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Determination of Antibiotic Resistance in *Salmonella Spp* Isolated from Raw Cow, Sheep and Goat's Milk in Chaharmahal Va Bakhtiyari Provience, Iran

[1]*Forough Tajbakhsh,* [2]*Elahe Tajbakhsh,* [3]*Ebrahim Rahimi and* [4]*Manochehr Momenii*

[1]Young Researchers Club, Islamic Azad University, Shahrekord Branch, Shahrekord-Iran
[2]Department of Microbiology, Faculty of Basic Sciences,
Islamic Azad University, Shahrekord Branch, Shahrekord-Iran
[3]Departments of Food Hygiene, Facultyl of Veterinary Medicine,
Shahrekord Branch, Islamic Azad University-Shahrekord-Iran, Shahrekord, Iran
[4]Biotechnology Research Center, Islamic Azad University,
Shahrekord Branch, Shahrekord-Iran

Abstract: The World Health Organization has recently pointed out an alarming increase in the incidence of antibiotic resistant strains of *Salmonella*, which are due to the use of antibiotics in intensive breeding. *Salmonella spp* is one of the main causes of food-borne illness worldwide. Conventional plating methods for the detection of these microorganisms in food are well established. This study was carried out to determine the prevalence and antibiotic resistance of *Salmonella* species from raw cow, sheep and goat's milk samples and molecular detection of *Salmonella typhi murium* in these samples in Chaharmahal va Bakhtiyari provience. A total of 550 raw cow, sheep and goat' milk samples were collected from commercial dairy herds in Chaharmahal va Bakhtiyari provience. Using cultural method, 20 of 550 samples (3.63%) were contaminated with *Salmonella spp*. The primers were selected from the *fliC* genes, specific for the detection of *Salmonella typhimurium* serotype. In this study a total of 20 *Salmonella spp*, (7samples) 1.27% were found to be contaminated with *Salmonella typhimurium.* In the present study *Salmonella* isolates showed resistance to: Nalidixic acid, Cephalothin, Ampicillin, Streptomycin, Neomycin, Chloramphenicol and Tetracycline.

Key words: Antibiotic Resistance · Salmonella · Raw milk

INTRODUCTION

Salmonella is a genus of Gram-negative rod-shaped bacteria of the family Enterobacteriaceae. They cause a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. Gastroenteritis associated with food-borne outbreaks is probably the most common clinical manifestation of the infection [1]. *Salmonella* species have been considered as one of the most important food-borne pathogens, all around the world [2].

Salmonellosis is the most common food- borne disease in both developing and developed countries, although incidence rates vary according to the country. Animals are the principal reservoir of this pathogen. Foods from animal Sources such as beef, poultry meat, egg and milk have been proved to carry these pathogens [2].

The infective dose of *Salmonella* can be as low as 15 to 20 cells, depending upon age and health of host. *Salmonella* causes one of the most common enteric (intestinal) infections in the world wide and is the second most common bacterial food-borne cause after Campylobacter infection. The number of salmonellosis cases has increased significantly throughout the past decade in several countries, *Salmonella enteritidis* has become the most common cause of salmonellosis [3-8]. Infections of humans can be acquired through direct contact with carrier domestic or wild animals or through the consumption of contaminated foods or water [9]. Infections with non typhoidal *Salmonella* (salmonellosis), are one of the most commonly recorded causes of gastroenteritis in humans. Strains of *Salmonella* sp. were classified into serovars in accordance with the Kauffmann–White scheme (1), which gave serovar status

Corresponding Author: Elahe Tajbakhsh, Department of Microbiology, Faculty of Basic Sciences,
Islamic Azad University, Shahrekord Branch, Shahrekord-Iran.

to each antigenic type on the basis of wide diversity observed in somatic (O), capsular (VI) and the flagellar (H) antigens [1].

