Prevalence of Antimicrobial Resistant Salmonellae Isolated from Bulk Milk of Dairy Cows in and around Debre Zeit, Ethiopia

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ABSTRACT

A cross sectional study to determine the prevalence and antimicrobial resistant profile of *Salmonella* isolates from 106 bulk milk of dairy cows was undertaken from December 2013 to April 2014 in supermarket, large and small holder's dairy farms in Debre Zeit, Ethiopia. The bacteria was isolated and identified according to standard methods and sensitivity tests were done by the Kirby-Bauer disc diffusion method. The study revealed an overall prevalence of 23.6% (25/106). The occurrence of *salmonella* in large and small scale farm was 20.4% and 27.3% respectively. The isolated *Salmonella* spp. was resistant to at least two or more antimicrobials which were used in this study. A higher proportion of the isolates (96%) were resistant to ampicillin and the lowest resistance was recorded for streptomycin (8%). Assay of antimicrobial resistance revealed that 96% of *Salmonella* isolates were resistant to two or more of the nine antimicrobials tested whereas 4% of the isolate was sensitive. The most common resistance was to Ampicillin 24 (96%), oxytetracycline 21 (84%), amoxicillin 12 (48%), Chloramphenicol 10 (40%). A significant proportion has developed resistance for routinely prescribed antimicrobial drugs both in veterinary and public health sectors. This poses considerable health hazards to the consumers unless prudent antimicrobial usage, adequate heat treatment, improvement of standards of hygiene and development and enforcement of suitable legislation, which safeguard consumers, are urgently instituted.

Key words: Isolates, *Salmonella*, Prevalence, Antimicrobial resistance, Bulk milk sample

INTRODUCTION

Salmonellosis is the most common food borne zoonotic disease in both developing and developed countries with different incidence rates (Addis et al., 2011). *Salmonella* species are the major pathogenic bacteria in humans as well as in animals which are leading causes of acute gastroenteritis in several countries (Hussein et al., 2014). Dairy cows are reservoir for Non Typhoidal *Salmonella* in industrialized countries and large outbreaks of *Salmonella* infection have been associated with foodborne transmission from milk and other dairy products (Threlfall, 2000). Some studies have reported that animal origin foods are considered to be the primary source of human salmonellosis (Acha and Szyfers, 2001). *Salmonellae* are infrequent cause of mastitis in dairy cows but several species of *Salmonella* have been documented to colonize udders and shed at levels of up to 2000 Organisms /ml (Liyuwork et al., 2013). According to Jayarao and Henning (2001) *Salmonella* was isolated from 6.1% of bulk tank milk samples from dairy herds in eastern South Dakota and western Minnesota, United States of America.

Members of the genus *Salmonella* are gram negative and rod shaped short bacilli bacteria belonging to the family *Enterobacteracae* (Ellermeier and Slauch, 2006). *Salmonella* are comprised of two central species, *Salmonella enterica* and *Salmonella bongori* (Brenner *et al*., 2000). Presently, six subdivisions of *Salmonella enterica* subspecies exist with over 2500 serovars currently identified and several common serovars to human clinical infections (Coburn, 2007). *Salmonella* is primarily intestinal bacteria that widespread in the environment and commonly found in farm effluents, human sewage and in any material subject to fecal contamination. Salmonellosis has been recognized in all countries but appears to be most prevalent in areas of intensive animal husbandry, especially poultry, dairy and swine production (Wray and Davies, 2003). The distribution of *Salmonella* can vary greatly depending on the Serovars. Generalist species

such as *Salmonella enterica* serotype *Enteritidis* and *Salmonella enterica* serotype *Typhimurium* have established global niches (Ellermeier and Slauch, 2006).

Salmonella is a leading cause of foodborne mortality and morbidity in worldwide (Morbidity and Mortal Weekly Report, 2008). The severity of disease depends heavily on host susceptibility and the virulence of the serovar. *Salmonella* virulence requires the coordinated expression of complex arrays of virulence factors that allow the bacterium to evade the host's immune system regardless of source and host status (Ohl and Miller, 2001). Pathogenicity is mediated by certain factors such as strain virulence, infectious dose, route of infection, and host susceptibility (Wray and Davies, 2000).

The World Health Organization (WHO) has reported as increase in the incidence of antibiotic resistant strains of *Salmonella* due to the use of antibiotics as treatment and prophylaxis frequently around the world WHO (2010). Resistance of *salmonella* to commonly used antimicrobials is increasing due to the use of antimicrobial agents in food animals irrationally both in the veterinary and public health sectors and has become worldwide problem (Tauxe, 1991 and D'Aoust et al., 1992). This is partly due to the use of antimicrobial agents in food animals at sub therapeutic level or prophylactic doses that may promote growth and markedly increase the human health risks associated with consumption of contaminated milk and meat product (Zewdu and Cornelius, 2009).

