Sindh Univ. Res. Jour. (Sci. Ser.) Vol. 50 (004) 647-652 (2018)

http://doi.org/10.26692/sujo/2018.12.00105

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Isolation and Antimicrobial Drug Susceptibility Profiling of *Salmonella* isolates from Raw Milk

Y. M. JALBANI, A.H. SHAH[++], G. S. BARHAM*, N. A. KOREJO**, Q. KALWAR***, S. G. TUNIO*

Shaheed Benazir Bhutto University of Veterinary and Animal Sciences and Sakrand and Institute of Quality Standards and Testing Technology for Agro Products

Received 09th March 2018 and Revised 1st November 2018

Abstract: The present study was conducted to determine the prevalence of *Salmonella* spp. in milk. The samples were collected from two intermediaries viz. farms (n=50) and retail shops (n=50) and antimicrobial susceptibility of the isolates were performed. The overall prevalence of *Salmonella* isolates at dairy farm was recorded as 28% amongst which 50% were *S. typhi*. Further, results revealed that eight dairy farms (80%) were positive, while two farms (20%) were detected as negative for *Salmonella*. The maximum prevalence was noted in Farm 7 (100%) whereas; milk samples from Farm 2 and Farm 10 were free from *Salmonella* contamination .None of the dairy shops found free of *Salmonella* carriage. The overall prevalence of salmonella was recorded as 44%. Among the salmonella isolates the prevalence of *S.typhi* was 54%. The maximum prevalence (60%) of *Salmonella* spp. in milk was noted in samples from five shops i.e. Shop 2, 4, 5, 9 and 10. More so, *S. typhi* was detected in 70% of milk samples from all seven shops except Shop 3, 6 and 9 which showed maximum carriage as 100%. Whereas, samples from three shops viz. Shop 1, 2 and 7 were free of *S. typhi* contamination. The organisms were found highly resistant to Neomycin and Kanamycin (84.21%) followed by Oxytetracycline (78.95%), Tetracycline (73.68%), Gentamycin (68.42%), Cefixime (52.63%), Cefoxitin (47.37%), Ciprofloxacin (36.84%) and Cefipime (32.11%). The overall MDR profile showed 68.4% *S. typhi* isolates were resistance to three and/or above antibiotics. A total of 26.31%, 21.04%, 15.78 and 5.26%, *S.typhi* showed resistance against three, four, five and six antimicrobial agents, respectively.

Keywords: Antimicrobial Drug Susceptibility, *Salmonella* isolates, raw milk

1. INTRODUCTION

Food security is a critical issue throughout the world owing to outbreaks of food origin illnesses which ultimately result in considerable expenses individuals, the food stuff manufacturers and the financial system. In spite of advances in food science technology, food borne diseases are still one of the key problem in relation to public health and cost-effective problem in both developed and developing countries. (WHO, 1995). The food safety is a major issue, where animal origin foods such as milk and meat products are generally regarded as high risk with respect to pathogen contents. It is supposed that the 75% of pathogens have been affecting humans worldwide from the last 10 years. It has been caused by pathogens acquiring from animals and animal origin foods (WHO, 2011). *Salmonella* species have been considered as one of the most important milk-borne pathogen for humans all around the world (Jamshidi *et al.*,2009). *Salmonella*spp. Still ranks to be at top position among the diarrhea causing bacteria. It is also to be known that typhoidal *Salmonella* causing diseases to a population of 0.2 billion with 1.3 billion cases of intestinal illness along with 3 million of death throughout the world annually (Gobum *et al.*, 2007). Salmonella is the second most commonly reported human zoonosis in spite of reduces in frequency in the last three years in EU (EFSA Journal, 2007). The presence of zoonotic pathogens in the food chain entails the necessity of intervention strategies as well as reliable and efficient control of production lines in food items. Antimicrobial drug resistance of *Salmonella* organisms is to be expected as a result of the use of antimicrobial drugs in animals, often used for food purpose. Such variety of drugs may be of farmer's interest because it can be either used therapeutically or prophylactically or as growth promoter (feed additives). An untoward effect of the wide scale use of antibiotics is to develop antibacterial resistance, a response adapted by microorganisms in which they are able to tolerate certain amount of drug to which it was previously susceptible. Such resistance response to inactive specific antibiotic drugs is mainly due to genetic the genetic variability and capability of microbial populations to adopt environmental conditions. Resistances mechanisms of both typhoidal and typhoidal *Salmonella* spp. have been also increased in developing countries. Various *Salmonella*s rovers are Able to resist the conventional antibiotics such as

