ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (1) 21 - 28
doi: 10.30466/vrf.2022.544308.3312
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Identification of *Salmonella* carriers by amplification of *FimA*, *Stn* and *InvA* genes and bacterial culture methods in fecal samples of buffalo

Abdulghaffar Ownagh[1]*, Navid Etemadi[2], Peyman Khademi[1], Hossein Tajik[3]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] DVM Graduate, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Article Info

Article history:

Received: 05 December 2021
Accepted: 29 August 2022
Available online: 15 January 2023

Keywords:

Feces
Ruminants
PCR
Salmonellosis
Virulent genes

Abstract

Salmonellosis is one of the most important bacterial diseases in human and animals. Rapid diagnosis and sub sequence accurate treatment of *Salmonella* carriers help reduce the salmonellosis in human and livestock animals. In this study, 420 fecal samples were taken during year 2019 from buffalo in the Urmia, Khoy and Piranshahr regions in west Azerbaijan province, Iran. Samplings were carried out in different seasons. Presence of *Salmonella* invasion genes (*FimA*, *Stn* and *InvA*) were evaluated by polymerase chain reaction. The bacterial culture and biochemical tests were performed on feces samples for isolation of bacterium *Salmonella*; however, all samples were negative in culture method. PCR findings showed that, 50 (11.90%) fecal samples were positive to the genes. The analysis of results showed that frequency of salmonellosis outbreak in different parts of west Azerbaijan province followed a similar pattern and the incidence of salmonellosis according to forecast in the warm seasons (spring and summer) was more than in cold seasons (autumn and winter). The prevalence of *Salmonella* in buffalo's feces based on warm and cold seasons were 32 (64.00%) and 18 (36.00%), respectively. The results showed significant difference between cold and warm season in the prevalence of salmonellosis. Therefore, the application of molecular technics is essential for the prevention and treatment of salmonellosis. The results also showed that specificity of PCR method was better than culture method for detection of *Salmonella* in feces sample.

Introduction

Salmonella spp. is gram-negative, anaerobic and toxin producing that belongs to the *Enterobacteriaceae* family.[1] Salmonellosis is one of the most important zoonotic infectious diseases in humans and animals.[2] The disease is usually associated with miscarriage, enterocolitis with diarrhea and foul-smelling and watery diarrhea followed by septicemia and death. Salmonellosis is usually associated with active and inactive carriers of the bacterium.[3] The role of carriers in the spread of infection is very important. Excoriation of *Salmonella* through feces has reported to lead to contaminate the environment, water, food and a wide range of human and animal populations.[4]

To date, 2,700 *Salmonella* serotypes have been reported with *S. typhimurium* being more important than the rest.[5] Food-borne diseases caused by non-typhoid *Salmonella* are a major public health problem worldwide.[6] In underdeveloped countries, more than one billion intestinal infections and up to 5.00 million deaths are reported annually.[7] Controlling the spread of *Salmonella* infection requires rapid detection, treatment of infected animals and separation of infected animal from others. However, different *Salmonella* servers may differ in their severity.[8] however, it means that all *Salmonella typhimurium* and *S. enterica* are pathogenic.[9] In particular, it is considered as the cause of human salmonellosis.[10] In southeast Asia, *S. enteric* is on the rise. The infectious dose of *Salmonella* for pathogenicity in the host is usually 10^6 to 10^8 CFU.[11] In the past, culture methods and biochemical tests were basically used to diagnose *Salmonella.* [12] However, due to the low efficiency and time consuming of these methods, nowadays molecular and serological methods have replaced.[13]

In the present study identification of *Salmonella* was confirmed by detection of different genes (*FimA, Stn and InvA*) by polymerase chain reaction.[14,15] The gene *InvA*

***Correspondence:**

Abdulghaffar Ownagh. DVM, DVSc
Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: a.ownagh@urmia.ac.ir

invading *Salmonella* is present in *Salmonella* isolates and is responsible for invading the epithelial tissue of the human and animal intestines.[16] The *Stn* gene is toxic to intestinal epithelial cells and leads to intestinal disorders. The reason for selecting this gene is it's approximately 99.00% abundance in different *Salmonella* serotypes. This gene is not only specific for *Salmonella* but is present in all pathogenic *Salmonella.*[17,18] In this study, feces sample were used to diagnose salmonellosis in buffaloes. The use of stool samples is important not only for the diagnosis of salmonellosis but also for diagnosis of carriers of this bacterium.

The aim of this study was to investigate the prevalence of *Salmonella* carriers in buffaloes of west Azerbaijan province. The Buffalo's meat and milk products are playing important role in food production in west Azerbaijan province. Therefore, this livestock can play an important role in spreading the prevalence of salmonellosis in urban and rural communities as well as other livestock herds. Also, sampling was done in different seasons of the year and in different parts of the province to determine the prevalence of salmonellosis seasonally and geographically. Also, in this study, the sensitivity of PCR method was compared to culture method.

