, 14]. Because the clinical manifestations of the disease are similar to those of other intestinal bacteria, it is not possible to diagnose Campylobacteriosis on the basis of clinical symptoms alone, thus, laboratory determination of the infection seems very important.

MATERIALS AND METHODS:

18 samples of water supply cisterns in Kermanshah (table 4, table 5) were taken including 1 spring, 1 mirage, 1 dam, and 124 deep wells that were concentrated in 18 storage tanks. Sampling was performed according to the standard water and sewage experiments book [15] with sterile sampling glass in the amount of two containers, which in each one was 2000ml, then it was carefully packed and transferred to the laboratory in a cold box [16].

Isolation of *Listeria monocytogenes* and *Campylobacter jejuni* by PCR:

Research steps:

1. Preparation of standard strain of *Listeria monocytogenes* and *Campylobacter jejuni*
2. Preparation of materials and equipment for DNA extraction, electrophoresis on an agarose gel, PCR, and cloning.
3. Primer Selection: By reviewing similar studies in this field, the best primer with the most reference was selected [22, 23] and its sequence was reviewed online on the NCBI site (table 2).

4. DNA extraction from *Listeria monocytogenes* and standard *Campylobacter jejuni* collected from water samples.
5. Optimization of PCR test with hly an and mapA genes target
6. Analysis of the collected water samples by PCR reaction using designed primers for hly and mapA genes on DNA extracted from these samples.
7. Investigation of the amplified gene fragments in water samples using electrophoresis.
8. Evaluation of LOD limits of detection of both genes by serial dilution method
9. Determining the specificity of the PCR test.
10. Purification of the PCR product
11. Cloning of the PCR product

Utilized primers in this study:

Table 2: Product size, the sequence of primers, and target genes

PCR Product size	Sequence of primer	Primer Name
589bp	**5'- CTA TTT TAT TTT TGA GTG CTT GTG-3'**	**c.jej-F**
	5'-GCT TTATTT GCC ATT TGT TTT ATT A-3'	**c.jej-R**
456 bp	**5'- GCAGTTGCAAGCGCTTGGAGTGAA-3'**	**hly A- F**
	5' - GCAACGTATCCTCCAGAGTGATCG- 3'	**hly A- R**

Isolation of *Listeria monocytogenes* and *Campylobacter jejuni* according to the standard of 30-MFHBP [13] was performed. Based on this method, 2 liters of prepared water samples from the 0.45-micron sterile filter were passed through a sterile filtration machine for *Listeria monocytogenes* and these steps were performed for *Campylobacter jejuni.*
Then the filter surface was washed with 1.5 cc of distilled water and transferred into 1.5-cc tubes and centrifuged at 12000rpm for ten minutes. Then the supernatant was discarded and the resulting pellet was dissolved in 200 microliters of double distilled water. DNA was extracted from 200 microliters of samples using Sina Clone DNG-Plus commercial kit. Also, standard strains of *Listeria monocytogenes* and *Campylobacter jejuni* were prepared. DNA was extracted from the strains by the DNG-Plus method to optimize the PCR test.
Isolation of *Listeria monocytogenes* and *Campylobacter Jejuni* by culture method:
Isolation of *Listeria monocytogenes* and *Campylobacter jejuni* was performed according to the standard of 30-MFHBP [13] and based on this method, 2 liters of the prepared water samples were passed through the 0.45-micron sterile filter for *Listeria monocytogenes* and these steps were also performed for *Campylobacter jejuni.*

A: Isolation of *Campylobacter Jejuni*:
Campylobacter Selective Agar culture medium (Quelab) was prepared according to protocol and antibiotic supplements including trimethoprim, polymyxin B, and vancomycin, as well as 7% sheep blood were added. Then, the filters were transferred to a specific culture medium and placed in an anaerobic jar to create micro aerophilic conditions in the incubator for 48 hours. Then, the plates containing the suspected colony were passed several times and oxidase and catalase tests were used for confirmation, and at the same time for the final confirmation, an antibiogram with cefalotin and nalidixic acid antibiotics was performed (table 1).

B: Isolation of *Listeria monocytogenes:*
Listeria selective agar and tryptic soy broth culture medium was prepared according to the protocol and then the filters were transferred into the enrichment TSB medium and then streak culture on the specific culture medium was performed and after 24 hours, some tests such as oxidase and catalase tests, SIM, MR, VP, CAMP tests and TSI, as well as fermentation of dextrose, rhamnose, and xylose sugars were performed (table 1).

RESULTS:

Using PCR, from 18 samples of water supply cistern in Kermanshah (table 4, table 5), Campylobacter was isolated from all samples and *Listeria monocytogenase* was isolated from 17 samples. Moreover, using culture method, Campylobacter was isolated from 4 samples and *Listeria monocytogenase* was isolated from 2 samples (table 3).