[++] Correspondence Author: Y. M JALBANI, and A.H. SHAH email: Ym_Jalbani@Yahoo.Com

*Department of Animal Products Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam

**Department of Veterinary Medicine, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam

***Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS, Beijing

ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and other newer antibiotics (quinolones and extended-spectrum cephalosporin) have been reported with increasing frequency in many areas of the world (Su *et al.*, 2004).

2. MATERIAL AND METHOD

Sample collection

A total of hundred (n=100) raw milk samples were collected from two points *i.e.* dairy farms (n= 50; five samples from each farm) and retail shops (n= 50; five samples from each farm) in and around the Hyderabad city. Sampling was done from April to September; however, the samples were collected at morning time. The milk sample was thoroughly mixed first before sample taken into sterilized sample bottles which were finally transported under chilled conditions (4^0C) within 4-6 hours to the department of Animal Products Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sind Agriculture University Tandojam for further processing.

Isolation of *Salmonella*

Milk sample was inoculated on selective agar i.e. *Salmonellashigella*agar (SS agar) using (Pore plate method) streak plate method for the isolation *Salmonella* spp. Black centered colonies were selected and picked using sterilized wire loop and sub cultured on *Salmonella* shigella agar in order to purify the organism.

Identification of *Salmonella*

The presumptive identification of isolated bacteria was done by various tests including colonial morphology, Grams staining, motility, oxidase, catalase, indoxyl acetate hydrolysis, triple sugar iron, urease, Simmon's citrate, coagulase, methyl red and vogus prausker tests according to Bergey's Manual of Systematic Bacteriology (Holt, 1994).

Antibiotic susceptibility profiling

Antibiotics of different groups **(Table-1)** used to test susceptibility of the *Salmonella* species by disc diffusion method following the Clinical Laboratory Standard Institute (CLSI 2010) protocol.

Table-1 List of antibiotics for antibiotic susceptibility testing of *Salmonella* isolates

S. No	Class/group of antibiotics	Antibiotic Disc	Disc potency (ug)
1.	Aminoglycosides	i. Gentamycin	10
		ii. Neomycin	30
		iii. Kanamycin	30
2.	Cephalosporins	i. Cefixime	5
		ii. Cefoxitin	30
		iii. Cefepime	30
3.	Quinolones	i. Ciprofloxacin	05
4.	Tetracycline	i. Tetracycline	30
		ii. Oxytetracycline	30

Preparation of McFarland standard

Inoculums obtained from the fresh culture plates of pure bacterial colonies derived from parent culture were prepared by mixing in Muller Hinton broth. Inoculums turbidity was adjusted to an equivalent of 0.5 McFarland standards ($1.5x10^8$cfu/ml). 1ml suspension from subsequent inoculum was transferred to Muller-Hinton agar plate supplemented with 5% sheep blood to produce a confluent lawn of bacterial growth.

Muller Hinton agar

Commercially available medium was weighed (38g) and suspended in one litter of distilled water. The medium was bring about to boil for proper mixing and dissolving. Prepared agar was autoclaved at121^0C for 15 minutes. About 25 ml of prepared media was poured in 90mm sterilized Petri dishes. Incubation of dishes was done at 37°C for 24 hours to confirm the sterility of medium.

Disc Diffusion Method

The antibiotic susceptibility pattern of *Salmonella typhi* was determined by using Disc Diffusion method as described by Bauer *et al.* (1966). The CLSI (clinical lab standard institute) criteria were adopted to interpret the diameter of the zones of different antibiotics. The plates were left for drying at 37°C for ~5 minutes before impregnation of antibacterial discs on to the prepared agar. Incubation of all the plates was set under aerobic condition at 37°C for 48 hours. Following incubations, the diameter of zones of inhibition was measured.

