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Isolation of Non-Typhoidal *Salmonella* from Sheep faeces in Eastern Hararghe, Ethiopia

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Abstract

A cross-sectional study was conducted from October 2014 to May 2015 on sheep faeces at Eastern Hararghe to isolate non-typhoid *Salmonella* spp. Non-typhoidal *Salmonella* (NTS) represents an important human and animal pathogen causing gastro-enteritis on global scale. Animals are the main reservoirs for the organism. A total of 113 sheep fecal samples were collected and processed bacteriologically according to the protocol recommended by the International Standardization Organization designed for isolation of *Salmonella* from food and animal feces (ISO-6579, 2002) with some modifications. Among a collected 113 fecal samples, 7(6.19%) were positive for *Salmonella*. However, the difference was not statistically significant (p-value>0.05). Based on ages groups the highest prevalence of *Salmonella* isolate was obtained from old sheep 2(12.5%) and the adult sheep yields the least. But, the difference was not statistically significant (p-value>0.05). In this study the higher prevalence of *Salmonella* isolate was pointed out from male with 7.69% than female sheep with 5.41%, even though the association between sexes was not statistically significant (P-value>0.05). The disease was found more prevalent in the animals living together with human. In conclusion, non-typhoidal *Salmonella* was more prevalent in Eastern Hararghe and this study indicates that the necessity of a further investigation on the isolation, identification, serotyping and antimicrobial susceptibility testing of non-typhoidal *Salmonella* in the study area.

Keywords: Feces; Non-typhoidal *Salmonella*; Sheep; Eastern Hararghe

Introduction

Ethiopia consist a huge and diverse livestock population. Livestock production in Ethiopia has a long traditional practice. Small ruminant population of Ethiopia is one of the largest in Africa [1]. Sheep population in Ethiopia is estimated to be around 25.9 million heads [2]. It is estimated that 1,078,000 sheep and 1,128,000 goats are used in Ethiopia for domestic consumption annually Majority of these animals are found in the highlands while a quarter of them are reared in the lowlands. Sheep are economically living banks for their owners and serve as source of immediate cash, meat, hair production and insurance against crop failure especially where land productivity is low and unreliable. In general, sheep helps in poverty alleviation schemes [3,4]. However, there are many constraints to sheep production system. Those constraints are considered diseases (viral, bacterial, parasitic and fungal diseases), feed shortage and lack of veterinary service. Bacterial diseases including Non-Typhoidal Salmonellosis (NTS) are becoming real threat to sheep production and zoonoses [5].

The genus *Salmonella* obtained its name from the American veterinarian Daniel Elmer Salmon, who first isolated *Salmonella enterica* serotype Cholerae suis from pigs in 1885 [6]. It is speculated that the genera of *Escherichia coli* and *Salmonella* diverged from a common ancestor [7]. Some writers estimate *Salmonella* diverged from the genus *Escherichia*, 120-160 million years ago [8].

Salmonellosis is the leading most common food borne bacterial zoonoses caused by organisms of the genus *Salmonella* causing gastroenteritis on a global scale. It causes significant morbidity and mortality in both humans and animals and restricts trade [9]. Salmonellosis can be typhoidal and non-typhoidal. Unlike typhoidal Salmonellosis caused by *Salmonella typhi* and *Salmonella Paratyphi*, whose only reservoir is humans; non-typhoid Salmonellosis is acquired from multiple animal reservoirs and represents an important human and animal pathogen. NTS infections in animals are not only of importance because of the direct economic impact diseased animals represent but also because diseased animals may serve as a source of infection for humans, although it can be spread from person to person [10].

The genus Salmonella comprises two species. Those are; (1) *Salmonella enterica*, which is divided into six subspecies: *Salmonella enterica* subspecies enterica, *Salmonella enterica* subspecies salamae, *Salmonella enterica* subspecies arizonae, *Salmonella enterica* subspecies diarizonae, *Salmonella enterica* subspecies houtenae and *Salmonella enterica* subspecies indica; and (2) *Salmonella bongori* (formerly called *Salmonella enterica* subspecies bongori). Recently, there are more than 2541 serotypes (serovars) based on their surface somatic (O), flagellar (H) and capsular (V) antigen [11]. *Salmonella* is a facultative anaerobic gram-negative rods within the family of Enterobacteriaceae [12,13] having peritrichous flagella except *Salmonella Pullorum* and *Salmonella Gallinarum* which lack flagella for mobility [14]. *Salmonella* grow optimally at 35°C to 37°C, PH for growth is between 4.0 and 9.0 (although it is tolerant to drying, it is not much resistant to heat like other enterobacteriaceae). They catabolize a variety of carbohydrates into acid and gas, use citrate as the sole carbon source, and produce hydrogen sulphide (H_2S) and decarboxylate lysine, but failed to metabolize lactose, sucrose and urea [15].

