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Molecular Epidemiology Of Mycobacterium Avium Subspecies Paratuberculosis In The Milk Of Animal Species

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Abstract

Mycobacterium subspecies paratuberclosis is an important zoonotic pathogen responsible for Johne's and Crohn's diseases in animals and humans population, respectively. Foods with animal origins may act as reservoirs of M. avium subsp. paratuberclosis. The present survey was done to assess the molecular distribution of M. avium subspecies paratuberculosis in the milk of cow, sheep, goat, donkey, buffalo, and camel species. In this study, a total of 300 raw milk samples were collected from Chaharmahal Va Bakhtiary and Isfahan province, Iran. Samples were subjected to DNA extraction. Extracted DNA was assessed for the M. avium subspecies paratuberculosis genome using the Polymerase Chain Reaction. Thirty out of 300 (10%) raw milk samples were positive with M. avium subsp. paratuberculosis. Raw buffalo milk samples harbored the highest contamination rate of M. avium subsp. paratuberculosis (17.50%), while raw donkey milk samples harbored the lowest (5%). Total prevalence of M. avium subspecies paratuberculosis amongst the raw milk samples collected from Isfahan and Chaharmahal Va Bakhtiary province were 66.66% and 33.33%, respectively. Statistically significant differences were obtained between types of samples and bacterial distribution ($P < 0.05$) and also geographic area of sampling and bacterial distribution ($P < 0.05$). Role of raw milk, particularly raw buffalo milk samples as reservoir of M. avium subsp. paratuberculosis have been determined. Using pasteurized and sterilized milk will decrease the risk of M. avium subsp. paratuberculosis.

Keywords: Mycobacterium avium subspecies paratuberculosis, Molecular epidemiology, Prevalence, Raw milk, Iran.

Introduction

Milk of animal species is a rich source of proteins, minerals, fats, vitamins and several supplementary and essential materials for the human health (1-5). Its consumption has diverse health benefitis in human, especially in neonate and elder people (6). In some parts of the world, raw milk is consumed routinely (7). They mainly didn't consume pasteurized or setrilized milk. In these cases, there is a risk of the occurrence of foodborne diseases due to the consumption of raw milk and meat (8-17).

Mycobacterium avium subspecies paratuberculosis (M. avium subsp. paratuberculosis) is the causative agents of Johne's disease (JD), which is a chronic gut infection progressively developed to complicated diseases, including prolonged diarrhea, poor digestion, and excessive weight loss (18). JD is mainly occurred in ruminants animals that provide milk and/or meat for human consumption, particularly cow, sheep, deer, goat, llamas, bison, and elk (19). Ruminants infected with M. avium subsp. paratuberculosis

excrete the bacterium in their feces and milk. In fact, M. avium subsp. paratuberculosis is excreted into the milk within the udder and also through fecal contamination during milking (20).

M. avium subsp. paratuberculosis also may be involved in Crohn's disease and type 1 diabetes in humans. However, the M. avium subsp. paratuberculosis role in human diseases has not been determined well, minimizing the exposure of humans to the organism is considered desirable as a precautionary measure (21). In this regard, the role of foods with animal origins, particularly milk as a reservoitrs of M. avium subsp. paratuberculosis have been estimated (22). Infected ruminants may shed M. avium subsp. paratuberculosis in milk, and the bacterium can become disseminated (23). As a result, M. avium subsp. paratuberculosis presence in raw milk and in natural waters is probable, but the M. avium subsp. paratuberculosis numbers in milk and waters should be decreased by bioling. Thus, the highest risk of infection through milk consumption is in communities that consume raw milk.

According to the hiogh importance of M. avium subsp. paratuberculosis and high consumption rate of raw milk in some parts of the world, the present survey was done to assess the molecular epidemiology of M. avium subsp. paratuberculosis in raw milk samples of cow, sheep, goat, buffalo, camel, and donckey species in Iran.

Materials and methods

Samples

A total of three-hundred raw milk samples, including cow (n= 80), goat (n= 60), sheep (n= 50), buffalo (n= 40), donkey (n= 30), and camel (n= 40) samples were collected from Chaharmahal va Bakhtiary and Isfahan province, Iran. Animal species were clinically healthy and their milk samples showed normal properties. Fiftyy mililiters per each animal case where collected using a sterile tubes. Initial dropletes of raw milk samples were thrown away and the samples were taken from the middle stream part of the milk. Samples were trasnferred to laboratory at 4 °C.