Molecular conformation of *Salmonella typhi*

For the molecular identification of *Salmonella* isolates, extracted DNA was used as template for PCR using primers, as described by Song *et al* (1993). The sequence of oligonucleotide primer sets were used, which are given in (**Table-2)**. The reaction was performed in volume of 50 µL which contained 5 µL of 10X Taq buffer, 4 µL 2.5 mM dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP), 38.5 µL of deionized distilled water was taken in a 1.5 micro centrifuge tube. Then 0.5 µL (25 pmol) each of gene specific forward (ST1) and reverse (ST2) primers were added. Afterwards 1 µL of extracted DNA was added and finally 0.5 µL Taq polymerase was added and contents of tube mixed well by pippetting. The tubes were placed in a thermal cycler and allowed to run by the following protocol by Massi *et al.* (2003) and Ambati *et al.* (2007). The cycling conditions were set as; denaturation at 94^0C (30 sec) which was followed by annealing at 54^0C for 30 sec, and finally elongation at 72^0C for period of one minute with a final extension at 72^0C for 5 min. This whole procedure was repeated for 30 cyles in a thermal cycler (Eppendorf, Germany). The PCR products were run on 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light and photographed.

Table-2 Oligonucleotide sequences for *Salmonella* typhic identification

Subject	Target gene	Sequence (5' – 3')	Band size (bps)	Nucleotide sequence
S. typhi	Flagellin gene-I	ST1 – 5'-TAT GCC GCT ACA TAT GAT GAG-3'	495	1036-1056
		ST2 – 5'-TTA ACG CAG TAA AGA GAG-3'		1513-1530
	Flagellin gene-II	ST3 – 5'-ACT GCT AAA ACC ACT ACT-3'	364	1072-1089
		ST4-5'-TGG AGA CTT CGG TCG CGT AC-3'		1416-1435

Table-3 Differential characteristics of *Salmonella* species

Test(s)	Biochemical Test Result	
	***Salmonella* spp.**	***S. typhi***
TSI	Alkine/Acidic	Alkaline/acidic
Citrate	+ve	-ve
Dulcitol	+ve	-ve
Rhamnose	+ve	-ve
Indole	-ve	-ve
Methyl Red	+ve	+ve
VP	-ve	-ve

Gel Electrophoresis and band visualization

One percent of agarose gel was prepared by adding 10 ml TAE buffer and dissolved in 490 ml of distilled water using microwave oven. The mixture was cooled slightly (45^{0}C) at room temperature. The gel was poured in gel tray with comb inserted in it and left till it was fixed completely. A volume of 450 ml TAE buffer was added in electrophoresis tray. The gel containing tray was placed in electrophoresis tray and comb was removed gently. The ladder of 1000 bp was loaded into first well. In all other wells, PCR products of samples were loaded.

In each well 6µL were added both from ladder as well as from samples. The electrophoresis unit was run at 100 volt for 35 minutes. After that gel containing tray was placed gently in gel documentation system, visualized in computer and clear bands were recorded (if any) and the band size was measure with the marker.

3. RESULTS

Identification of *Salmonella typhi*

The differential characteristics of other species of the genus *Salmonella* vs *S. typhi* on the basis of biochemical tests are presented in Table-1. Morphologically, the organisms showed transparent colonies with black centers on SS agar, whereas, biochemically, *Salmonella typhi* showed negative reaction on Citrate, Dulcitol, and Rhamnose while other spp. Of Salmonella showed positive reaction. In case of Methyl Red, Indol and Vogues Prospkuer all spp. were found to be having similar type of reactions. For confirmation *Salmonella typhi*, all presumptively identified isolates were subjected to nested polymerase chain reaction. *Salmonella* spp. suspected as *S. typhi* were confirmed by generation of band size 495 (in first round) and 364 bps (in second round) by targeting or amplification of flagellin gene I and II, respectively.

The overall results regarding the *Salmonella* isolates **(Fig. 3)** from dairy farms revealed that eight dairy farms (80%) were positive whereas two farms (20%) were detected as negative (0%). The maximum prevalence was noted at Farm 7 (100%) followed by Farm 1, 4, 8 and 9 (20%). However, the milk samples from farm-2 and farm 10 were free of *Salmonella* contamination. Furthermore, among the samples analyzed for *Salmonella* spp., *S. typhi* were detected in 50% of the samples.