Materials and Methods

Sampling. In 420 buffaloes 15.00 gr of feces sample were taken from each animal by sterile gloves during 2019 in Urmia, Khoy and Piranshahr regions, West Azerbaijan province, Iran (140 samples from each region). Also, these samples were taken in different seasons of the year so that 70 samples were taken in warm season (spring and summer) and 70 samples in cold season (autumn and winter). Samples were collected directly during defecation and placed in sterile packages in plastic bags. The samples were transferred to the microbiology laboratory in the Faculty of Veterinary Medicine, Urmia University and kept at – 20.00 ˚C until to onset of culture and molecular methods.

Isolation of *Salmonella* by culture method. Each sample (5.00 g) was transferred to test tubes containing 4.00 mL of selenite F Broth (Merck, Darmstadt, Germany) enrichment solution. After mixing with vortex, the tubes were incubated at 37.00 ˚C for 24 hr. Then, samples were cultured on Salmonella-Shigella agar (Merck). The suspected colonies were tested biochemically on urea Broth, peptone water, sulfur-indole-motility, citrate and triple sugar iron media (Merck). The growth of bacterium on culture medium was evaluated after 24 hr incubation at 37.00 ˚C.[19]

DNA extraction for detection of *Salmonella* by PCR method. Enriched feces samples in selenite F medium were removed from the incubator and vortexed for about a few seconds to homogenize. Then 500 µL of enriched samples were transferred to a micro centrifuge (Eppendorf, Hamburg, Germany) tube with of 500 µL DNase-RNase (DNA Zist Asia, Mashhad, Iran) free distilled water. The cell suspension was centrifuged (Sigma, Ostode am Hrz, Germany) for 10 min at 14,000 *g*. The supernatant was discarded carefully. The pellet was suspended in 300 µL of DNase-RNase free distilled water (DNA Zist Asia) by vortexing (Fanavaran Sahand Azar, Tehran, Iran). The tube was centrifuged at 14,000 *g* for 5 min and the supernatant was discarded carefully. The pellet was suspended in 200 µL of DNase-RNase-free distilled water by vortexing. The micro centrifuge tube was incubated for 15 min at 100 ˚C and immediately chilled on ice. The tube was centrifuged for 5 min at 14,000 *g* at 4.00 ˚C. The supernatant was carefully transferred to a new micro centrifuge tube (Eppendorf) and incubated again for 10 min at 100 ˚C and chilled immediately on ice. An aliquot of 5.00 µL of the supernatant was used as the template DNA in the PCR (http://www.pcr.dk/DNA-purification.htm).

Primers and PCR. After making sure that the primers (NCBI) were suitable, they were sent to Pishgam Co. (Tehran, Iran) for synthesis. For the molecular detection of *Salmonella* PCR targeting the *16S rRNA* and transposon (*InvA*, *FimA* and *Stn*) genes were employed. The primers for the PCR used in this study were previously described by Revolledo and Ferreira, and Quan *et al.*[14,20] To perform the PCR, Taq DNA Polymerase Master Mix RED (Amplicon, Sten huggervej, Denmark) was used. The PCR reaction was carried out in 25.00 µL volume comprising 5.00 µL of extracted DNA, 50.00 pmol of each primer (*InvA*-F-R, *FimA*-F-R, and *Stn*-F-R), 12.50 µL of master mix. The PCR thermal programs were set on the thermal cycler device (Quanta Biotech, Byfleet, UK). During PCR, Water DEPC-Treated (California, Santa Cruz, USA) was used as negative control of extraction. *S. typhimurium* (ATCC 1730) was used as positive control of DNA amplification procedure. Time and temperature for amplification of the selected genes in PCR method were detailed as follow: *InvA* gene, the first denaturation was performed in 94.00 ˚C for about 3 min, 35 temperature cycles including second denaturation fulfilled in 94.00 ˚C for about 30 sec. and annealing was performed in 53.00 - 69.30 ˚C for about 30 sec and elongation in 72.00 ˚C for about 30 sec. Final elongation was carried out in 72.00 ˚C for about 3 min. For *Stn* gene the first denaturation was performed in 94.00 ˚C for about 3 min, 35 temperature cycles including second denaturation fulfilled in 94.00 ˚C for about 30 sec and annealing was performed in 55.00 ˚C for about 30 sec and elongation in 72.00 ˚C for about 30 sec. Final elongation was carried out in 72.00 ˚C for about 10 min. For *Fim* gene the first denaturation was performed in 94.00 ˚C for about 3 min, 45 temperature cycles including second