Salmonella enterica serovar *Typhimurium* and *Salmonella enterica* serovar *Enteritidis* are the most frequently isolated serovars from food-borne outbreaks throughout the world. According to the antigenic profile of Salmonella species they show different disease syndromes and host specificity. Therefore, it is necessary and important to discriminate *Salmonella* serovars from each other in order to ensure that each pathogen and epidemiology is correctly recognized [2].

Antimicrobial-resistant *Salmonella* are increasing due to the use of antimicrobial agents in food animals at sub-therapeutic level or prophylactic doses which may promote on-farm selection of antimicrobial resistant strains and markedly increase the human health risks associated with consumption of contaminated meat products [9, 10].

Cattle have been implicated as a source of human infection with antimicrobial resistant *Salmonella* through direct contact with livestock and through the isolation of antimicrobial resistant *Salmonella* from raw milk, cheddar cheese and hamburger meat traced to dairy farms. Antimicrobial use in animal production systems has long been suspected to be a cause of the emergence and dissemination of antimicrobial resistant *Salmonella* [11]. The ultimate aim of this study was to determine the level of *Salmonella* contamination in raw cow, sheep and goat's milk samples in Chaharmahal Va Bakhtiyari provience, Iran and determination of antimicrobial resistance of *Salmonella spp*. isolated from raw milk.

MATERIALS AND METHODS

Sample Collection: A total of 550 raw cow (n=200), sheep (n=175) and goat' milk (n=175) samples were collected from commercial dairy herds in Chaharmahal Va Bakhtiyari provience (Iran). The samples of milk were transported to the laboratory after being collected in a portable cooler container with ice packs (at 4°C) and microbiological analysis was carried out immediately.

Isolation and Identification: Samples were examined for the presence of Salmonella by Iranian National Standard method No.1810 recommended by the Institute of Standards and Industrial Research of Iran (ISIRI) [12]. Twenty-five ml of milk sample was aseptically added to 225 ml of buffered peptone water (Merck) and incubated at 37°C for 24h and constituted the pre-enrichment stage of the isolation. Subsequently, 10 ml of this pre-enrichment culture was added to 100 ml of selenite cystine broth (Merck) as selective enrichment medium and incubated for 24 h at 37°C. After incubation, a loopful of the enriched cultures was streaked onto Salmonella-Shigella agar (Merck) plates and incubated at 37°C for 24-48 h. Non lactose fermenting colonies with or without black centre on Salmonella-Shigella agar were the suspected *Salmonella* spp. Such colonies were picked out and sub-cultured for biochemical tests and were identified according to Soltan dalal *et al*. [3].

DNA Extraction: A total of 20 *salmonella* strains isolated from raw cow, sheep and goat' milk samples, were used. Bacterial DNA was extracted with the phenol-chloroform method as previously described by Sambrook *et al*. [13]. Briefly, the bacteria, grown on nutrient agar plates for overnight at 37°C, were suspended in Tris-EDTA buffer (10 mMTris-HCI and 1 mM EDTA) and centrifuged at 2000g for 10 min. Following centrifugation, the pellet was incubated with Tris-EDTA buffer and proteinase K (Fermentas) for overnight at 55 °C. The following day, the DNA was extracted by phenol and chloroform extraction method and suspended in Tris-EDTA buffer. This product was stored as DNA template at -20°C until they were used in the PCR (13).

The sequence of primers used in this study was shown in Table 1. The *Fli*15 and *Tym* primers are specific for the *fliC* gene of *Salmonella typhimurium* [14]. Reactions with these primers were carried out in a 25µl amplification mixture consisting of 2.5µl 10x PCR buffer (500mM KCl, 200mM Tris HCl), 1.25µl dNTPs (10mM), 1.5µl MgCl2 (50mM), 0.5µl of each primer, 0.5µl of Taq DNA polymerase (Fermentase) and 2µl of extracted DNA [14]. Amplification was performed in a gradient thermocycler. The cycling condition was as follows: an initial incubation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 30 seconds and final extention priod for 10 minutes at 72°C. Amplified products were electrophoresed in 1.2% agarose gel and a 100bp DNA ladder was used as a size reference. After staining with ethidium bromide the gel was documented with a gel documentation apparatus [14].