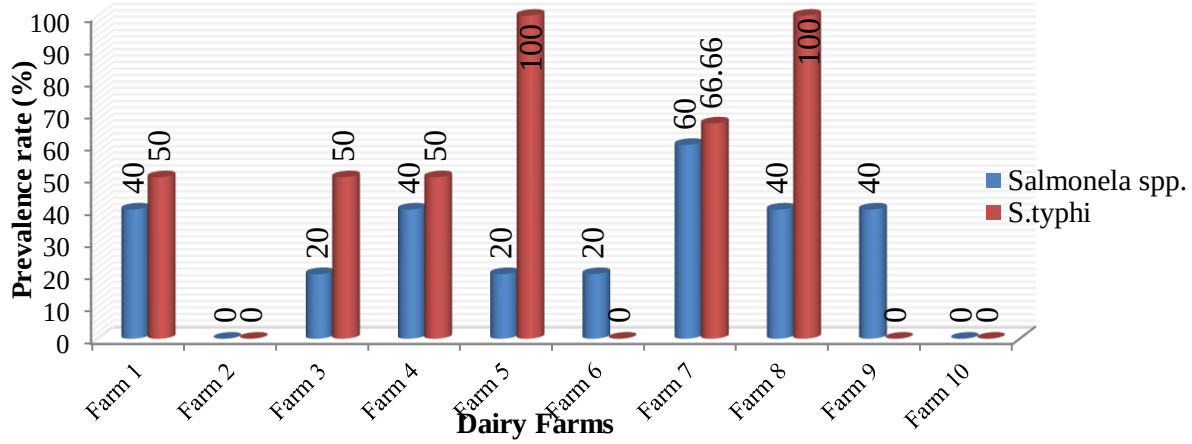

Fig. 3: Prevalence of *Salmonella* Isolates at farm level.

(Fig.4) demonstrates that none of the dairy shops found free of salmonella carriage. The maximum prevalence (60%) of *Salmonella* spp. in milk was noted in samples from five shops i.e. Shop 2, 4, 5, 9 and 10 followed by shop 3, 7 and 8 (40%) and shop 1 and 6 (20%). More so, *S. typhi* was detected in 70% of milk samples from all seven shops except Shop 3, 6 and 9 which showed carriages 100%. Whereas, samples from three shops viz. Shop 1, 2 and 7 were free of *S. typhi* contamination.

Fig. 4 Prevalence of Salmonella in dairy milk at retail level

Antimicrobial Susceptibility Profile of *Salmonella typhi* isolates from raw milk.

The results regarding the antimicrobial susceptibility patterns of *S. typhi* was noted as resistance and/or sensitive and are presented in **(Fig. 5)**. A total of nineteen (19) *S. typhi* isolates detected from milk were further tested for antimicrobial susceptibility pattern profiling. The organisms were found highly resistant to Neomycin and Kanamycin (84.21%) followed by Oxytetracycline (78.95%), Tetracycline (73.68%), Gentamycin (68.42%), Cefixime (52.63%), Cefoxitin (47.37%), Ciprofloxacin (36.84%) and Cefipime (32.11%). In contrast to resistance, the isolates found sensitive to Ciprofloxacin (63.16%) followed by Cefepime (57.89%), Cefoxitin (52.62%), Cefixime (47.38%), Gentamycin (31.58%), Tetracycline (26.32%), Oxytetracycline (21.05%), Neomycin (15.79%) and Kanamycin (15.79).

