World Journal of Medical Sciences 11 (3): 342-347, 2014
ISSN 1817-3055

DOI: 10.5829/idosi.wjms.2014.11.3.8537

Antimicrobial Resistance of Salmonella Serovars Isolated from Food of Bovine Origin in Selected Woredas of Tigray, Ethiopia

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Abstract: Salmonella species are food borne pathogen and the leading causes of acute gastroenteritis in several countries. The situation is aggravated by the ever increasing rate of antimicrobial resistance strains. The study was designed to determine antibiogram Salmonella serovars isolated from food of bovine origin. A total of 384 of milk (n_1=192) and meat samples (n_2=192) were collected from selected Woredas of Tigray, Ethiopia. The samples were pre-enriched with buffered peptone water and incubated at 37°C for 18hrs. Aliquots were inoculated into Selenite-Cysteine broth. A loopful of broth was streaked on Brilliant Green agar. then sub-cultured on Biolog Universal Growth Agar. The bacteria were further identified by BiOLOG identification system. Antimicrobial resistance of Salmonella serovars was done by disk diffusion using twelve antimicrobials and ranges for minimum inhibitory concentration was determined. The study revealed that out of 384 samples, *S.typhimurium 40* (10.4%)*, S. enteritidis 33* (5.7%) *and S. newport 1 (0.2%)* were isolated. Antibiogram of the isolates (n=63) revealed high resistance to cephalothin (74.6%), Chloramphenicol (71.4%), tetracycline(74.6%), gentamicin(54%) whereas low resistance to sulphoxazoletrimethoprim (17.5%), Neomycin(17.5%), streptomycin(27%), Kanamycin(25.4%), Ciprofloxacin(11.1%), Nitrofurantoine(4.8%), Norfloxon(7.9%) and Ciftriaxon (*15.9* %). Multidrug resistance observed in (71.4%) of *Salmonella serovars.* The study reveals *Salmonella serotypes* originating from food of bovine origin and its multi-drug resistance. This poses a concern to design prevention and control methods.

Key words: Antibiograms · Meat · Milk · Salmonella

INTRODUCTION

Food borne pathogens cause important economic and health problems in the world. In recent years, Salmonella has been one of the most common causes of food born disease [1]. Salmonella species are leading causes of acute gastroenteritis in several countries and salmonellosis remains an important public health problem worldwide, particularly in the developing countries [2]. Salmonellosis often occurs through contaminated food, especially food products with an animal origin such as meat, milk, egg, animal foods and sometimes vegetables in the food chain [3, 4]. In developing countries, estimation of Salmonellosis is difficult because there has not been sufficient surveillance [5, 6]. Therefore, globally, many studies have been performed reporting that the prevalence and kind of Salmonella serotypes are different based on geographical regions [7, 8]. *Salmonella enterica serovars enteritidis* and *typhimurium* were reported to be the two most frequent serotypes of Salmonella in the world [9, 10]. During the two past decades, the emergence of antibiotic-resistant *Salmonella* has become a serious problem worldwide. Wide usage of antibiotics in the diet of domestic animals has made drug resistant bacteria which could be transferred to human beings [11].

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

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associated with consumption of contaminated meat products [12, 13]. 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 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 [14]. This practice promotes the development of drug-resistant bacteria that can spread to humans. Thus food borne diseases, when associated with resistant bacteria, are harder to treat, resulting in longer hospitalization, higher mortality and morbidity, decreased productivity and increased costs [15]. Likewise, antimicrobial resistance is like a fluid and constantly evolving challenge. Further transfer of antimicrobial resistant bacteria to humans via food chain has been reported [16]. Studies made on salmonella isolation from food of bovine had not been clearly documented sofar in Ethiopia [17]. A limited number of investigation have been studied the presence of antimicrobial resistance of salmonella serovars in food animals in Ethiopia [18, 19]. The finding of the present study on antimicrobial resistance of *Salmonella serovars* will provide useful information for the development of public health policy in food animal production. Thus the study was carried out with aim to isolate Salmonella serotypes from food of bovine origin and to determine its antibiogram.

MATERIAL AND METHOD

Study area and Design: A cross-sectional study was conducted from November 2012 to June 2013 in three districts of Tigray, Mekelle; Alamata and Adigrat. These districts were selected mainly because of their difference in the altitudes that may help us to obtain reliable evidence about the magnitude and epidemiology of disease in the region [20].

Sample Size and Sampling Technique: A total of 384 samples were collected from bovine raw milk and meat in the selected Woredas of Tigray, Ethiopia. The sample size was determined according the formula given by Thrusfield [21] using 50% prevalence so that the maximum sample size could be achieved. Accordingly, the calculated value for sample size was 384. Then equal number of milk (n_1=192) and meat (n_2=192) samples were included purposefully. In sampling of milk and meat samples, simple random sampling technique was applied until sample size achieved.