Table 1: Sequence of oligonucleotides used as primers in the PCR.

Primer	Sequence	Target gene	Amplicon fragment	References
Fli15	CGGTGTTGCCCAGGTTGGTAAT	*fliC*	559	14
Tym	ACTCTTGCTGGCGGTGCGACTT			

Susceptibility to antimicrobial agents was tested using the disk diffusion method on Mueller Hinton agar (Merck) plates according to the National Committee for Clinical Laboratory Standards Guidelines [12]. The following 17 different antimicrobial agents were examined against *Salmonella* isolates recovered from raw cow's milk samples: Trimethoprim (5µg), Furazolidone (10µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Imipeneme (10µg), Cephalothin (20µg), Cefexime (5µg), Ceftazidim (30µg), Streptomycin (10µg), Ampicillin (10µg), Neomycin (30µg), Tobramycin (20µg), Kanamycin (20µg), Amikacin (30µg), Gentamicin (10µg), Chloramphenicol (30µg) and Tetracycline (30µg). The diameters of zones of inhibition were recorded to the nearest millimeter and classified as susceptible, intermediate and resistant.

RESULTS

In this study, 20 samples (3.63%) out a total of 550 raw cow (n=200), sheep (n=175) and goat' (n=175) milk samples were identified as *Salmonella spp.*

Performing PCR assay 20 *Salmonella spp* isolated from raw milk, a 559 bp sequence of the *fliC* gene was amplified showing that 7 samples (1.27%) were contaminated with *Salmonella typhimurium* (Fig-1).

In this study, 7 samples (1.27%) were found to be contaminated *Salmonella typhimurium*. *Salmonella* isolates showed resistance to: Nalidixic acid, Cephalothin, Ampicillin,, Streptomycin, Neomycin, Chloramphenicol and Tetracycline. The results were shown in Table 2.

DISCUSSION

Salmonellosis is one of the most important zoonotic bacterial pathogen of food-borne Infection all around the world. The most important serotypes of *Salmonella* are *Salmonella typhimurium* and *Salmonella enteritidis* [15]. Therefore, it is important to control microbial food. Microbial agents can cause food spoilage and disease. Salmonella spp can cause gastrointestinal disease. The main sources of transmission are water, meat, eggs and raw foods [16].

Salmonellosis is not a reportable disease in Iran; therefore, the actual incidence of this infection in Iran is unknown. Therefore, the ultimate aim of this study was to determine the level of *Salmonella* contamination in raw cow, sheep and goat's milk samples in Chaharmahal va Bakhtiyar provience, Iran.

In this study the prevalence of *Salmonella* in raw milk was 3.63%. One of the most important findings of this study is 1.27% prevalence of *Salmonella typhimurium* in milk samples.

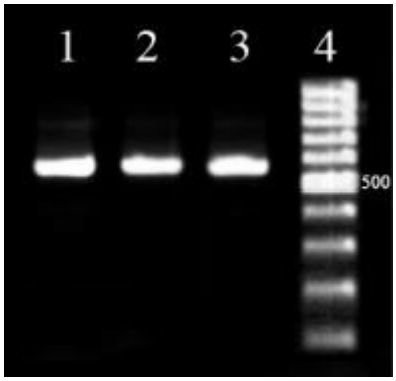

Fig. 1: Detection of Salmonela typhi murium serotype in raw milk samples. Lane 4, Molecular weight marker 100 bp DNA, Lane 1, 2, 3: positive samples

Table 2: Resistance of *Salmonella spp* isolated from raw cow milk to different antibiotics.

