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Biochemical and Molecular Identification of *Listeria monocytogenes* and *Escherichia coli* in the Raw Milk Samples Delivered to the Dairy Farms in Golestan Province, Iran

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ABSTRACT

Background: Milk is an essential human nutrient, and the monitoring of its sanitation is vital during transportation and storage. The present study aimed to assess bacteriological contamination with *Escherichia coli* and *Listeria monocytogenes* in the raw milk samples of the dairy farms in Golestan province, Iran.
Methods: In total, 100 samples were collected from dairy farms in hot and cold seasons. The frequency of *E. coli* and *L. monocytogenes* was determined using biochemical tests and polymerase chain reaction (PCR).
Results: The biochemical tests indicated that 28% and 27% of the samples were contaminated with *E. coli* and *L. monocytogenes* based on the culture-dependent methods, respectively. In addition, 35 and 40 samples were contaminated with *E. coli* and *L. monocytogenes* based on PCR, respectively. PCR had higher sensitivity compared to the biochemical tests ($P < 0.05$). *E. coli* and *L. monocytogenes* contamination was significantly higher in traditional dairy farms than industrial dairy farms ($P < 0.05$). However, seasonal sampling and geographical region had no significant effects on the contamination load.
Conclusion: According to the results, *E. coli* and *L. monocytogenes* were highly frequent in the raw milks samples. However, the microbial loads had no significant differences in hot seasons and traditional dairy farms.

1. Introduction

Milk is an abundant source of essential nutrients, such as proteins, lipids, carbohydrates, vitamins, and minerals [1]. The consumption of milk and other dairy products has been on the rise in most regions across the world, especially in developing countries. It has been proven that raw milk has higher nutritional value compared to processed milk as it contains higher amounts of vitamins and minerals. Although milk is considered to be a wholesome meal and all the essential elements for growth, it is not safe from contamination with various microorganisms, such as *Escherichia coli* and *Listeria monocytogenes*. The microbial contamination of milk is associated with the severe health risks, threatening the life of the consumers [2].

Although most *E. coli* strains are saprophytic, some strains cause disorders in humans or animals [3]. Intestinal infections such as diarrhea, sepsis, urinary tract infection, and mastitis have been reported to be caused by *E. coli*. Furthermore, *E. coli* is the most common cause of travelers'

diarrhea [4].

L. monocytogenes is a gram-positive coccobacillus, which infects both normal and immunocompromised hosts and is frequently detected in the intestinal tract of animals and humans [4]. This microorganism could grow at wide temperature ranges, as well as refrigeration temperatures. In addition, *L. monocytogenes* could spread in the environment and is transmitted to the human body through the consumption of contaminated food [4]. This bacterium is a causative agent of listeriosis, which is observed in noninvasive and invasive forms. The noninvasive form often emerges as diarrhea and vomiting. The clinical complications caused by invasive listeriosis include sepsis, meningitis, meningoencephalitis, spontaneous abortions, stillbirth, premature labor, and neonatal diseases [5].

Microbial culture methods are mainly used to identify pathogenic microorganisms in raw milk samples. However, these methods are not sufficiently sensitive in the identification of contaminations. Today, use of molecular and serological diagnostic methods is increasing. Polymerase chain reaction (PCR) is a method based on the amplification of a specific sequence in the target genome with high specificity and sensitivity [6,7]. In the present study, both culture and PCR methods were used to detect *E. coli* and *L. monocytogenes* in raw milk samples.

As the use of traditional raw milk is increasing and raw milk has high potential for contamination with pathogens, which threatens human health, the present study aimed to investigate the contamination of raw milk with *L. monocytogenes* and *E. coli* using culture-independent (PCR) and dependent methods to compare the findings. Furthermore, the effects of various factors on the frequency of raw milk contamination were evaluated, including seasonal sampling, geographical region, and type of farms.

2. Materials and Methods

2.1. Sampling

In total, 100 raw milk samples were randomly collected from a wide area in Golestan province, Iran (34, 32, and 34 samples from the west, center, and east, respectively). The collection sites were traditional (48 samples) and industrial dairy farms (52 samples), and sampling was performed in the summer of 2017 (50 samples) and winter of 2018 (50 samples). Raw milk samples were collected in sterile conditions using sterile syringes, poured into 50-milliliter tubes, and transferred to the laboratory while preserved in ice [8].