Epidemiologically, Salmonellosis has been recognized in all countries but appears to be most prevalent in areas of intensive animal husbandry, especially poultry and swine production [16]. The season has a great influence on the infection rate of an animal. Studies from tropical Africa have consistently shown that NTS is more common during the rainy season [17]. *Salmonella* spp. are transmitted by the faecal-oral route by either consumption of contaminated food or water [18,19], person-to-person contact, or from direct contact with infected animals, and inclusion of infected sheep to the flocks [20].

Salmonella avoid host defense in the stomach and reach the intestines, and the bacteria interact with the non-phagocytic cells such as the epithelial cells of the intestinal mucosa [21]. They adhere to the intestinal epithelial cells by adhesive structures (fimbriae) and invade epithelial cells to provoke gastroenteritis. The organisms have virulence factors such as virulence-plasmids, toxins, fimbriae and flagella that help in establishing an infection [22]. Infection in a given host may or may not be clinically apparent [23]. The severity of disease depends on infecting dose, health of the host, and *Salmonella* strain. In subclinical form, the animal may have a latent infection; remain as carriers and eliminate the agent in its fecal material. Clinical disease usually appears when animals are stressed by factors such as transportation, crowding, food deprivation, weaning, parturition, and concurrent viral or parasitic disease. In the clinical form of the disease animals may exhibit septicemia, and acute or chronic enteritis. The septicemic form usually common in lambs is characterized by depression and fever (40.5-41.5°c) which may end in death in 2 days. The enteric form is common in adults and manifested by fever, in appetence, diarrhea, pungent smelling mucus feces, abortion in ewes, and emaciation [24]. Diagnosis is based on clinical sign and laboratory examination of the feces, tissues from affected animal, feed and water [25].

The emergence of antimicrobial resistance has complicated the treatment and management of enteric fever [26]. The risk of introducing Salmonellosis into a herd/flock can be decreased by buying animals from *Salmonella*-free sources, isolating newly acquired animals, avoiding contamination of feed and water and removing aborting ewes, fetus, and placenta. To control human infections, wear protective gloves, wash hands carefully after handling aborted or diarrheic animals. Food should be well cooked [23].

Non-typhoidal Salmonellosis results from infection by *Salmonella serovars* such as *S. typhimurium*, *S. dublin* and *S. newport* in cattle; *S. typhimurium*, *S. dublin*, *S. anatum* and *S. montevideo* in sheep; *S .typhimurium* and *S. choleraesuis* in pigs; and *S. typhimurium*, *S. anatum*, *S. newport*, *S. enteritidis* and *S. arizonae* in horses. Pullorum disease (caused by *S. pullorum*) and fowl typhoid (caused by *S. gallinarum*) are found in chickens [27].

Ethiopia is known for a specific NTS serotype, *Salmonella Concord*. *Salmonella Concord* were first identified in Ethiopia more than two decades ago in a bone-processing factory and in human patients in Addis Ababa [28]. Recent reports on severe *Salmonella Concord* infections in adopted Ethiopian children and their foster families in many countries of Europe and the U.S.A have alerted the public health importance of the disease [29,30]. The initial confinement of *Salmonella Concord* to Ethiopia suggests that environmental, ecological or cultural factors such as local food habits may play an important role in the transmission dynamics. However, little is known about risk factors, zoonotic reservoirs and antibiotic resistance profiles of *Salmonella Concord* in Ethiopia. In addition, other studies conducted in Ethiopia indicated the presence of *Salmonella* in various food animals [31]. However, information on non-typhoidal Salmonellosis in sheep and goat is very limited [19]. In the light of unknown environmental and animal reservoirs and the scarcity of data available on *Salmonella*, it is imperative to establish surveillance to isolate the agent. Therefore; this study is conducted to isolate and determine the prevalence of non-typhoidal *Salmonella* from sheep feces at Eastern Hararghe, Ethiopia.