DNA extraction and analysis

Genomic DNA was extracted from the raw milk of animal species using the DNA extraction kit (Dneasy tissue kit, Qiagen, Hilden, Germany). Before DNA extraction, raw milk samples were centrifuged (1000 g, 15 min) and the supernatant discarded. The resultant pellet was washed using phosphate buffer saline (PBS, pH 7.3, Merck, Germany) and centrifuged again (500 g, 15 min). The pellet was suspended in 1 mL of PBS, centrifuged and resuspended in 100 mL of 0.2 N NaOH. In the initial procedure, the enzymatic digestion was done. For this purpose, lysozyme buffer (lysozyme (18 mg/ml), Tris–HCl pH 8.0 (15 mM), EDTA (1 mM), and Triton X100 (1%)) and proteinase K (Qiagen, Hilden, Germany) were applied. According to the manufacture's instruction, the procedure of the DNA extraction was done.

Quality assessment of DNA samples

The purity (A260/A280) and concentration of the extracted DNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA) (24-28). Furthermore, the DNA's quality was assessed on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany) (29-33).

Polymerase Chain Reaction (PCR)

To identify the presence of M. avium subspecies paratuberculosis amongst the DNA extracted from raw milk samples, the PCR was used. For this purpose, a set of primer (F: 5'-GTTCGGGGCCGTCGCTTAGG-3' and R:5;-GAGGTCGATCGCCCACGTGA-3' (400 bp)) was used (34). The reaction was done using 50 µl volume containing 2.0 mM MgCl2, 60 mM Tris-HCl buffer (pH 8.8), 10 pmol of each of the primers, 0.2 mM of each of the four dNTPs (Thermo Fisher Scientific, St. Leon-Rot, Germany), 0.5 U Taq DNA polymerase, and 5 mL DNA. A positive control (Positive DNA of M. avium subsp. paratuberculosis, 250 fg), and a negative control (PCR-garde water) (Thermo Fisher Scientific, St. Leon-Rot, Germany), were also used in each reaction. A programmable DNA thermocycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions. Amplication of products were done at 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and a final 1 cycle of 72°C for 5 min.

Gel electrophoresis

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in a 2.5% agarose gel stained with 0.1% ethidium bromide (0.4 µg/ml) (35, 36). Besides, UVI doc gel documentation systems (Grade GB004, Jencons PLC, London, UK) were used to analyze images (37, 38).

Statistical analysis

Data analysis was performed by SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA) (39). Chi-square and Fisher's exact two-tailed tests were performed to assess any significant relationship between the M. avium subspecies paratuberculosis prevalence amongst the examined samples (40). Besides, P-value < 0.05 was considered statistically significant (41).

Results

The present survey was done to assess the molecular distribution of M. avium subspecies paratuberculosis in the milk of cow, sheep, goat, donkey, buffalo, and camel species. Figure 1 shows the PCR amplification gel electrophoresis of M. avium subspecies paratuberclosis.

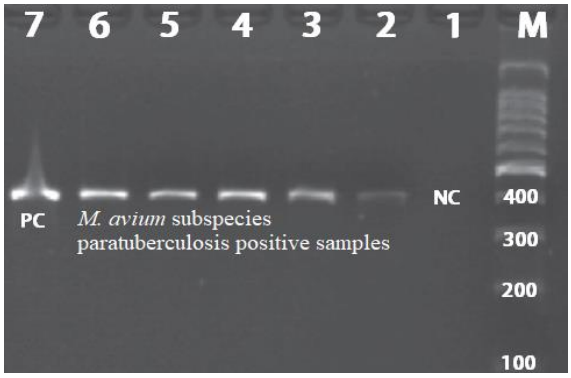

Figure 1. M. avium subspecies paratuberculosis prevalence amongst the collected raw milk samples. M: Ladder 100 bp, 1: Negative control, 2-6: Positive samples ofr the bacteria, and 7: Positive control.

Table 1 shows the M. avium subspecies paratuberculosis prevalence amongst the collected raw milk samples. Thirty out of 300 (10%) raw milk samples were contaminated with M. avium subspecies paratuberculosis. Raw buffalo milk samples harbored the highest rate of contamination with M. avium subspecies paratuberculosis (17.50%), while raw donkey milk samples harbored the lowest (5%). Statistivcally, significant differences were obtained between type of raw milk samples and M. avium subspecies paratuberculosis distribution (P <0.05).

Table 1. M. avium subspecies paratuberculosis prevalence amongst the collected raw milk samples.

Types of raw milk samples	N. collected	N. positive for *M. avium* subspecies paratuberculosis (%)
Cow	80	8 (10)
Sheep	50	6 (12)
Goat	60	4 (6.66)
Donkey	30	1 (5)
Buffalo	40	7 (17.50)
Camel	40	4 (10)
Total	300	30 (10)

Figure 2 shows the M. avium subspecies paratuberculosis prevalence based on the geographical area of sampling procedure. Accoring to obtained data, total prevalence of M. avium subspecies paratuberculosis amongst the raw milk samples collected from Isfahan and Chaharmahal Va Bakhtiary province were 66.66% and 33.33%, respectively. Statistivcally, significant differences were obtained between geographical area of sampling and M. avium subspecies paratuberculosis distribution (P <0.05).