Development of antimicrobial resistant *Salmonella* has economic as well as public health importance. Studies made elsewhere indicated that dairy items are important vehicles of salmonella for raw consumers (Jay, 2000). In Ethiopia, limited studies were carried out on milk and milk products. The objectives of this study were to determine the prevalence and antimicrobial resistant profile of *Salmonella* isolated from bulk milk of dairy cows.

MATERIALS AND METHODS

Study area

The study was carried out in100 dairy farms found in Debre Zeit and the surrounding areas from December 2013 and April 2014. Debre Zeit town is located Misraq shewa zone of the oromia region, some 47.9 km south east of Addis Ababa and has total human population of 95000. It is located at latitude and longitude of 10° 35' 0" N and 35° 48' 0" E respectively. The area lies at an altitude of 1850 meter above sea level and experiences the mean annual rainfall, maximum and monthly temperatures ranges between 801.3mm, 25.5 0 C, 23.7 in July & 27.70 C in may, respectively. The mean annual minimum temperature is 10.50 C, monthly values ranges between 7.4 in December and 12.10 C in July and August.

Study design

The study was cross sectional and the targets were government and private holdings dairy farms and supermarkets. The list of all the currently operational large dairy farms registered in Debre Zeit was collected from the city municipality. The minimum sample size was calculated by using the following formula according to Thrusfield (2007): n = Z^2 *p* exp (1-*Exp*xp)/ d^2 where n = sample size, Exp (expected prevalence) = 2.1% (Tesfaw *et al.*, 2013), d (absolute precision) = 0.05 at 95% confidence interval. Accordingly the sample size was calculated to be 32. To maximize the precision of the estimate the calculated sample size was inflated more than three times and a total of 106 samples were considered.

About 30 ml of milk from bulk milk was collected by using sterile screw capped labeled universal bottles and transported to the microbiology laboratory of the college of veterinary medicine and agriculture, Addis Ababa university, Debre Zeit, by using an ice box. Upon arrival, the samples were immediately processed or stored over night in a refrigerator at 4°c until processed in the following day.

Isolation and identification of *Salmonella*

A total of 106 bulk milk sample from cross and local breed cows were included in the study from different households/small holders, supermarket, and large sized dairy farms found in Debre Zeit and the surrounding area, Ethiopia. A sampling unit consisting of 30ml of bulk milk was taken aseptically at random from milk tank container using screw capped universal bottles. The isolation of *Salmonella* was performed according to the standard operating procedure set by the global *salmonella* surveillance and laboratory support project of the WHO and the National Health Services for Wales (NHS), in which both procedures use ISO-6579 (ISO, 2002). Overnight frozen samples were allowed to thaw for 3-5 hours at room temperature before analysis. The bacteriological media used for the study were prepared following the instructions of the manufacturers.

Five ml of each sample were pre-enriched in 45ml Buffered Peptone Water (BPW) (Himedia) in a ratio of 1:9 and incubated for 16 to 24 hours at 37°c. Following this, 1 ml and 0.1 ml aliquot of the pre-enrichment broth was transferred

aseptically into 10 ml of selenite- F(Himedia) and 10 ml of Rappaport-Vassilliadis (RV) broth(oxiod), mixed and then incubated for 24 and 48 hours at 37ºC and 42ºC respectively. Following incubation, a loopful of each culture was streaked onto one plate of Brilliant Green Agar (oxiod) and another plate of Xylose Lysine Deoxycholate (XLD and BGA) medium and incubated at 37ºC for 24 to 48 hours. Most *Salmonellae* give an alkaline reaction in BGA medium and have pink colonies. On XLD (Himedia) medium the majorities of *Salmonella* serotypes produces hydrogen sulphide and have red colonies with a black (H2S) center (Quinn *et al.,* 1994). A total of four suspected colonies from each sample were plated on nutrient agar for further biochemical tests.