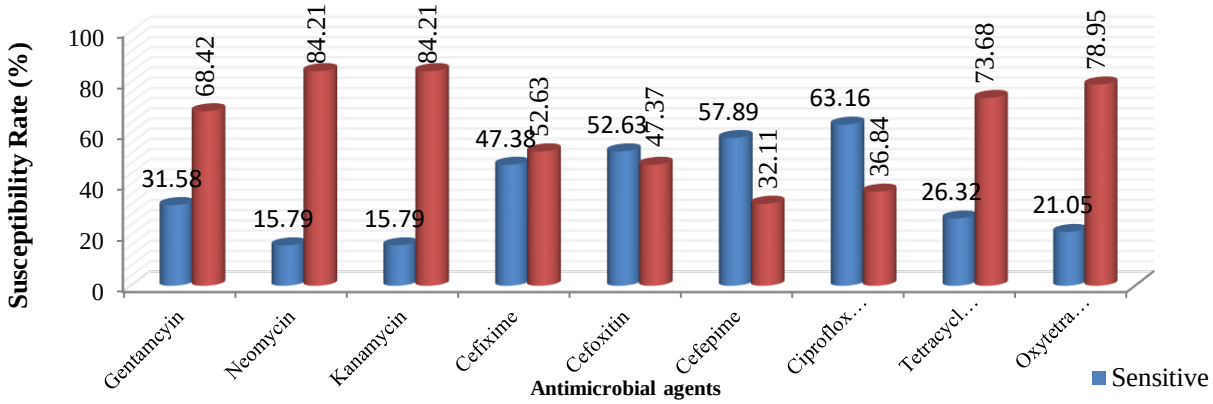

Fig. 5 Antimicrobial Susceptibility profile of *S. typhi* isolates from raw milk

Muti-drug Resistant patterns of *S. typhi*

Fig. 6 representing the multi-drug resistant pattern of *S. typhi* isolates. The overall multidrug resistance pattern (MDR) profile showed 68.4% *S. typhi* isolates were resistance to three and/or above antibiotics. A total of 5.26% organisms showed resistance against six antimicrobial agents while 15.78% isolates were resistant to five antibiotics. Similarly 21.05% showed resistance to four antibiotic combinations. The maximum percentage (26.31%) of resistance was noticed against combination of three antimicrobial drugs. Furthermore 21.05% isolates showed resistance against two antibiotics and 10.5% organisms were resistance to only single drug.