2.2. Samples Culture and Biochemical Tests

Initially, a serial dilution was used to obtain the appropriate dilution for culturing. To do so, the samples were plated on *E. coli* and *L. monocytogenes* CHROMagar™ (Chromagar Company, Paris, France) for 24 hours. Afterwards, the *E. coli* cultures were incubated in aerobic conditions at the temperature of 37 °C, and the *L. monocytogenes* cultures were incubated in microaerophilic conditions in 10% carbon dioxide using a CO_2 incubator at the temperature of 30 °C. After 24 hours of incubation, suspected colonies were selected, and bacterial gram staining was carried out.

The biochemical tests included indole, methyl red, Voges-Proskauer (VP), citrate, and culturing on triple sugar iron agar (TSI), which were used to identify *E. coli*. Furthermore, catalase, oxidase, hemolysis, and Christie-Atkins-Christie-Munch-Petersen test (CAMP) were used to identify L. *monocytogenes* [8].

2.3. Polymerase Chain Reaction (PCR)

At this stage, genomic DNA was extracted directly from the samples using a DNA extraction kit (GeneAll Biotechnology, Seoul, Korea) in accordance with the instructions of the manufacturer. Afterwards, PCR was used to amplify the specific sequences of each bacterial DNA, and 16s rRNA and inlA were applied to identify *E. coli* and *L. monocytogenes,* respectively [9, 10]. Table 1 shows the primer sequences used in the study.

The PCR reaction (2× Mastermix Red; Ampliqon, Odense, Denmark) was performed at the final primer concentration of 500 nM) and template DNA of one microgram in the final volume of 50 microliters using a thermocycler (BioRad, California, USA). The PCR program for each of the genes is presented in Table 2. Finally, the PCR product was detected via agarose gel electrophoresis.

2.4. Statistical Analysis

Data analysis was performed in SPSS version 20, and the effects of the study variables (seasonal sampling, diagnostic methods, types of dairy farms, and geographical region) were assessed using generalized linear models at the significance level of $P < 0.05$. Moreover, the odds ratio (OR) was calculated to determine the significance of the associations between the variables.

3. Result and Discussion

3.1. The Frequency of Bacterial Contamination

The frequency of bacterial contamination is shown in Tables 3 and 4. According to the biochemical tests, 28 (28%) and 27 samples (27%) were contaminated with *E. coli* and *L. monocytogenes*, respectively. On the other hand, the results of PCR indicated that 35 (35%) and 40 samples (40%) were contaminated with *E. coli* and *L. monocytogenes* (Figure 1). The obtained results demonstrated a significant difference in the identification of *L. monocytogenes* ($P < 0.05$) between the biochemical tests and PCR, while the difference was not significant in the case of *E. coli* ($P > 0.05$).

Table 1: Primer sequence

Bacteria	Gene	Primer Sequence	Product Length	Reference
Escherichia coli	16srRNA	F: 5′-AGC ACT GAA TGA CGC CGG AAT TGA GAC A-3′ R: 5′-TCT GAG GGA CCT TAA TTT TCC CTG ATT CTC-3′	971	Miyamoto *et al.* (2002)[9]
Listeria monocytogenesis	inlA	F: 5′-AGCCACTTAAGGCAAT-3′ F: 5′-AGTTGATGTTGTGTTAGA-3′	760	Niederhauser *et al.* (1992)[10]

Table 2: PCR program used in this study

		E. coli	*L. monocytogenes*
Initial denaturation		2 min, 95 °C	5 min, 95 °C
30 Cycles	Denaturation	30 sec, 94 °C	45 sec, 94 °C
	Annealing	15 sec, 58 °C	45 sec, 58 °C
	Extension	60 sec, 68 °C	60 sec, 72 °C
Final Extension		10 min, 68 °C	5 min, 72 °C

In addition, PCR was observed to be more sensitive compared to the culture method in the identification of *E. coli* and *L. monocytogenes*, so that the OR of the samples contaminated with *E. coli* and *L. monocytogenes* in the culture method and PCR was estimated at 0.74 and 0.42, respectively.

In the studies by Vahedi et al. (2013), Asmahan Ali et al. (2011), and Fadaei et al. (2008), contamination of raw milk with E. coli was higher compared to the present study (42%, 63%, and 70%, respectively) [11-13], which demonstrated the variations in thecontamination of raw milk in various regions.

In addition, the mentioned studies evaluated raw milk contamination a few years ago, and advanced equipment have been introduced for the supply and transfer of raw milk ever since. In recent years, the better control and supervision in livestock farms have also resulted in the decreased rate of contamination as observed in the present study compared to the previous findings in this regard. Similar results have also been reported by Yarahmadi et al. (2006), indicating that the microbial load in raw milk samples has decreased in recent years compared to the past [14].