Biochemical tests

Colonies from the nutrient agar were tested by the following biochemical tests: indole, methyl red, Voges proskauer, urea broth, lysine iron agar (oxiod) and Triple Sugar Iron (TSI) (oxiod) (ISO 6579, 2002). All four suspected pure culture isolates colonies were inoculated on TSI agar, lysine iron agar, urea broth, Tryptone broth, methyl red Voges proskauer(MR-VP)broth (Himedia) and incubated for 24 or 48 hours at 37ºc. Colonies producing an alkaline slant with acid (yellow color) butt on TSI with hydrogen sulphide production and gas formation, alkaline slant, butt hydrogen sulphide production on lysine iron agar, negative for urea hydrolysis (yellow color), negative for tryptophan utilization (indole test) (yellow-brown ring), negative for Voges-Proskauer (yellow color of the media) were considered to be *Salmonella* positive (ISO 6579, 1998 and Quinn et al., 1994).

Antimicrobial tests

The antimicrobial susceptibility test was performed according to the National Committee for Clinical Laboratory Standards (NCCLS, 2007) by using the Kibry-Bauer disk diffusion method. The antimicrobials included Streptomycin (STR, 25µg), Ceftriaxone (CRO, 30µg), Oxytetracycline (OT, 30µg), Sulfamethoxazole (SXT, 25µg), Chloramphenicol (C, 30µg), Ampicillin (AMP, 10µg), Nalidixic acid (NA, 30µg), Amoxicillin (AMC, 30µg) and Kanamycin (KA, 30µg) (Oxoid). From each isolate, four to five biochemically confirmed well isolated colonies grown on nutrient agar were transferred into tubes containing 5ml of brain heart infusion broth (Oxoid, England). The broth culture was incubated at 37ºC for 8hrs until it achieved the 0.5 McFarland turbidity standards. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller Hinton agar plate (Oxiod CM 0337 Basingstoke, England). The plates were held at room temperature for 30minutes to allow drying. Antibiotic discs were placed on the inoculated agar surface at about two cm apart. The plates were incubated at 37ºc overnight and diameter of the zones of inhibition was measured. The measurements were compared with zone size interpretative chart furnished by Clinical Laboratory and Standard Institute (CLSI) guideline M100-S17 and the zones were graded as sensitive, intermediate and resistant.

Statistical analysis

The data were entered into Microsoft office excel spread sheet and analyzed using SPSS statistics (Version 20.0) software. The Chi-square test was used to assess the significant differences among breed, source of milk sample and amount of milk. Effects were reported as statistically not significant since the P value is greater than 5% since P value was 0.77.

RESULTS

Prevalence of *Salmonella*

Of the 106 bulk milk samples 25 (23.6 %) were positive for *Salmonella*. Ninety eight of the samples were from intensive farms and *Salmonellae* were isolated from 23(23.5%) of the samples. The occurrence of *Salmonella* in large and small scale farms was 20.4% and 27.3 % respectively. The bacteria was isolated from 18(22.0%) and 2(25%) bulk milk samples of cross bred and local cows respectively. The occurrence of the bacteria were not significantly different among variables/factors such as sample source/farm size, breed and milk yield/day since P value is greater than 5 % (Table 1).

Mono drug resistance

The mono drug resistant features of the isolates are presented in table 2. Only one isolate was sensitive to all of the drugs tested. A higher proportion of the isolates (96%) were resistant to ampicillin and the lowest resistance was recorded for streptomycin (8%). Resistance to ceftriaxone and nalidixic acid were observed in 7(28%) and 4(16%) of the isolates respectively. Except for ampicillin and co-trimoxazole intermediate resistance was recorded for all antimicrobials.

Multi drug resistance

The Multi Drug Resistance (MDR) profiles of the isolates are shown in Table 3. All isolates were resistant to two or more drugs except one (Table 3). Of the 14 MDR profiles, the AMP OT AMC phenotype was recorded in 5 isolates, seven isolates displayed resistance to three drugs, one isolate was resistant to six drugs and two isolates were resistant to seven drugs.

Table 1. Prevalence of *salmonellae* isolated from bulk milk of dairy farms in Debre zeit, Ethiopia

Factors	Number of sample examined	Positive (%)	X^2	df	P value
Source					
Large farm	49	10 (20.4)	0.608	2	0.738
Small holder	44	12(27.3)			
Supermarket	13	3(23.1)			
Breed					
Cross	82	18(22)	0.652	2	0.77
Local	8	2(25)			
Mixed	16	5(31.2)			
Milk amount					
8-50	46	12 (26.1)	0.646	2	0.724
51-150	13	2(15.4)			
>150	47	11(23.4)			

X^2=chi-square; df=degree of freedom

Table 2. Antimicrobial sensitivity test results of *Salmonella* isolates from bulk milk of dairy farms in Debre zeit, Ethiopia