denaturation fulfilled in 94.00 ˚C for about 1 min and annealing was performed in 55.00 ˚C for about 30 sec and elongation in 72.00 ˚C for about 1 min. Final elongation was carried out in 72.00 ˚C for about 10 min. Eventually, in order to replicate the *16S rRNA* gene the following steps were taken: The first denaturation was performed in 95.00 ˚C for about 5 min, 35 temperature cycles including second denaturation fulfilled in 94.00 ˚C for about 1 min and annealing was performed in 60.00 ˚C for about 30 sec and elongation in 72.00 ˚C for about 1 min Final elongation was carried out in 72.00 ˚C for about 10 min. The presence of 284, 260, 85 and 660 base pairs indicated the presence of *InvA*, *Stn*, *FimA* and *16S rRNA* genes, respectively. Moreover, primer sequences were as follow: *InvA* gene (F-5- GTGAAATTATCGCCACGTTCGGG CAA-3, R-5- TCATCGCA CCGTCAAAGGAACC-3), *Stn* gene (F-5- CTTTGGTCGTAAAATAAGGCG-3, R-5- TGCCCAAAG CAGAGAGATTC-3), *FimA* gene (F-5-CCTTTCTCCATCGTC CTGAA-3, R-5- TGGTGTTATCTGCCTGACCA3) and *16S rRNA* gene (F-5- GGAACTGAGACACGGTCCAG-3, R-5- CCAGGTAAGGTTCTTCGCGT-3).[14,19]

Electrophoresis of PCR products. The PCR products (*16S rRNA, InvA* and *Stn* genes) were electrophoresed on a 1.50% agarose gel containing safe stain and then visualized using Ingenius Gel Documentation (Syngene Bio Imaging, Cambridge, UK). The PCR product for *FimA* gene was electrophoresed in 3.00% agarose gel.

Sequencing of DNA isolates. For confirmation of *Salmonella* genus, two samples of the isolated DNA were sent to the Pishgam Company, Tehran, Iran.

Results

The results showed that isolation of *Salmonella* in culture method was negative in all samples. In contrast, PCR method indicated the presence of *Salmonella* genes (*16S rRNA, InvA*, *Stn* and *FimA* in 50 samples (11.90%). The number of positive samples in Urmia, Khoy and Piranshahr were 14 (28.00%), 20 (40.00%) and 16 (32.00%), respectively. The analysis of positive results showed that out of 16 positive samples in Piranshahr, 10 (62.50%) samples were belonged to warm season and 6 (37.50%) samples were related to cold season. Also, in Urmia and Khoy regions, the frequencies of positive samples in warm season were 8 (57.10%) and 14 (70.00%), respectively, and in cold season were 6 (40.90%) and 6 (30.00%), respectively. In whole samples, the frequencies of positive samples in the warm and cold seasons of the year were 32 (64.00%) and 18 (36.00%), respectively. All positive samples were belonged to buffaloes whose fecal consistency had a normal appearance and did not show clinical signs of salmonellosis. In Figures 1, 2, 3, and 4, the PCR results based on *FimA*, *Stn, InvA* and *16S rRNA* genes of the samples are shown on gel electrophoresis.

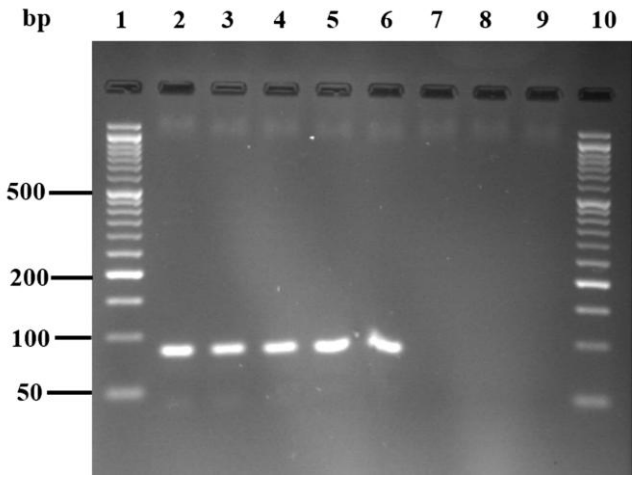

Fig. 1. Agarose gel image of amplified fragment of *Salmonella FimA* gene (85 bp) using PCR. Lanes 1, and 10: 50 bp molecular ladder (Smobio Technology Inc., Hsinchu, Taiwan), Lane 2: Positive control (*S. typhimurium* ATCC 1730), Lanes 3, 4, 5, and 6: Positive samples to *Salmonella*, Lanes 7, and 8: Negative samples to *Salmonella*, Lane 9: Negative control.