Sample Collection, Transport and Handling

Milk Samples: Milk samples were collected according to the National Mastitis Council Guideline [22] by principal investigator. Milk samples were aseptically collected directly from teats of lactating cows (n=64) and from distribution sites (shop=64 and restaurant=64) using sterile sample bottle. Samples were transported using icebox to Microbiology Laboratory of College of Veterinary Medicine, Mekelle University, Ethiopia. Milk samples were immediately cultured or stored at 4°C for a maximum of 24 hr until the samples were cultured.

Meat Samples: Raw meat from slaughter house (n=64) during slaughtering and non pre-packed meat samples from beef were purchased randomly from selected butcher shops (n=64) and restaurant (n=64). Sections of meat (10cm × 10cm × 3cm) from neck of each carcass were aseptically removed and placed in separate sterile plastic bags to prevent spilling and cross contamination. It was immediately transported with ice box to Microbiology Laboratory of College of Veterinary Medicine, Mekelle University, Ethiopia. After culture, the prepared samples were transported with icebox to Microbiology Laboratory of Institute Biodiversity Conservation, Addis Ababa for further confirmatory identification process.

Culture and Identification

Milk Sample: Bacteriological examination was done according to the CLSI [23]. About 0.1 ml of milk inoculated into selective enrichment liquid media at a ratio of 1:10 in Selenite-Cysteine broth. A loopful of broth was streaked on plates of Brilliant Green agar, (Oxoid, UK). Then pure colony was taken and sub-cultured on BUG(BiOLOG Universal Growth Media) at 37°C for 18-24 hours as a primary and secondary culture. Well-isolated fresh colonies from BUG (Biolog, USA) media were inoculated into 18-20 inoculation fluid to have bacterial suspension with turbidity equivalent to 20% transmittance as measured by turbidity meter. This suspension was poured into micro plates with multi-channel pipettes. The Micro Plates were loaded into Omnilog tray to be incubated, analyzed and interpreted for 18-24 hours as per guidelines of BiOLOG User Guide [24] and finally identified bacteria was printed out.

Meat Sample: The microbiological examination of each meat sample, 25 g was homogenized with 1 g of the homogenate and added to 5 mL of buffered peptone water (BPW-HiMedia Laboratories, Mumbai, India) and incubated at 37°C for 18h. Aliquots from pre-enrichment were inoculated into selective enrichment liquid media

at a ratio of 1:10 in Selenite-Cysteine broth. A loopful of broth was streaked on plates of Brilliant Green agar, (Oxoid, UK). Then pure colony was further sub-cultured on BUG at 37°C for 18-24 hours as a primary and secondary culture. Well-isolated fresh colonies from BUG (Biolog, USA) media were inoculated into 18-20 inoculation fluid to have bacterial suspension with turbidity equivalent to 20% transmittance as measured by turbidity meter. This suspension was poured into Micro plates with multi-channel pipettes. The micro plates were loaded into Omnilog tray and incubated, analyzed and interpreted for 18-24 hrs as per guidelines of BiOLOG User Guide [24] and finally identified bacteria were printed out.

Antimicrobial Susceptibility Test: Antimicrobial susceptibility test was performed for all *Salmonella serovars* isolates according to the criteria of the CLSI [23]. For susceptibility test, a pure culture of all identified *Salmonella* were taken from BUG media and transferred to a tube containing 5 ml of sterile normal saline and mixed gently to make homogenous suspension which was adjusted to a turbidity equivalent to a 0.5 Mc Farland standard as measured by turbidity meter. The bacterial suspension was inoculated on to Muller–Hinton agar (Oxoid, UK) with the sterile swab to cover the whole surface of the agar. The inoculated plates were left at room temperature to dry. The plates were prepared as per the manufacturer's instructions and checked for sterility before inoculation by incubating the plates over night at 37°C.

Before using the antimicrobial disks, they were kept at room temperature for one hour and then dispended on the surface of media. Following this, the plates were incubated aerobically at 37°C for 24 hrs.