Antimicrobial drug	%Resistance of isolates from raw cow milk
Trimethoprim (5µg)	0%
Furazolidone (10µg)	0%
Nalidixic acid (30µg)	78.57%
Ciprofloxacin (5µg)	0%
Imipeneme (10µg)	0%
Cephalothin (20µg)	21.42%
Cefexime (5µg)	0%
Ceftazidim (30µg)	0%
Streptomycin (10µg)	21.42%
Ampicillin (10µg)	42.58%
Neomycin (30µg)	21.42%
Tobramycin(20µg)	0%
Kanamycin (20µg)	0%
Amikacin (30µg)	0%
Gentamicin (5µg)	0%
Chloramphenicol (30µg)	21.42%
Tetracycline (30µg)	42.58%

Previous studies have shown a wide range of estimates for the prevalence of *Salmonella* in bulk tank milk. Steele *et al*. [17] detected *Salmonella* in only 0.17% of bulk tank samples from Ontario, Canada. Murinda *et al.* [18] found *Salmonella* spp. in 2.24% of milk samples from the bulk tanks of 30 Tennessee farms.

The detection of *Salmonella* in 3.63% of the samples tested indicates that the degree of prevalence of the pathogen in raw milk in Chaharmahal va Bakhtiyar provience is relatively higher than originally believed. Although contamination of dairy products currently accounts for a small percentage of foodborne?? illness, it is clear that raw milk consumption and the consumption of products made with raw milk present some risk. Although proper pasteurization minimizes these risks to the public, there is a small but growing group of people that consume unpasteurized milk or milk products, either

for practical (e.g., farm families) or cultural (e.g., soft ethnic cheeses) reasons, or because of perceived health benefits [9]. Although the levels of *Salmonella* in the milk samples tested here seemed to be very low and the infectious dose for this organism is low, the potential for this organism to grow in improperly stored raw milk and in products made from raw milk presents a public health risk, particularly to susceptible members of the population. Continuing surveys of milk will help to estimate the true level of risk associated with these practices and may help to identify dairy management practices that minimize the contamination of bulk tank milk with zoonotic food-borne pathogens.

Karns *et al*. [9] reported a prevalence of 11.8% from bulk tank milk by PCR method which is higher than the present report. In the other study by Addis *et al*. [19], from 195 dairy cows tested 28.6% were positive from milk samples. Akoachere *et al*. [20] in Cameroon reported a high prevalence (27%) of *Salmonella* among cattle. This may be due to the difference in the living condition, like housing conditions, feeding habits, types of feed given for the cattle.

Salmonella enterica serovar *typhimurium* and *Salmonella enterica* serovar *enteritidis* cause the most important food-borne diseases that several studies have been done in the field to isolate these serovars. Jamshidi *et al*. [2] reported a prevalence of 1.6% *Salmonella typhimurium* infection in poultry carcasses in Mashhad.

In the study conducted by Soltan Dalal *et al*. [3], in Tehran, 47.8% of meat chicken and 28.8% of beef meat samples were contaminated with Salmonella. In this study, the predominant serotypes reported were *S. thompson* (54.9%) and *S. enteritidis* (9.8%) [1].

Antibiotic resistance in *Salmonella* is an emerging problem during the last decades. The intensive use of antibiotics in both human and veterinary medicine, as well as in agriculture has caused bacteria to develop resistance mechanisms against therapeutic drugs [21].

In this study *Salmonella* isolates showed antimicrobial resistance was toNalidixic acid, Cephalothin, Ampicillin, Streptomycin, Neomycin, Chloramphenicol and Tetracycline with percentages 78.57, 21.42, 42.58, 21.42, 21.42, 21.42 and 42.58% respectively.

Results of the current study indicated that ciprofloxacin and Imipeneme are good antimicrobials showing 100% activity against *Salmonella* spp isolated from cow. This is also comparable with the result reported by Morshed and Peighambari in Iran [22]. The issues of food safety attract more attention from the government and public worldwide in recent years. The incidence of salmonellosis outbreak cannot be neglected due to the overwhelming effects to human. The knowledge about *Salmonella* and its evolution is important to ensure the safety and quality of food.

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