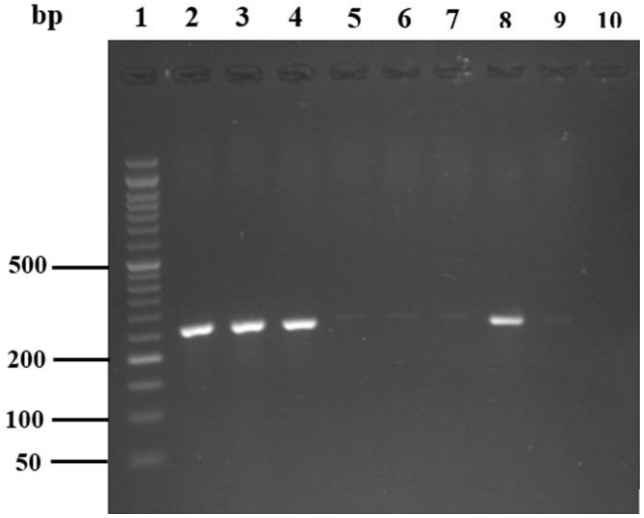

Fig. 2. Agarose gel image of amplified fragment of *Salmonella Stn* gene (260 bp) using PCR. Lane 1: 50 bp molecular ladder (Smobio Technology Inc.), Lane 8: Positive control (*S. typhimurium* ATCC 1730), Lanes 2, 3, and 4: Positive samples to *Salmonella*, Lanes 5, 6, 7, and 9: Negative samples to *Salmonella*, Lane 10: Negative control.

Phylogenetic analysis of *Salmonella* species *InvA*, and *16S rRNA* genes sequences of two isolates of DNA obtained from animals showed that they were belonged to the genus *Salmonella* and all of them were closely related to each other with less significant genetic distance (99.9:100) and less significant branches. The similarity between the Iranian isolates and other sequences obtained from the gene bank was slightly different and this might be attributed to the differences of the host, source and locality. Depending on the obtained results, phylogenetic

analysis and testing of the genetic distances, the Iranian *Salmonella* isolates from animals were closely related. The sequences were registered in NCBI with the accession numbers *ON954830* and *ON968482*).

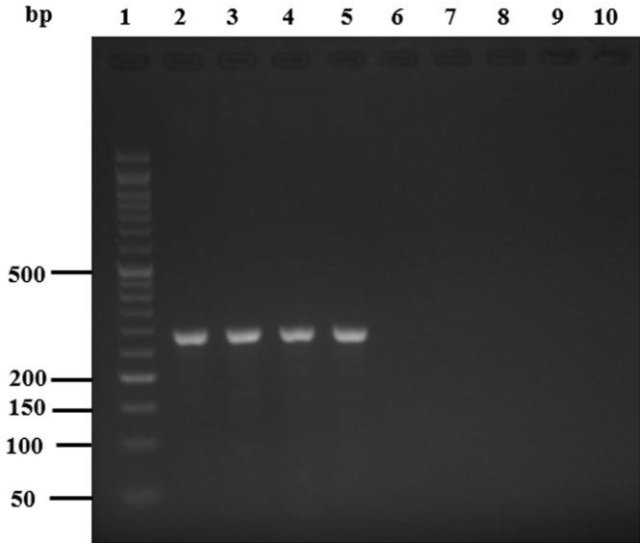

Fig. 3. Agarose gel image of amplified fragment of *Salmonella InvA* gene (284 bp) using PCR. Lane 1: 50 bp molecular ladder (Smobio Technology Inc.), Lane 2: Positive control (*S. typhimurium* ATCC 1730), Lanes 3, 4 and 5: Positive samples to *Salmonella*, Lanes 6, 7, 8 and 9: Negative samples to *Salmonella*, Lane 10: Negative control.

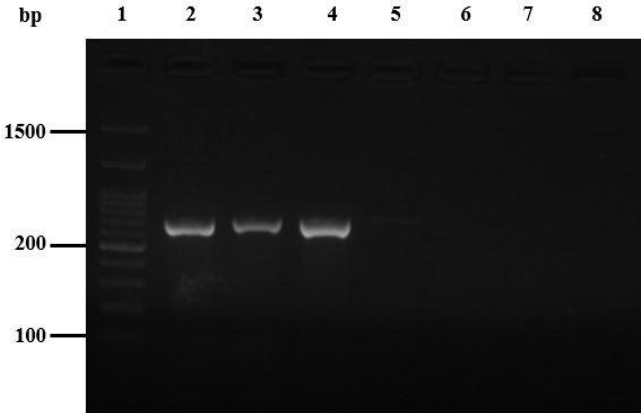

Fig. 4. Agarose gel image of amplified fragment of *Salmonella 16S rRNA* gene (660 bp) using PCR. Lane 1: 50 bp molecular ladder (Smobio Technology Inc.), Lane 2: Positive control (*S. typhimurium* ATCC 1730), Lanes 3, 4, and 5: Positive samples to *Salmonella*, Lanes 6 and 7: Negative samples to *Salmonella*, Lane 8: Negative control.