Antimicrobials	Resistant No. (%)	Intermediate No. (%)	Susceptible No. (%)
AMC	12(48)	9 (36)	4(16)
AMP	24(96.0)	-	1(4)
C	10(40)	5(20)	10(40)
CRO	7(28)	9(36)	9(36)
K	5(20)	5(20)	15(60)
NA	4(16)	5(20)	16(64)
OT	21(84)	1(4)	3(12)
STR	2(8)	2(8)	21(84)
SXT	9(36)	-	16(64)

AMP=Ampicillin; CRO=ceftriaxone; K=kanamycin; NA=Nalidixic acid; STR=Streptomycin; OT=Oxytetracycline; SXT= Trimethoprim-sulfamethoxazole; C=Chloramphenicol; AMC=Amoxicillin

Table 3. Multi drug resistance profiles of *Salmonella* isolates (n = 24)

Number of Antimicrobials	Resistance Pattern	No. of isolates (%)
Two	AMP OT(5)	5(20.8)
Three	AMP NA SXT (1)	7(29.2)
	AMP OT AMC(5)	
	AMP K AMC(1)	
Four	AMC CRO OT C(3)	5(20.8)
	AMP NA OT AMC(1)	
	AMP OT SXT C(1)	
Five	AMP OT AMC SXT C(1)	4(16.7)
	AMP K NA SXT C(1)	
	AMP S OT SXT C(1)	
	AMP OT C AMC SXT(1)	
Six	AMP CRO STR OT SXT C(1)	1(4.2)
Seven	AMP K NA OT SXT C AMC(1)	2(8.4)
	AMP CRO K OT SXT C AMC(1)	

AMP=Ampicillin; CRO=ceftriaxone; K=kanamycin; NA=Nalidixic acid; STR=Streptomycin; OT=Oxytetracycline; SXT= Trimethoprim-sulfamethoxazole; C=Chloramphenicol; AMC=Amoxicillin

DISCUSSION

In this study an overall prevalence of 23.6% was estimated. The present estimates are higher than reports from finding of 8.9% estimate in east tennessee and southwest virginia (Rohrbach et al., 1992) and the 6.1% estimate in south eastern dakata and western minnesota according to Jayaroo and Henning (2001). Previous studies on milk samples collected from lactating dairy cows at sebeta, Ethiopia (Abrham et al., 2013) and Addis Ababa (Addis et al., 2011) reported prevalence estimates of 16% and 28.6% respectively. According to Liyuwork et al (2013) and Robert S Barlow et al (2015) the prevalence estimate of salmonella in milk of lactating dairy cows and fecal samples from beef, dairy and veal calf of cattle were 2.1% and 14.4% in Addis and Ababa, Ethiopia and Australia respectively. The difference in prevalence between different studies might be associated with difference in the hygienic and farm management practices. Epidemiological patterns of Salmonella differ between geographical areas depending on climate, population density, farming practice, food harvesting and processing technologies and consumer habits (Radostits et al., 1994). The prevalence of foodborne pathogens in milk is influenced by numerous factors such as farm size, hygiene, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies used, geographic location, and season (Oliver et al., 2005). The higher occurrence of *Salmonella* poses a significant health risks to humans. It is generally accepted that the occurrence of any *Salmonella* isolate in food items should be regarded as a risk human being (Fathi et al., 1994). Thus raw milk from farms and markets predispose the household and the community to *Salmonella* infections and could result in food poisoning outbreaks. All *Salmonella* isolates but one was resistance to two or more antimicrobials. Resistance to most these drugs was also reported in earlier studies in Ethiopia (Addis et al., 2011; Teshome and Anbessa, 2012; Abrham et el., 2013 and Tesfaw et al., 2013) and elsewhere (D'Aoust et al., 1992 and White et al., 2001). The isolates were resistant to antimicrobials commonly used in veterinary and public health settings. The reasons for the emergence of antimicrobial resistant *Salmonella* isolates was most likely due to the indiscriminate use of antimicrobials (Guthrie, 1992), medications without prescriptions and administration of sub therapeutic dose of antimicrobials (Acha and Szyfers, 2001).

CONCLUSION

The occurrence of *Salmonella* in bulk milk samples is considerable and a potential source of food borne salmonellosis. Assay of antimicrobial resistance revealed that almost all isolates of *Salmonella* were resistant to two or more of the antimicrobials tested whereas only a few isolate was sensitive. The significantly high proportion of multidrug resistant isolates poses a serious threat to raw milk consumers and suggests the need to consume only cooked milk. The currents study indicated the necessity of a further investigation on the prevalence and antimicrobial susceptibility pattern of *Salmonella*, by considering it as a potential food borne pathogen, starting from the farm to table.

Competing interests
The authors have no competing interests to declare.

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