Material and Methods

Study area description

The study was conducted using simple random sampling from October 2014 to May 2015 on sheep faeces at Eastern Hararghe. Eastern Hararghe was located in Oromia regional state which is 500 km far to East of Addis Ababa, the capital city of Ethiopia. Geographically, is situated at 410 51' 58" N latitude and 90024'10"S longitude. The area is located at 2000 m altitude above sea level and receives an average annual rain fall of approximately 900 mm, with a bimodal distribution pattern, peaking in mid-April and mid-August. There are four seasons, such as a short rainy season (from mid-March to mid-May), a short dry season (from end May to June), a long wet season (July to mid-October) and a long dry season (end of October to February). Main pasture production is expected after the short rainy season, continuing until the end of the long wet season. Mixed type agriculture is the main occupation of the population of the area. Ecologically, the area has 65% midland and 35% lowland zones [32]. The two predominant soil types are 60% rigo soils and 40 % heavy black clay soil. The mean annual temperature ranges from 10°c to 18°C with a relative humidity of 65%. The husbandry system was sedentary system and the animals are reared mainly for marketing (HADB, 2010).

Study animals

The study animals were randomly selected sheep from small holder farm by simple random sampling method. Animals of both sexes were sampled.

Study design

A cross-sectional study involving microbiological analysis was conducted from October 2014 to May 2015 with consecutive sampling to isolate and determine the prevalence of non-typhoidal *Salmonella* (NTS) in sheep feces based on the protocol described by International Standardization Organization (ISO) 6579.

Sample size determination

Sample size was determined by using the average (3%) of four *Salmonella* prevalence, 2.1% [19], 4.8% [31], 2.1% [33] and 3.3% [34] obtained from researches conducted in the country. The absolute precision was decided to be 5% at 95% confidence level and the sample size was calculated according to Thrusfield [35] shown below:

Where, n=required sample size, P_{exp}=expected prevalence, d^2= desired absolute precision

Thus, based on the above formula the calculated sample size was 113. A total of 113 sheep were randomly selected to isolate and determine the prevalence of non-typhoidal *Salmonella* from sheep feces.

Study Methodology

Sample collection and transportation

Two peasant associations called Bechake and Yifa bate were selected from the Eastern Hararghe randomly. Fecal samples were collected aseptically from the rectum by placing a gloved hand directly in the rectum. Then, it was immediately placed in sterile disposable plastic bottle. During sampling age, sex, source, housing conditions of each animal and address of the owners were recorded. Age of the sheep was determined based on the eruption of incisor teeth cited by Gatenby [5] and grouped as young (less than 1year), adult (1 year 1 month to 3 year) and old (more than 3 years). The bottles were labeled by source, age, and sex, and put in a cool ice box with ice pack. Then, it was transported to Microbiology Laboratory and bacteriologically processed up on the arrival within a maximum of one to two hours.

Salmonella isolation procedures

The isolation procedure was conducted at Microbiology laboratory based on ISO 6579 protocol designed for isolation of *Salmonella* species in animal feces and environmental samples (ISO 6579) [36] with some modification. The isolation technique involves three steps; non-selective pre-enrichment, selective enrichment and plating out described as follows; Twenty five grams of feces from individual sheep was crashed in a sample bottle by wooden spatula. Twenty five grams of feces was crashed in a sample bottle by wooden spatula. For pre-enrichment it was added into a disposable plastic container with 225 ml of buffered peptone water (BPW) (Oxoid, CM 0509, Basingstoke, Hampshire, England). The sample mixture was homogenized well by shaking and incubated at 37°C for 18-24 hours. After pre-enrichment, a portion of 1 ml culture was transferred into a tube containing the selective enrichment media of 10 ml Selenite Cysteine (SC) broth (SC: Difco TM, Becton, Dickinson, USA) and incubated at 37°C for the next 18-24 hours. Another 0.1 ml portion was transferred into 10 ml of Rappaport-Vassiliadis medium with soya (RVS broth) (RVS; LABM, LAB 86, Lancashire, UK) for enrichment and incubated at 41.5°C ± 1°C for 24 hours. Following enriched, the culture was mixed well with vortex mixer and a loop-full of inoculum was inoculated on to Xylose lysine deoxycholate agar (XLD agar) (XLD; Oxoid, CM 0469, Basingstoke, England), *Salmonella Shigella* Agar (SSA) (SSA; LABM, LAB 052, Lancashire, UK) and Brilliant green agar (BGA) (BGA; Difco TM, Becton, Dickinson, USA) plates prepared according to the manufacturer direction (Annex 8.3) with steak method separately. The plates were incubated at 37°C for 18-24 hours in an inverted position [37]. After 18-24 hours, the plates were detected for typical *Salmonella* colony growth characteristics. The incubation period was prolonged to 48 hrs for those that did not show any growth during the 18-24 hrs. On XLD a typical *Salmonella* colony has a slightly transparent zone of reddish color and a black centre; a pink-red zone also seen in the media surrounding the colonies. Typical *Salmonella* colonies on a BGA agar plate cause the colour of the medium red/pink. The colonies were grey-reddish/pink. Typical colony grown on SSA has a black centre with lightly pale zone.