Discussion

Salmonellosis is a common disease of humans and animals and is economically important in the world. Livestock and livestock products are the most important reservoir of bacteria to human being.[21] Therefore, the diagnosis of salmonellosis in livestock is very important. The common habitant of *Salmonella* is the gastrointestinal tract. The carrier animals excrete bacterium through the feces daily. Usually, these animals have no obvious clinical symptoms; therefore, it can be important as the public health.[22] Ruminants such as buffalo up to 10 weeks and horses up to fourteen months can excrete bacteria to environment. This long period excretion leads to the contamination of the environment and the creation of new hosts.[22] Therefore, in salmonellosis, asymptomatic carriers are more important than infected animals with clinical symptoms. Prompt diagnosis and subsequent appropriate treatment of carriers are very important in preventing the further spread of salmonellosis and its risk.[23] There are various methods for diagnosing *Salmonella* including microbial culture and using various biochemical tests, immunological and serological methods as well as the detection of nucleic acid of *Salmonella* in suspected sample.[24] Long-standing methods of diagnosing *Salmonella* in feces or food are slow and time consuming.[25] However, the polymerase chain reaction method is a sensitive and specific method that relies on an effective gene present in all *Salmonella* strains and specific to this genus such as the *InvA* gene, effective in attacking and invading *Salmonella*. In recent years, promising results have been shown in the diagnosis of *salmonella* in cattle, dogs and horses.[26]

In the present study, *Salmonella* species were isolated at the same from from buffaloes. On the contrary, higher infection rate was reported in buffaloes (5.70%) than in cows (5.00%) in another study in Egypt.[27] Much higher percentage (22.50%) of *Salmonella* isolation was reported in cows in another study in USA.[19] Regarding the infection in buffaloes, much higher percentage (2.16%) was reported in another study in Egypt.[28] *S. enteritidis* was the prevalent species among cows and *S. typhimurium* was the prevalent species among buffaloes. Conversely, other investigators reported that nearly similar serovars of *Salmonella* were recovered from both cows and buffaloes.[29] The variation in the reported *Salmonella* species in cows and buffaloes in different studies might be due to the difference in the prevalent species in the studied area.

Regarding the recovery of *S. enteritidis* from healthy buffaloes in this study, it is well-documented that animals may act as carriers of *Salmonella* species and the herd carrier status is positively correlated to the herd size.[30] The inability to isolate *Salmonella* from buffaloes with diarrhea might be attributed to infection of buffaloes by another enteric microorganism rather than *Salmonella* or because of the intermittent shedding of *Salmonella* in the feces of animals. Contrary to this finding, the higher *Salmonella* infection rate was recovered from diarrheic cows (7.69%) compared to apparently healthy cows (0.97%).[31] The differences in the isolation rates reported in different studies might be due to differences in the ecological conditions, type of samples and/or culture methods.[32]

The *InvA* gene encodes *Salmonella's* inner membrane proteins to invade host intestinal epithelial cells. Sequences of this gene are present in all species of *Salmonella* and are also specific to this genus and do not exist in other bacteria.[33] Rahn *et al*. were able to identify an intrinsic component of the *InvA* gene that play key roll to *Salmonella* invasion in cell culture. This method is completely economical and is specific in the diagnosis of *Salmonella* and other bacteria.[34] According to previous studies, excretion of *Salmonella* through feces is the most common way of contaminating the environment, water and food, and probably the most appropriate approach to confirm *Salmonella* infection in live animals is stool sampleing.[7,11,15,18,35] The presence of *Salmonella* has been studied in meat, urine, blood and water samples.[36] Contamination of meat, urine, blood and other tissues as well as water and the environment can lead to environmental contamination and generalization of bacteria in the body (septicemia and bacteremia).[36]

However, considering the main place of bacteria in the body, it could be claimed that to confirm the presence of *Salmonella*, none of these samples are as important as feces, and feces is the main cause of environmental and animals' contamination.[37] Time is the important factor in the diagnosis and treatment of Salmonellosis. The advantage of PCR method, in addition to being specific, is the high speed of this method in diagnosing *Salmonella* in different samples such as urine, blood, milk, feces, meat and other body tissues as well as water.[38] This method is able to detect the lowest number of *Salmonella* per gram of sample. PCR is a reliable, sensitive and effective method for detecting the *InvA* gene in *Salmonella* species.[39] In 2010, Ahmadi *et al*. used the *InvA* gene and the primers to detect *Salmonella* in bovine and buffalo feces. They also used the same primers in 2009 to diagnose *Salmonella* in feces.[1] In 2011, the prevalence of *Salmonella* in German shepherd dogs in Garmsar, Semnan province, was determined using the mentioned genes and primers.[38] In 2003, Malorny *et al*. used a variety of primers (P1 / P2, S18 / S19, ST11 / ST15, and S139 / S141) to detect *Salmonella* in fecal samples.[35] They found S139 and S141 primers more suitable than the others. However, detecting *Salmonella* with traditional methods such as microbial culture requires repeated sampling and a long time for different cultures and specific media. Sometimes there may be specimens containing large amounts of dead mass that the presence of *Salmonella* in these specimens cannot be detected by microbial culture. However, using PCR method, these samples can also be examined for the presence of *Salmonella*. Various studies have been performed comparing culture and PCR methods in the diagnosis of salmonellosis. In 2001, Feder *et al*. showed that the accuracy of PCR in the detection of *Salmonella* in water was 80.00%. In the same study, however, the culture method detected only 44.00% of *Salmonella*-contaminated water samples.[40] In a study by Chaudhary *et al*., out of 37 *Salmonella* isolates, all isolates contained virulence genes (*FimA*, *Stn*, *InvA*). In another study by Kadry *et al*., 8 *Salmonella* isolates contained the *InvA* gene.[41,42] Also in a study, phylogenetic analysis showed that *Salmonella* isolated in humans and eggs were the same. Cohen *et al.* showed similar results in *Salmonella* enteritidis from equine feces.[43] High sensitivity and specificity of PCR method in the study of Pusterla *et al*. were reported 100% and 98.00%[44] and in the study of Gentry-Weeks *et al*. 80.00% and 98.60%, respectively.[45] Shanmugasamy *et al*. also mentioned the sensitivity and specificity of this method for the diagnosis of *Salmonella* as 99.60% and 100%, respectively.[46]