For susceptibility test, antimicrobials which were used for treatment of bovine mastitis or considered as important antimicrobial agents for human was selected for antibiogram based on the criteria of Clinical and Laboratory Standards Institute (2008). Thus, antimicrobials used in this study were cephalothin (30µg), sulphoxazole-trimethoprim (25µg), neomycin (5 µg), streptomycin(10µg), kanamycin (30 µg), chloroamphenicol (30mg), (tetracycline (30µg) and gentamicin (10µg) (Oxoid, UK). Antimicrobials not used for treatment of bovine mastitis but important for human were ciprofloxacin (5 µg), nitrofurantoine (300 µg), norfloxon (10 µg), ciftriaxone (30 µg) (Oxoid, UK). The diameters of the zone of inhibition around the disks were measured to the nearest millimeter using calibrated rulers and the isolates were classified as susceptible, intermediate and resistant according to the interpretative standards of CLSI [23] In addition, minimum inhibitory concentration (MIC) was determined using broth dilution method with an antimicrobial concentration ranging from 0.25-512 µg/µL, in accordance with the guidelines of CLSI [23]. Those isolates with minimum inhibitory concentrations (MIC) higher than the breakpoint for the respective antimicrobial agents were regarded as resistant, while those with MIC equal to or lower than the breakpoint were regarded as susceptible. Moreover, isolates showing resistance to three or more antimicrobials subclass were considered as multidrug resistant.

Quality Control: Confidence in the reliability of test results was increased by adequate quality assurance procedures and the routine use of control strains. Thus, *E. coli* ATCC-25922 was taken as an important part of quality control for culture, BiOLOG identification and antimicrobial susceptibility through this study.

Variables: Independent variables such as types of samples were interpreted against dependent variable of Salmonella *serovars* and antimicrobial sensitivity pattern of each isolates.

Ethical Issues: Verbal consent was obtained from dairy farms, abattoirs and butcher shop owners/managers.

Statistical Analysis: The collected data was entered into EPI data version 3.1 and exported to SPSS version 16 computer soft ware then the data was analyzed. Accordingly, descriptive statistics such as percentages and frequency distribution were used to describe /present bacterial isolates and antimicrobial susceptibility which were expressed as percent of resistant and susceptible. In addition, the proportion of bacteria resistant to at least one of the antibiotics and resistant two or more were calculated.

RESULTS

Prevalence of Salmonella Serovars Isolated from Milk and Meat Samples of Bovine Origin: The total number of *Salmonella serovars* isolated from milk and meat sample of bovine origin shown in Figure 1. *S.typhimurium 40* (10.4%)*, S. enteritidis 33* (5.7%) *and S. newport 1 (0.2%)* were detected from both milk and meat samples of bovine origin.

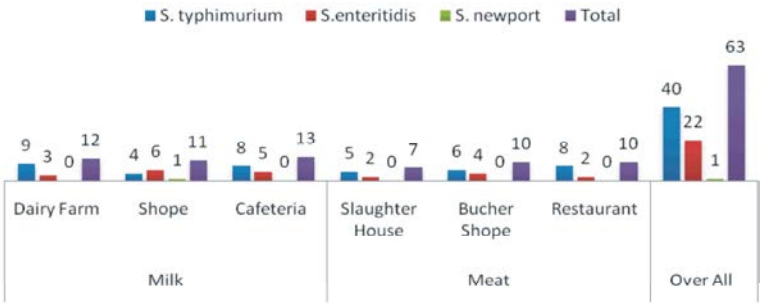

Fig 1: Prevalence of Salmonella Serotpes Isolated from Milk and Meat of Bovine

Table 1: Antimicrobial susceptibility pattern and MIC range of Salmonella serotypes isolat*ed* from milk and meat sample of bovine origin

	S.typhimurium				*S. enteritidis*				*S. newport*							
	Milk(n=21)		Meat(n=19)		Milk(n=14)		Meat(n=8)		Milk(n=1)		Meat(n=0)		overall(n= 63)		MIC(µg/µl)	
Antimicrobials	S(%)	R (%)	S(%)	R(%)	S (%)	R (%)	S (%)	R (%)	S(%)	R (%)	S (%)	R (%)	S (%)	R (%)	MIC50	MIC90
cephalothin (30µg	28.6	71.4	26.3	73.7	35.7	64.3	0.0	100	0	100	0	0	25.4	74.6	MIC50	MIC90
SXZ(25µg)	90.5	9.5	78.9	21.1	100.0	0	50	50	0	100	0	0	82.5	17.5	64	512
Neomycin(5 µg	76.2	23.8	89.5	10.5	92.9	7.1	62.5	37.5	100.0	0	0	0	82.5	17.5	*32*	*128*
streptomycin(10µg)	66.7	33.3	73.7	26.3	92.9	7.1	50	50	100	0	0	0	73	27	*64*	*512*
kanamycin (30 µg)	71.4	28.6	78.9	21.1	85.7	14.3	50	50	100.0	0	0	0	74.6	25.4	*128*	*512*
Chloramphenicol (30mg)	33.3	66.7	26.3	73.7	28.6	71.4	25	75	0	100	0	0	28.6	71.4	*128*	*512*
Tetracycline (30µg)	28.6	71.4	21.1	78.9	35.7	64.3	12.5	87.5	0	100	0	0	25.4	74.6	*512*	*512*
Gentamicin (10µg)	42.9	57.1	52.6	47.4	42.9	57.1	37.5	62.5	100	0	0	0	46	54	*128*	*512*
Ciprofloxacin(5 µg)	85.7	14.3	89.5	10.5	92.9	7.1	87.5	12.5	100	0	0	0	88.9	11.1	*512*	*512*
Nitrofurantoine(300µg)	95.2	4.8	94.7	5.3	92.9	7.1	100	0	100	0	0	0	95.2	4.8	*64*	*128*
Norfloxon(10 µg)	90.5	9.5	89.5	10.5	92.9	7.1	100	0	100	0	0	0	92.1	7.9	*16*	*64*
Ciftriaxon(30 µg)	90.5	9.5	78.9	21.1	85.7	14.3	75	25	100	0	0	0	84.1	*15.9*	*32*	512