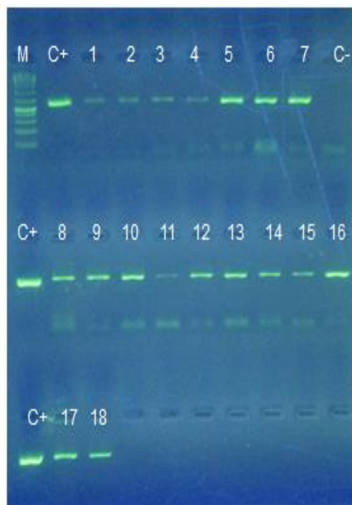
Table 3: Cultivation results are as follows

Row	Storage cistern name	Cultivation results of *Listeria monocytogenes*	Cultivation results of *Campylobacter jejuni*
1	Miyan Darband	Negative	Positive
2	Sarab Qanbar	Negative	Negative

3	Chaman	Negative	Negative
4	Zeynabie	Positive	Positive
5	Zafar	Negative	Negative
6	Moalem	Negative	Positive
7	Jomhori	Negative	Negative
8	Shahid Nazari Refinery, Deh pahn Entrance	Positive	Negative
9	Shahid Nazari Refinery, Exit	Negative	Positive
10	Shahid Nazari Refinery, Gavoshan Dam Entrance	Negative	Negative
11	Ferdowsi	Negative	Negative
12	Taqe Bostan Fountain	Negative	Negative
13	Olfati nia	Negative	Negative
14	Mosalla	Negative	Negative
15	Kosar	Negative	Negative
16	Zibashahr	Negative	Negative
17	Pardis	Negative	Negative
18	Zamzam	Negative	Negative

22/2% were positive for *Campylobacter jejuni* (table 3) and 11/1% were positive for *Listeria monocytogenes (table 3).*

PCR results of water Storage cistern in Kermanshah City:



M: size marker 1kb DNA ladder bioflux
C+: positive control
C-: negative control
1-18 : positive samples

Figure 1: C Jejuni Monoplex tests on the samples

Table 4: samples water

Row	Storage cistern
1	Jomhori
2	Pardis
3	Zibashahr
4	Sarab Qanbar
5	Ferdowsi
6	Olfati nia
7	Mosalla
8	Gavoshan Dam
9	Shahid Nazari, Deh pahn Entrance
10	Shahid Nazari Exit
11	Miyan Darband
12	Zamzam
13	Zafar
14	Moalem
15	Chaman
16	Kosar
17	Taqe Bostan Fountain
18	Zeynabie

PCR test on samples L.mono 456bp
C+: Positive Control (456bp)
M: 1Kb DNA ladder (bioflux)
C-: Negative control
1-10,12-18: Positive samples
11: Negative sample

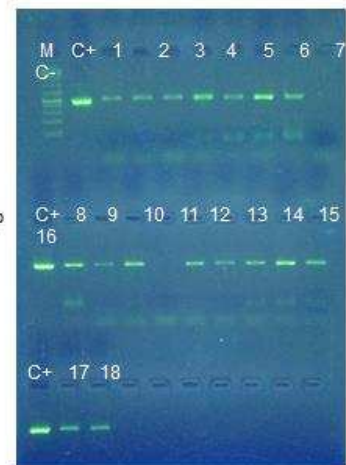


Figure 2: PCR test on sample L.mono 456bp

Table 5: samples water

Row	Storage cistern

1	Jomhori
2	Pardis
3	Zibashahr
4	Sarab Qanbar
5	Ferdowsi
6	Olfati nia
7	Mosalla
8	Gavoshan Dam
9	Shahid Nazari, Deh pahn Entrance
10	Shahid Nazari Exit
11	Miyan Darband
12	Zamzam
13	Zafar
14	Moalem
15	Chaman
16	Kosar
17	Taqe Bostan Fountain
18	Zeynabie

PCR test results:

100% of the samples were positive for *Campylobacter jejuni* (Figure 1) and 94.4% contained *Listeria monocytogenes (Figure 2).*