In the studies conducted by Fulya et al. (2011), Crump et al. (2002), and Murinda et al. (2002), the contamination rate of raw milk with E. coli was lower compared to our findings (10%, 13%, and 1.46%, respectively) [15-17], which could be attributed to the sanitary surveillance of the products. On the other hand, Murinda et al. (2002) reported that the raw milk samples in storage tanks were contaminated with animal faeces during the sampling process. Although the frequency of E. coli contamination based on PCR was higher compared to the biochemical tests in the current research, the difference in this regard was not considered significant ($P > 0.05$). However, Wang et al. (2014) and Omiccioli et al. (2009) claimed that molecular methods were more sensitive in the diagnosis of E. coli in dairy samples [18, 19]. In the present study, the rate of listeria contamination was estimated at 40%. As for the other studies in this regard, L. monocytogenes contamination of raw milk has been reported to be 25.3%, 6%, and 13.5% by Nero et al. (2008), Rahimian Zarif (2010), and Rahimi et al. (2010), respectively [20-22]. The differences in the findings may be due to the diagnostic methods applied to detect the bacteria and variations in geographical and climatic conditions [20].

Table 3: The frequency of *E. coli* contamination in different geographical area, dairy farm, and seasonal sampling

Dairy farm	Season	Culture (Positive)	PCR (Positive)
Industrial	Hot	6	10
	Cold	3	5
Traditional	Hot	10	12
	Cold	9	8
		Total: 28	Total: 35

Table 4: The frequency of *L. monocytogenes* contamination in different geographical area, dairy farm, and seasonal sampling.

Dairy farm	Season	Culture (Positive)	PCR (Positive)
Industrial	Hot	2	5
	Cold	5	7
Traditional	Hot	12	16
	Cold	8	12
		Total: 27	Total: 40

For instance, Nero et al. (2008) and Rahimian Zarifi (2010) employed biochemical methods, while in the study by Rahimi et al. (2010) these methods were used to detect *L. monocytogenes.* According to the results of the present study, diagnostic methods affect the detection of the microbial load, so that the odds of contamination diagnosis in biochemical methods has been estimated to be 0.42 higher compared to PCR ($P < 0.05$).

According to the results obtained by Thomas et al. (1991), Nogva et al. (2000), and Aslam et al. (2003), PCR could be used to identify *L. monocytogenes* in raw milk samples [23-25]. Furthermore, the aforementioned studies have demonstrated that PCR is able to detect small levels of *L. monocytogenes* contamination in raw milk. Consistent with our findings, Aznar and Alarcón (2003) have reported that PCR has higher ability to identify L. monocytogenes in raw milk samples compared to biochemical methods [26].

3.2. Effect of Geographical Area on Raw Milk Contamination

In the current research, the E. coli contamination rate of the raw milk samples collected from the east, center, and west of the study area was estimated at 11%, 14%, and 10%, respectively. However, no significant difference was observed between the areas in this regard ($P > 0.05$). On the other hand, *L. monocytogenes* contamination in the samples collected from the east, center, and west was determined to be 16%, 11%, and 13%, respectively, and no significant difference was observed between the areas in this regard ($P > 0.05$). In contrast with the results of the present study, Asmahan Ali et al. (2011) and Fedaie et al. (2008) reported a significant difference in the contamination rate of raw milk with *E. coli* within various geographical regions in Charmahal-Bakhtiari province (Iran) and Khartoum states (Sudan), respectively [12, 13].

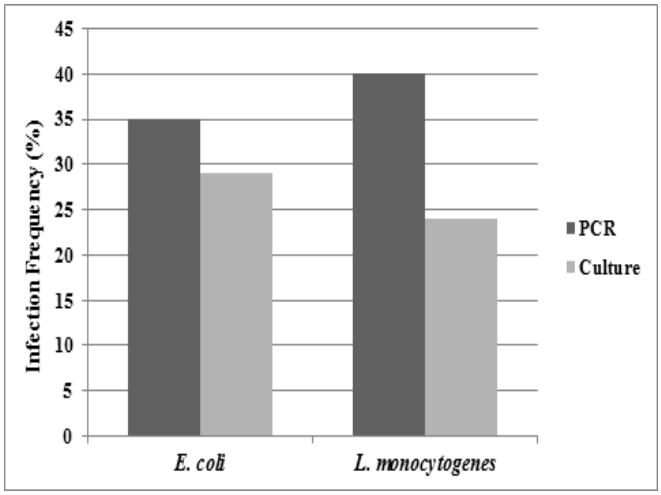

Figure 1: Raw milk contamination with E. coli and L. monocytogenes based on diagnostic methods