S: susceptible R: Resistant MIC: Minimum inhibitory concentration, SXZ: Sulphoxazole-trimethoprim

Table 2: Percentages of number of antimicrobials resistance of *Salmonella serotypes* isolated from milk and meat sample of bovine origin

	S.typhimurium		*S. enteritidis*		*S. newport*		
Number of antimicrobials	Milk(n=21)	Meat(n=19)	Milk(n=14)	Meat(n=8)	Milk(n=1)	Meat(n=0)	overall(n=63)
One(%)	23.8	21.1	38.5	0	0	0	28.6
MDR(%)	76.2	78.9	61.5	100	100	0	71.4

MDR: Multi-drug resistance

Antimicrobial Resistance Profile of Salmonella Serovars Isolated from Milk and Meat Samples: Analysis of serovars specific resistance rates indicated for the samples isolated from milk and Meat was shown on Table 1. All Salmonella *serovars stain* isolates showed high percentage resistance to cephalothin, chloramphenicol, tetracycline and gentamicin except *S. newport* that were susceptible to gentamicin. On the other hand, most Salmonella *serovars* isolates were susceptible to sulphoxazole-trimethoprim, neomycin, streptomycin, kanamycin, ciprofloxacin, nitrofurantoine, norfloxon and ciftriaxon.

The overall multiple antimicrobial resistance rate was 71.4%. The resistances against two or more antimicrobial agents were observed in *S.typhimurium* 76.2%, *S. enteritidis* 61.5% and *S. newport* 100% isolated from milk showed multiple drug resistance (Table2).

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 *S.typhimurium, S. enteritidis and S. newport* were identified in the present study. The present study, the prevalence of salmonella serotypes isolated from food of bovine origin was 16.4%. Similar prevalence (17.8%) was recorded by Mohammad *et al.* [25] and Aaku *et al.* [26]. Lower results were reported by Molla *et al.* [12] and Abouzeed *et al.* [27], who reported that the prevalence of *Salmonella* in 4.6% and 4.2 % respectively. Abouzeed *et al.* [27] recorded that the most prevalent serotypes of animal Salmonellae is *Serovars typhimurium* which similar to the present investigation. This result is significantly high to be a potential source of food borne Salmonellosis.

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.

In this study, *Salmonella* isolates showed high resistance to cephalothin, chloramphenicol, tetracycline and gentamicin. This similar with study conducted by Teshome and Anbessa [17] and Forough *et al*. [28].

Results of the current study indicated that Salmonella strains are highly susceptible to trimethoprime-sulfamethoxazole, neomycin, streptomycin, kanamycin, ciprofloxacin, nitrofurantoine, norfloxon and ciftriaxon. This is also comparable with the result reported by Morshed and Peighambari [29] in Iran. if you have year add.

High proportion (71.4%) of Salmonella isolates were resistant to two or more of the antimicrobials that are commonly used in the veterinary and public health set up. This may pose difficulties in the treatment of human clinical cases and other bacterial diseases. *S.typhimurium was* found out to be more resistant two or more antimicrobials than *S. enteritidis* and *S. newport.* This is similar with finding of Berrang *et al*. [30] conducted in USA.

CONCLUSIONS

The current study revealed presence of *Salmonella serotypes* originating from food of bovine origin and multi-drug resistance Salmonella serovars. This poses a concern to design prevention and control methods. In view of this research finding, there is a need to develop comprehensive policies to ensure safe food.

ACKNOWLEDGEMENT

Authors are grateful to Mekelle University, Ethiopia-NORAD project III for giving financial support to the present investigation and also thankful to Institute of Biodiversity for identification Salmonella serotypes identification.

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