However, Salehi *et al*. noted that PCR and the *InvA* gene could not be used to detect a specific serotype of *Salmonella.*[47] Cohen *et al*. in their studies in 1995 and 1996 were able to detect *Salmonella* in a stool sample for 24 hr using PCR.[43,48] However, the use of culture method requires much more time and also its accuracy and specificity are less than PCR method. It is important and essential to enrich the samples before direct DNA extraction by PCR. In the present study, the samples were incubated in Selenite-F Broth at 37.00 ˚C for 18 hr. This method, while being cheap, leads to an increase in the number of living bacteria and also prevents the growth of other organisms. In West Azerbaijan province, buffalo is one of the most important domestic livestock in villages and even industrial and semi-industrial farms. Milk, meat, wool and other by- products of this animal are used in this province. Contamination of this animal with *Salmonella* leads to the spread of infection in urban and rural communities as well as livestock herds.[1]

Therefore, considering the importance of this livestock in this province and other provinces makes it necessary to study the prevalence of salmonellosis and rapid diagnosis and subsequent appropriate treatment of carriers. In the present study, samples were taken from different regions of the province to determine the prevalence of salmonellosis in the province to some extent, which according to the results of the pattern of *Salmonella* prevalence in different parts was almost the same. In the same study conducted by Ahmadi *et al*., the rate of salmonellosis in buffaloes in Urmia was reported to be 3.00%.[1] Meanwhile, in the present study, this rate was 28.00% in the same city and 11.90% in the total of three cities being sampled.[1] This is an important figure in the province and requires more attention to prompt and appropriate treatment of salmonellosis. Shekarforoush *et al.* Reported contamination of buffalo milk with *Salmonella* is 7.00% in Ahvaz city, Khuzestan province.[49] Also, in a study on slaughtered chickens in Mashhad city by Afshari-Nic *et al*., the *Salmonella* positive samples were 11.90%.[50] Therefore, due to the long cold season in West Azerbaijan province, the growth and distribution of *Salmonella* should

be less than other regions. In the results of our study, this issue was quite obvious and the number of positive samples taken in the warm seasons was much more than the cold seasons of the year. Due to this issue, it is recommended to study the prevalence of *Salmonella* in different provinces of Iran.

The results of this study showed that, buffaloes, like other ruminants and other domestic animals, can carry *Salmonella* and excrete it in the feces. The rate of *Salmonella* contamination was 16.00%. Due to role of this animal and its products in human societies, the transmission of *Salmonella* from buffalo to humans is important. In general, the presence of carriers in livestock populations should be taken into consideration seriously because the issue of public health and prevention of further outbreaks of the disease is directly related to carriers. Therefore, rapid and appropriate diagnosis, isolation and treatment of carriers seems absolutely necessary.

Acknowledgments

The authors would like to thanks the Urmia University for Financial support and Mr. Ali Pirnejad and Mr. Ali Kazemnia for their technical assistances.

Conflict of interest

The authors declare that they have no competing interests.