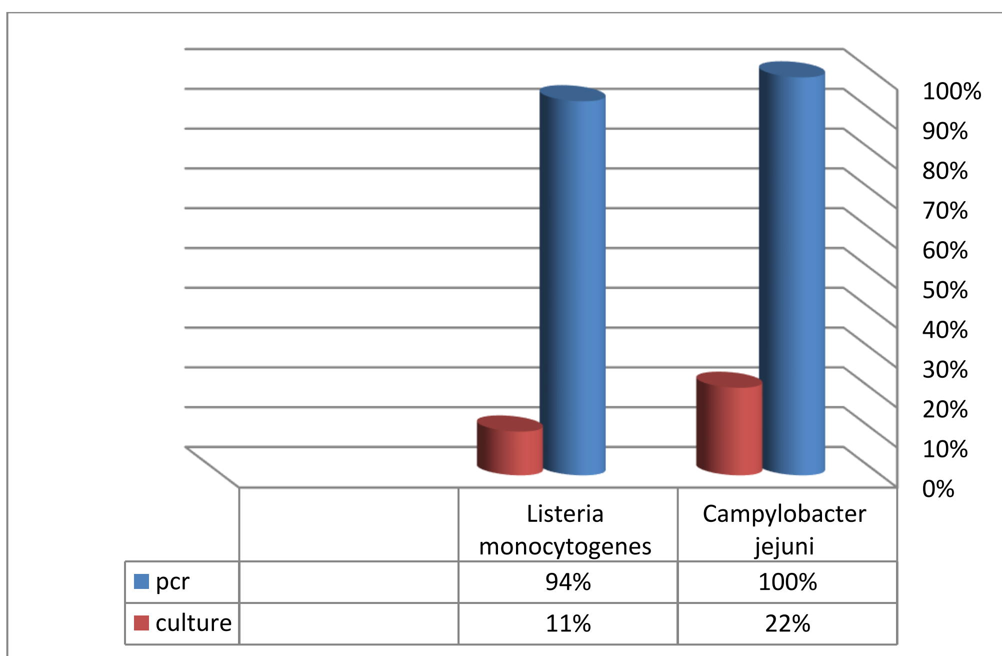


Chart 1: Comparison of culture separation percentage and PCR

DISCUSSION:

Campylobacter jejuni is one of the leading causes of enteritis in the world. In addition, side effects such as enteritis caused by these bacteria have been reported many times [17, 18]. *Campylobacter jejuni* is one of the main causes of Campylobacteriosis in humans, which has been introduced as the most common disease in humans and animals in the European Union. The most important pathogenic species of Campylobacter in humans include *Campylobacter jejuni* and *coli* [19]. Important manifestations after this infection are Guillain-Barree syndrome and reactive arthritis. Listeria is also one of the bacteria that is widely distributed in the natural environment and its main sources are dust and soil, vegetables, animal excrement, water, sewage, and sludge [3, 4]. Listeria is in a group of aerobic bacteria, short bacilli, gram-positive, spore-free, motile, catalase-positive, and has 6 species. The main species that causes listeriosis in humans and animals is *Listeria monocytogenes*. Listeriosis is considered a foodborne disease and its manifestations in humans occur in the form of meningoencephalitis, sepsis, and abortion. Transmission of the disease in humans is through the consumption of milk, meat, vegetables, and generally contaminated food and water, inhalation of airborne bacteria, insects, direct contact with vector human feces and infected animal products [4, 20]. In 1997, Arva Nitoda et al. conducted a study in northern Greece to identify Listeria and Salmonella species in the surface water. In this study, 128 water samples were prepared from 4 rivers and 1 lake. The results showed that 5 samples (3.9%) were positive for the presence of *Listeria monocytogenes* [21], which was not consistent with the present study. In 2009, in a study by Kargar and Ghasemi, %91.7 of the isolated bacteria from the cheese samples, had the hly a gene, which was

consistent with the present study. In 2012, Shamloo Agakhani studied the prevalence of Listeria species in raw milk samples in Isfahan using the hly a gene. Out of 91 isolated samples, 4 samples were positive for *Listeria monocytogenes*. So, it was not consistent with the present study that the rate of *Listeria monocytogenes* is %94. In 2014, Momeni et al. studied the hly a gene frequency in fresh vegetables. In this study, which was performed on 145 samples of fresh vegetables in Shahrekord, %56 were positive for *Listeria monocytogenes* by culture and (%67) had hly a gene, which was consistent with the present study. Khalili et al. studied on 470 stool samples of patients with gastroenteritis referred to Nikopour Clinic in Yazd. They demonstrated that 16 cases (%5.6) were infected with *Campylobacter jejuni*. So, it was not consistent with the present study, which had %22.2 *Campylobacter jejuni* [22]. A study by Ali et al. in Pakistan found that %18 of diarrhea samples were infected with Campylobacter, which was almost consistent with the present study [23]. A study conducted in Thailand by Bodhidatta on 623 children with acute dysentery, *Campylobacter jejuni* was isolated as the most common microbial agent in %28 of patients. That was almost consistent with the present study [24]. Another study by Sari et al. in 2011 found that about 100 samples used in the study, %76 were positive by the hippurate test, while using the PCR method, %28 were positive [25]. This indicates that PCR is a better and more accurate method than traditional identification methods.

CONCLUSION:

Polymerase chain reaction (PCR) is a rapid and sensitive reaction to detect DNA as an indicator of the presence of a microorganism in very small amounts. This method has a higher sensitivity and specificity compared to other diagnostic methods, including culture, and unlike the culture method, there is no need for bacterial viability in the sample [16]. Traditional methods of identifying Campylobacter and Listeria based on culture medium generally take 4 days and to confirm bacterial species take 6-7 days (table 3). Therefore, the polymerase chain reaction in this study had high accuracy, speed, sensitivity, and specificity, so that it is not able to react with other bromatoxism organisms or microbial pathogens. On the other hand, it is done in less than 3 hours, which indicates the appropriate time to use it in laboratories (chart 1).

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