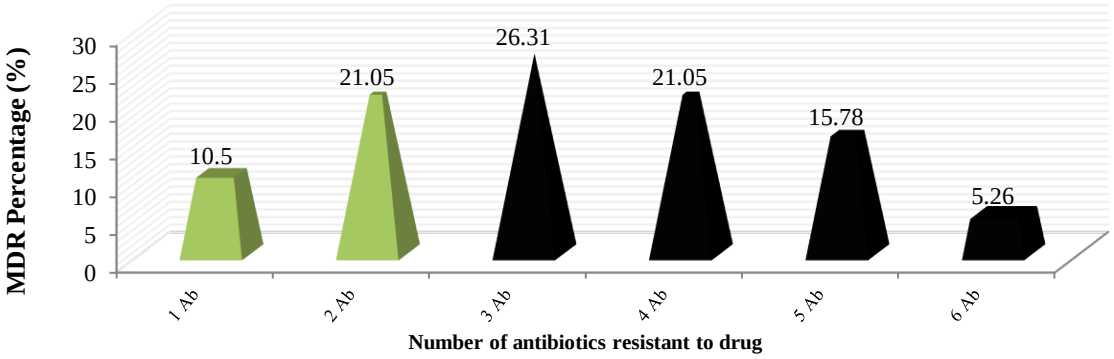

Fig. 6 Multi-drug Resistant patterns of *S. typhi* isolated from raw milk

4. DISCUSSION

Food borne pathogens are considered as the solemn problem, affecting public health worldwide. *Salmonella* isolates in animal origin foods are alarming signals for public health. In the present study, the prevalence of *Salmonella* in milk was determined and results revealed that 50% samples from dairy farms carried *Salmonella spp*. These results are in accordance with those reported in India by Zagare *et al.* (2012) who reported that *Salmonella* was found as predominant in all milk and dairy samples. From Ethiopia, Zelalem *et al.* (2012) reported the prevalence of *Salmonella*(47.8%) at dairy farms, however, prevalence varied with scales of farms *i.e.* 57.1%, 33.33% and 83.33% at small, medium and large farms, respectively. Further studies showed that 54% of the samples from dairy retail shops carried *Salmonella* spp. Lubote *et al* .(2014) in Tanzia further corroborate this study by reporting *Salmonella* prevalence in street vendors as 43.75% compared to dairy farms (33.33%). In USA, Karns *et al.* (2005) reported a quite low prevalence (11.8%) from bulk tank milk by using polymerase chain reaction method. The difference in results might be due to due to conditions where animals are reared like housing conditions, feeding habits, types of feed given to the animals. The variations in results could be Lack of cooling facilities, contaminated water usage; improper handling of milk storage equipments and lack of knowledge on good hygienic practices are key features. Vahedi *et al.*(2013) stated that the long transportation period is a major cause of contamination of milk. In order to reduce milk contamination the utensils and equipments must be cleaned and rinsed using boiled water proper detergents and disinfected immediately after use (Chye *et al.*, 2004). Enteric fever is one of the serious persistent health troubles, Inspite of wide usage of antibacterial drugs and even with the development of extended spectrum antibacterial drugs. This is due to the rapidly gained resistance to different antibiotics by *Salmonella typhi* (Butt *et al.*,2003). The results of the present study revealed that the maximum resistance to Kanamycin and Neomycin (84.21%). Resistance to Aminoglycosides is in accordance with Oluyege *et al.* (2009) in Nigeria, they reported the resistance of Gentamycin as (71.8%). Furthermore in Ethiopia, the findings of Tesfew*et al.* 2013) opposes the results of present study for susceptibility of Gentamycin. In Mexico, very low resistance pattern for Gentamycin (3.2%) and Kanamycin (4.3%) of *Salmonella* isolates was detected by Bywater *et al.* (2004). Tajbaksh *et al.* (2013) from Shahkhord, Iran observed the resistance pattern of Neomycin and Kanamycin as (21.42%), which is quite different result in comparison with present study. Antimicrobial drugs are the greatest contribution of the twentieth century therapeutics. As a class, they are most frequently used as well as misused drugs (Tripathi, 2010). Strainssensitive to antimicrobial agents in human medicine constitutes 50% (the other half is in animals) and 80% of human use antibacterial drugs as home practice means out of hospitals (Miller and Pegeus, 2002).

Multi-drug resistance is a major clinical problem in food borne pathogens. Multi-drug resistance (≥4 drugs) is one of the major threats to humans and animals which limit therapeutic selection of antibiotics (Kurincic *et al.*, 2005). A total of 5.26%, 15.78%, and 21.05% *S. typhi* were found resistant against six, five, and four antibiotics, respectively. According to Adabara *et al.* (2012) the multiple drug resistance strain of *S. typhi* was observed. Similarly, Tesfaw *et al.* (2013) confirmed the resistant to one or more of the antimicrobial drugs were used against all the isolates.

5. CONCLUSSION

It is concluded that antibiotic-resistant *Salmonella*, in particular multi-drug resistant *Salmonella typhi* frequently found in milk collected at farm and retail level is an alarming signal for public health perspective.

REFERENCES:

Adabara, N. U., B.U. Ezugwu, A. Momojimoh, A. Madzu, Z. Hashiimu D. Damisa, (2012). The Prevalence and Antibiotic Susceptibility Pattern of *Salmonella typhi* among Patients Attending a Military Hospitalin Minna, Nigeria. Advances in Preventive Medicine, 1-4.

Addis, Z., N. Kebed, Z. Worku, H. Gezahegn, A. Yirsa, T. Kassa, (2011). Prevalence and Antimicrobial Resistance of *Salmonella* isolated from lactating cows and in contact humans in dairy farms of Addis ababa. BMC. Infectious Diseases, (11): 222-228.

Ambati, S. R., G. Nath, B. K. Das (2007). Diagnosis of typhoid fever by polymerase chain reaction. Indian Journal of Pediatric, (74): 909-913.

Bauer, A. W., M. Turck, (1996). Antibiotic susceptibility test by standardized single disk method. American Journal of Clinical Pathology, (36): 493-496.

Butt, T., R. N. Ahmad, A. Mahmood, S. Zaidi, (2003). Ciprofloxacin treatment failure in typhoid fever case in Pakistan. Emerging Infectious Diseases, 9(16): 21-22.

Bywater, R., H. Deluker, E. Deroover, A. De Jong, H. Marion, M. McConville, T. Rowan, T. Shryock, D. Shuster, V. Thomas, M. Valle, A. Walters, (2004). European survey of antimicrobial susceptibility among Zoonotic and Commensal bacteria isolated from food producing animals. Journal of Antimicrobial Chemotherapy, (54):744-754.

CLSI, (2010). Performance standards for antimicrobial susceptibility testing. Twelfth Informational Supplement M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute, 30(1).

Goburn, B., G. A. Grassl, B. B. Finlay, (2007). *Salmonella*, the host and disease: A brief review. Immunological Cellular Biology, (85): 112-118.