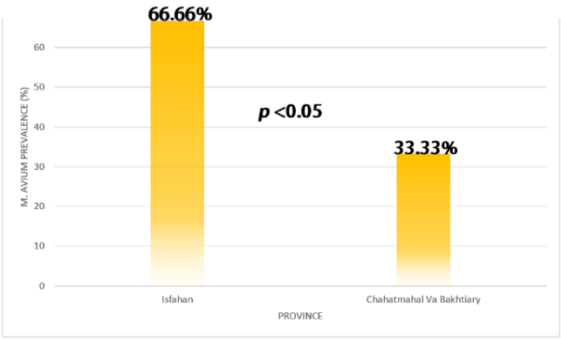

Figure 2. M. avium subsp. paratuberculosis prevalence based on the geographical area of sampling procedure.

Discussion

The present survey was aimed to assess the PCR-based prevalence of M. avium subsp. paratuberculosis amnongst the raw milk samples of animal species. Findings showed that the M. avium subsp. paratuberculosis prevalence amongst the raw cow, sheep, goat, donkey,

buffalo and camel milk samples were 10%, 12%, 6.66%, 5%, 17.50%, and 10%, respectively. Geographical distribution was also recorded with the high prevalence in Isfahan province.

As diverse kinds of animal species, particularly cow, sheep, goat, deer, and other ruminants may be the source of M. avium subsp. paratuberculosis dessimination in the nature, close contact of other species such as buffalo, donkey and camel with them may be the main reason for the presence of bacteria in their milk samples. Additoionally, some kinds of wild ruminants, such as deer, llamas, bison, and elk may also be reservoirs of M. avium subsp. paratuberculosis. Thus, contact of domestic animals with wild ruminants or even their infected samples, such as feces and milk, may dessiminate the infection in the environment. According to the high prevalence of M. avium subsp. paratuberculosis in examined raw milk samples, consumption of these types of milk may cause severe Crohn's disease in human population. Thus, well boiling of raw milk samples before consumption and also consumption of pasteurized and sterilized milk may decrease the risk of M. avium subsp. paratuberculosis in the community.

Scarce studies have been focused on the detection of M. avium subsp. paratuberculosis in raw milk of animal species. Shirvani et al. (2019) (42) reported that the M. avium subsp. paratuberculosis prevalence amongst the raw bovine, ovine, caprine, camel, buffalo and donckey milk samples were 15.83, 10, 8.18, 9.41, 25, and 6.66%, respectively. According to previous meta-analysis review, the M. avium subsp. paratuberculosis amongst the dairy farms of Iran, USA, Austrlia, Mexico, Czech Republic, Cyprus, Argentina, Denmark, Brazil, Switzerland, and Germany ranged between 0.0% to 0.80% (43). In Colombia (44), an apparent M. avium subsp. paratuberculosis herd-level prevalence was 4.1% (12/292; 95% CI: 1.8–6.4). In Canada (45), an apparent M. avium subsp. paratuberculosis cow level prevalence was 1.7% and 2.6% on the milk and serum ELISA, respectively. In Italy (46), 155 out of 780 dairy herds (19.9%) were found positive by ELISA and/or real time PCR. The estimated prevalence of M. avium subsp. paratuberculosis amongst the milk samples varies from region to region between 2.8 and 5.5%. In Switzerland (47), 4.2% of the raw milk cheese samples tested positive for M. avium subsp. paratuberculosis with the F57-based real-time PCR. Another survey in Canada (48) showed that 8.1, 1.2 and 2.0% of cattle were positive for M. avium subsp. paratuberculosis using IS900 qPCR, F57 qPCR and bacterial culture, respectively. Furthermore, 14% of collected environmental samples, but no dust samples, were test-positive for M. avium subsp. paratuberculosis.

Such a large variation across countries in M. avium subsp. paratuberculosis prevalence rate from meat and animal carcasses may show real regional differences or may be affected by the use of various detection techniques. Additional developments in the M. avium subsp. paratuberculosis detection techniques in foods are desirable.

Conclusion

Role of raw milk of animal species, particularly raw cow, sheep, goat, donkey, buffalo and camel milk samples were determined as reservoirs of M. avium subsp. paratuberculosis. This matter will be an important public health threat regarding the consumption of raw milk of animal species. Increase knowledge about the epidemiology of M. avium subsp. paratuberculosis in foods with animal origins will decrease the risk of M. avium subsp. paratuberculosis expansion in the community.

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