References

1. Ahmadi M, Dalirnaghadeh B, Shirzad Aski H, et al. Comparison of polymerase chain reaction (PCR) and conventional cultivation methods for detection of carriers of Salmonella spp. in cattle and buffalo. Comp Clin Pathol 2009; 19(3): 251-255.
2. Oloya J, Theis M, Doetkott D, et al. Evaluation of Salmonella occurrence in domestic animal and humans in North Dakota. Foodborne Pathog Dis 2007; 4(4): 551-563.
3. Zha L, Garrett S, Sun J. *Salmonella* infection in chronic inflammation and gastrointestinal cancer. Diseases 2019; 7(1): 28. doi: 10.3390/diseases7010028.
4. Lanzas C, Davies K, Erwin S, et al. On modelling environmentally transmitted pathogens. Interface Focus 2020; 10(1): 20190056. doi: 10.1098/rsfs. 2019.0056.
5. Dong N, Li Y, Zhao J, et al. The phenotypic and molecular characteristics of antimicrobial resistance of *Salmonella enterica* subsp. enterica serovar Typhimurium in Henan Province, China. BMC Infect Dis 2020; 20(1): 511. doi: 10.1186/s12879-020-05203-3.
6. Newell DG, Koopmans M, Verhoef L, et al. Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge. Int J Food Microbiol 2010;139 Suppl 1: S3-S15.
7. Majowicz SE, Musto J, Scallan E, et al. The global burden of nontyphoidal Salmonella gastroenteritis. Clin Infect Dis 2010; 50(6): 882-889.
8. L Plym F, Wierup M. Salmonella contamination: a significant challenge to the global marketing of animal food products. Rev Sci Tech 2006; 25(2): 541-544.
9. Kortman GA, Boleij A, Swinkels DW, et al. Iron availability increases the pathogenic potential of *Salmonella typhimurium* and other enteric pathogens at the intestinal epithelial interface. PloS One 2012; 7(1): e29968. doi: 10.1371/journal.pone.0029968.
10. Gilchrist JJ, MacLennan CA, Hill AV. Genetic susceptibility to invasive Salmonella disease. Nat Rev Immunol 2015; 15(7): 452-463.
11. Makendi C, Page AJ, Wren BW, et al. A phylogenetic and phenotypic analysis of Salmonella *enterica* serovar weltevreden, an emerging agent of diarrheal disease in tropical regions. PLoS Negl Trop Dis 2016; 10(2): e0004446. doi: 10.1371/journal.pntd.0004446.
12. Ehuwa O, Jaiswal AK, Jaiswl S. *Salmonella*, food safety and food handling practices. Foods 2021; 10(5): 907. doi: 10.3390/foods10050907.
13. Alexandre DL, Melo AMA, Furtado RF, et al. A rapid and specific biosensor for *Salmonella typhimurium* detection in milk. Food Bioprocess Technol 2018; 11(4): 748-756.
14. Revolledo L, Ferreira AJ. Salmonella antibiotic-mutant strains reduce fecal shedding and organ invasion in broiler chicks. Poult Sci 2010; 89(10): 2130-2140.
15. Liaquat S, Sarwar Y, Ali A, et al. Virulotyping of Salmonella enterica serovar Typhi isolates from Pakistan: Absence of complete SPI-10 in Vi negative isolates. PLoS Negl Trop Dis 2018; 12(11): e0006839. doi: 10.1371/journal.pntd.0006839.
16. Borriello G, Lucibelli MG, Pesciaroli M, et al. Diversity of Salmonella spp. serovars isolated from the intestines of water buffalo calves with gastroenteritis. BMC Vet Res 2012; 8: 201. doi: 10.1186/1746-6148-8-201.
17. Cheng RA, Eade CR, Wiedmann M. Embracing diversity: differences in virulence mechanisms, disease severity, and host adaptations contribute to the success of nontyphoidal *Salmonella* as a foodborne pathogen. Front Microbiol 2019; 10: 1368. doi: 10.3389/ fmicb.2019.01368.
18. Li X, Singh N, Beshearse E, et al. Spatial epidemiology of Salmonellosis in Florida, 2009- 2018. Front Public Health 2021; 8: 603005. doi: 10.3389/fpubh. 2020.603005.
19. Cummings KJ, Warnick LD, Alexander KA, et al. The incidence of salmonellosis among dairy herds in the northeastern United States. J Dairy Sci 2009; 92(8): 3766-3774.