Jamshidi, A., M. R. Bassami, S. Afshari-Nic, (2009). Identification of *Salmonella* spp. and *Salmonella typhimurium* by a multiplex PCR-based assay from poultry carcasses in Mashhad- Iran. International Journal of Veterinary Research,(3): 43-48.

Karns, J. S., V. Kassel, B. J. McKluskey, M. Perdue, (2005). Prevalence of *Salmonella* from US dairies as food safety by Polymerase Chain Reaction. Journal of Dairy Sciences, (88): 3475-3479.

Kurincic, M., I. Berce, T. Zorman, S. S. Mozina, (2005). The prevalence of multiple antibiotic resistance in *Campylobacter* spp. from retail poultry meat. Food Technology Biotechnology, 43(2): 157-163.

Levy, S. B. (1997). Antibiotic resistance: an ecological imbalance. In: Antibiotic resistance: Origins, evolution, selection and spread, Ciba Foundation Symposium 207, Chadwick I and Goode Journal Wiley, Chichester, 1-14.

Lubote, R., F. Shahada, A. Matemu, (2014).Prevalence of *Salmonella* spp. and Escherichia coli in raw milk value chain in Arusha, Tanzania. American Journal of Research Communication, 2(9): 1-13.

Massi, M. N., T. Shirakawa, A. Gotoh, M. Kawabata, (2003). Rapid diagnosis of typhoid fever by PCR assays using one pair of primers from flagellin gene of S. typhi. Journal of Infection and Chemotherapy, (9): 233-237.

Miller, S. I., D. A. Pegeus, (2002). Principles and practice of infectious diseases, 6th edition, Churchill Living stone, New York, 2346.

Nia, S. S., F. Rostami, F. S. Dehkordi, E. Rahimi, E. Yahaghi, E. K. Darian, M. B. Moghadam, (2014). Isolation and Evaluation Virulence Factors of *Salmonella typhimurium* and *Salmonella* enteritidis in Milk and Dairy Products. Iranian Journal of Medical Microbiology, 8(1): 54-61.

Oluyege, A. O., A.C. Dada, A. M. Ojo, E. Oluwadare, (2009). Antibiotic resistance profile of bacterial isolates from food sold on a University campus in south western Nigeria. African Journal of Biotechnology, 8 (21): 5883-5887.

Song, J. H., H. Cho, H.B. Moon, C.H. Pai, (1993). Detection of *Salmonella typhi* in the blood of patients with typhoid fever by polymerase chain reaction. Journal of Clinical Microbiology, (31): 1439-1443.

Su, L.H., C.H. Chiu, C. Chu, J.T. Ou, (2004). Antimicrobial resistance in nontyphoid *Salmone lla*serovers: a global challenge. Clinical Infectious Diseases, 39(4):546-51.

Tesfaw, L., B. Taye, S. Alemu, H. Alemayehu, Z. Sisay, H. Negussie, (2013). Prevalence and antimicrobial resistance profile of *Salmonella* isolates from dairy products in Addis Ababa, Ethiopia. African Journal of Microbiology Research, 7(43):5046-5050.

Tripathi, K. D, (2010). Essentials of medical pharmacology, 6th edition, Jaypee brothers Medical Publishers (P) ltd, New Delhi. 668Pp.

Vahedi, M., M. Nasrolahei, M. Sharif, A. Mirabi, (2013).Bacteriological Study of Raw and Unexpired Pasteurized Cow's Milk Collected at the dairy Farms and Super Markets in Sari City in 2011. Journal of Preventive Medicine and Hygiene,(54):120-123.

WHO, (1997).The medical impact of the use of antimicrobials in food animals.Report of a WHO meeting, Berlin, Germany, October 1997. WHO, Geneva, 13-17.

WHO, 2011.http://www.who.int/zoonosis/vph/en.

Zagare, M. S., A. M. Deshmukh, S. S. Patil, (2012). Analysis of Dairy Pack Food for Presence of Bacterial Pathogens. International J. of Science, 1(1):5536-5541.

Zelalem, Y., B. Faye, (2006). Handling and microbial quality of raw and pasteurized cow's milk and Irgo-Fermented milk collected from different shops and producers in Central Highlands of Ethiopia, 6(2): 67-82.