In the study by Rahimian Zarif (2010), the contamination rate of raw milk with *L. monocytogenes* was investigated in various cities in Kurdistan province (Iran) [21]. Inconsistent with our findings, the mentioned study indicated a significant difference in the contamination rate of *L. monocytogenes* in various regions of the province. In some cities, the contamination rate was 30%, while no contamination was observed in the other areas. In the present study, the division was carried out based on city, and 10 cities and 10 dairy farms were evaluated in each city. It seems that in the study by Rahimian Zarif (2010), the sample size in each city was not sufficient, which significantly affected the obtained results.

3.3. Effect of Seasonal Sampling on Raw Milk Contamination

According to the current research, *E. coli* contamination rate was 13% in the samples collected in cold seasons, while the rate reached 22% in the samples collected in hot seasons. In fact, the odds of *E. coli* contamination in the samples collected in cold seasons was 0.56 higher compared to hot seasons, while the difference in this regard was not considered statistically significant ($P > 0.05$). In the case of *L. monocytogenes*, the raw milk contamination rate was 19% and 21% in cold and hot seasons, respectively. However, no significant difference was observed between the samples in this regard ($P > 0.05$; OR: 0.72).

According to the results of the present study, the odds of raw milk contamination with *E. coli* were lower in cold seasons compared to hot seasons (OR: 0.56), while the difference in this regard was not significant ($P > 0.05$). Several studies have indicated that the risk of raw milk contamination by microorganisms is higher in hot seasons [11, 13, 27]. Considering that the temperature of 37°C is the optimal growth temperature for *E. coli*, it seems that the environmental and climatic conditions are appropriate for the growth of bacteria in raw milk in hot seasons, which in turn led to the higher rate of contamination in the samples. However, no significant difference was denoted in raw milk contamination with *L. monocytogenes* between cold and hot seasons. *L. monocytogenes* not only grows at the temperature of 37 °C, but it also could grow at lower temperature (e.g., 4 °C). Some studies have reported the rate of *L. monocytogenes* contamination in raw milk samples to be higher in cold seasons [28].

3.4. Effect of Dairy Farms on Raw Milk Contamination

Among 100 raw milk samples, 20 cases were contaminated with *E. coli*, which were collected from traditional dairy farms. Moreover, 15 contaminated samples were collected from industrial farms. Therefore, it could be concluded that the odds of contamination in traditional farms was 2.23 times higher than industrial farms, which was considered statistically significant ($P < 0.05$).

According to the results of the present study, 28 samples contaminated with *L. monocytogenes* were obtained from traditional dairy farms, and 12 cases were collected from industrial farms. Therefore, it could be inferred that the odds of contamination in traditional dairy farms was 3.8 times higher than industrialized dairy farms, which was considered statistically significant ($P < 0.05$).

The findings of the current research demonstrated the higher contamination rate of raw milk in traditional dairy farms compared to industrialized farms, which could be mainly due to the increased microbial load in traditional dairy farms compared to industrialized farms as more advanced equipment are available in various stages of supplying and transferring raw milk in the latter. These equipment reduce the use of hands during milk supply, thereby remarkably decreasing the risk of contamination.

4. Conclusion

According to the results, the contamination rate of raw milk with *E. coli* and *L. monocytogenes* in Golestan province dairy farms was relatively high. Although geographical area has no effect on the microbial load of the samples within the province, the contamination rate was higher in hot seasons in the samples collected from traditional dairy farms. Since the PCR is more sensitive in the identification of *E. coli* and *L. monocytogenes* in raw milk, it seems that such molecular methods could provide more accurate data compared to the conventional methods in laboratories and supervisory agencies. Since the rate of raw milk contamination was higher in traditional farms compared to industrialized farms, it is proposed that PCR be used to monitor the contamination of raw milk in traditional dairy farms. Furthermore, it is essential to monitor traditional dairy farms to raise their health level, while also educating and encouraging livestock breeders to industrialize these farms. As the rate of *E. coli* contamination was higher in hot weather, controlling raw milk contamination is recommended in hot seasons. Furthermore, the consumers of raw milk must be urged to heat and sterilize the product before use.

Authors' Contributions

M.Kh., and M.R., designed the manuscript, M.R., and S.K., carried out the experiments, M.E., performed the statistical analysis, and M.R., drafted the manuscript. All the authors read and approved the final manuscript.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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