20. Quan G, Xia P, Zhao J, et al. Fimbriae and related receptors for Salmonella Enteritidis. Microb Pathog 2019; 126: 357-362.
21. Rahman MT, Sobur MA, Islam MS, et al. Zoonotic diseases: etiology, impact, and control. Micro-organisms. 2020; 8(9): 1405. doi: 10.3390/ microorganisms8091405.
22. MacLennan CA, Martin LB, Micoli F. Vaccines against invasive Salmonella disease: current status and future directions. Hum Vaccin Immunother 2014: 10(6): 1478-1493.
23. Humphries RM, Linscott AJ. Practical guidance for clinical microbiology laboratories: diagnosis of bacterial gastroenteritis. Clin Microbiol Rev 2015; 28(1): 3-31.
24. Lee KM, Runyon M, Herrman TJ, et al. Review of *Salmonella* detection and identification methods: aspects of rapid emergency response and food safety. Food Control 2015; 47: 264-276.
25. Park SH, Aydin M, Khatiwara A, et al. Current and emerging technologies for rapid detection and characterization of Salmonella in poultry and poultry products. Food Microbiol 2014; 38: 250-262.
26. Aguilera-Herce J, Zarkani AA, Schikora A, et al. Dual expression of the Salmonella effector SrtJ in mammalian cells and plants. Front Microbiol 2017; 8: 2410. doi: 10.3389/fmicb.2017.02410..
27. Zahran R., El-Behiry A. Prevalence, molecular identification and virulence attributes of Salmonella serovars isolated from feces of diarrheic cow and buffalo-calves. Int J Curr Microbiol App Sci, 2014; 3(11): 9-27.
28. Hassanain NA. Detection of antibodies against zoonotic food borne pathogens in sera of food handlers. Glob Vet 2008; 2: 285-289.
29. Aydin F, Umur S, Gokce G, et al. Isolation and identification of bacterial and parasitic agents from diarrheal calves in Kars region [Turkish]. Kafkas Univ Vet Med J 2001; 7(1): 7-14.
30. Hume ME, Edrington TS, Looper ML, et al. Salmonella genotype diversity in non-lactating and lactating dairy cows. J Food Prot 2004; 67(10): 2280-2283.
31. Mohamed ON, Farid AF, Abaza AF, et al. Fecal shedding of non-typhoidal Salmonella species in dairy cattle and their attendants in Alexandria Suburbs. J Am Sci 2011; 7(9): 623-631.
32. Enticott G. Risking the rural: nature, morality and the consumption of unpasteurized milk. J Rural Stud 2003; 19(4): 411-424.
33. Bell RL, Jarvis KG, Ottesen AR, et al. Recent and emerging innovations in Salmonella detection: a food and environmental perspective. Microb Biotechnol 2016; 9(3): 279-292.
34. Rahn K, De Grandis SA, Clarke RC, et al. Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. Mol Cell Probes 1992; 6(4): 271-279.
35. Malorny B, Hoorfar J, Bunge C, et al. Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. Appl Environ Microbiol 2003; 69(1): 290-296.
36. Cabral JPS. Water microbiology. Bacterial pathogens and water. Int J Environ Res Public Health 2010; 7(10): 3657-3703.
37. Lin JS, Tsen HY. Development and use of polymerase chain reaction for the specific detection of Salmonella Typhimurium in stool and food samples. J Food Prot 1999; 62(10): 1103-1110.
38. Salehi TZ, Badouei MA, Madadgar O, et al. Shepherd dogs as a common source for Salmonella enterica serovar Reading in Garmsar, Iran. Turk J Vet Anim Sci 2013; 37(1): 102-105.
39. Cohen HJ, Mechanda SM, Lin W. PCR amplification of the fimA gene sequence of Salmonella typhimurium, a specific method for detection of Salmonella spp. Appl Environ Microbiol 1996; 62(12): 4303-4308.
40. Feder I, Nietfeld JC, Galland J, et al. Comparison of cultivation and PCR-hybridization for detection of Salmonella in porcine fecal and water samples. J Clin Microbiol 2001; 39(7): 2477-2484.
41. Chaudhary JH, Nayak JB, Brahmbhatt MN, et al. Virulence genes detection of Salmonella serovars isolated from pork and slaughterhouse environment in Ahmedabad, Gujarat. Vet World 2015; 8(1): 121-124.
42. Kadry M, Nader SM, Dorgham SM, et al. Molecular diversity of the *invA* gene obtained from human and egg samples. Vet World 2019; 12(7): 1033-1038.
43. Cohen ND, Wallis DE, Neibergs HL, et al. Detection of Salmonella enteritidis in equine feces using the polymerase chain reaction and genus-specific oligonucleotide primers. J Vet Diagn Invest 1995; 7(2): 219-222.
44. Pusterla N, Byrne BA, Hodzic E, et al. Use of quantitative real-time PCR for the detection of Salmonella spp. in fecal samples from horses at a veterinary teaching hospital. Vet J 2010; 186(2): 252-255.
45. Gentry-Weeks C, Hutcheson HJ, Kim LM, et al. Identification of two phylogenetically related organisms from feces by PCR for detection of *Salmonella* spp. J Clin Microbiol 2002; 40(4): 1487-1492.
46. Shanmugasamy M, Velayutham T, Rajeswar J. Inv A gene specific PCR for detection of Salmonella from broilers. Vet World 2011; 4(12): 562-564.
47. Salehi TZ, Mahzounieh M, Saeedzadeh A. Detection of *InvA* gene in isolated *Salmonella* from broilers by PCR method. Int J Poult Sci 2005; 4(8): 557-559.
48. Cohen ND, Martin LJ, Simpson RB, et al. Comparison

of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples. Am J Vet Res 1996; 57(6): 780-786.
49. Shekarforoush S, Rokni N, Karim G, et al. Study on the overview on food borne bacteria in foodstuffs with animal origin in Iran; Part two: meat and meat products [Persian]. Food Hyg 2012; 2(3): 1-14.
50. Afshari-Nic S, Zahraei-Salehi T, Jamshidi A. Detection of Salmonella spp contamination of carcasses slaughtered in poultry abattoir in Mashhad, Iran. Arch Razi Inst 2007; 62(4): 229-233.