Biochemical confirmation

Two to three typical suspect colonies per plate were sub-cultured on nutrient agar plates (Oxoid CM 0003, Basingstoke, England) and incubated at 37°c for 18-24 hours. Finally, pure colonies were confirmed biochemically by using Kligler Iron Agar (KIA) (KIA; LABM, LAB 059, Lancashire, UK), O-Nitrophenyl Galactopyronidase (ONPG), Lysine Decarboxylate (LDC), Citrate (Difco, Detroit, USA), Urease and Indole test. Urease and LDC tests were performed by using Urease and LDC (LDC; DiatabsTM, Rosco diagnostica) diagnostic tablets (DIATABS) respectively. Indole test was done from LDC result after adding kovac's reagent [38]. Then, the results were seen after incubation time. *Salmonella* colonies produce an alkaline slant (red) with acid (yellow color) but on KIA with or without hydrogen sulphide production (black color or not), positive for lysine (purple color), negative for urea hydrolysis (red color), negative for tryptophan utilization (yellow-brown ring), and positive for citrate utilization (blue color) [39].

Data analysis

Data collected in the study and the results of laboratory investigations were entered into Microsoft Excel, edited, coded and analyzed by statistical methods using proper statistical analysis (STATA) version 11.0. Descriptive statistics was used to determine the prevalence of non-typhoidal *Salmonella* in the study area. The association between explanatory variables (Peasants association, sex, age and housing system) was computed by fisheries exact test. Statistically significant associations between variable is considered if the calculated p-value is less than 0.05 with 95% confidence interval.

Results

In the present study a total of 113 fecal samples were collected from different sheep within two peasant associations (PAs) of eastern Hararghe to isolate non-typhoid and determine the prevalence of *Salmonella* from sheep feces. Out of the total of 113 samples, a total of overall 7(6.19%) samples were positive for *Salmonella*.

Risk factors		No. examined	No. of positive (%)	95% Confidence interval	X^{2*}	p-value*
PAs	Yifa Bate	57	5(8.77%)	2.91-19.30	0.438	0.226
Age	Bacheke	56	2(3.57%)	0.44-12.31	-	-
	Young	19	1(5.26%)	0.13-26.03	0.407	-
	Adult	78	4(5.13%)	1.42-12.61	-	-
	Old	16	2(12.50%)	1.55-38.35	-	-
Housing	Together*	58	5(8.62%)	0.44-12.53	0.242	0.432
	Alone	55	2(3.64%)	2.86-18.98	-	-
Sex	Female	74	4(5.41%)	1.49-13.27	0.691	0.457
	Male	39	3(7.69%)	1.62-20.87	-	-

: Together with human and other animal; X^{2*}: fisher's exact; P-value: 1-sided fisher's exact

Table 1: Prevalence of *Salmonella* based on different risk factors.

There was an increase in the number of positive samples from Yifa Bate as compared to Bacheke. However, there was no significant statistical association (p-value>0.05) between two PAs. The higher *Salmonella* infection rate was detected in old sheep feces (12.5%), followed by young (5.26%) and adult (5.13%) sheep. Despite, the

highest prevalence in old sheep, there was no significant statistical association between different age groups (Table 1).

In this study, the prevalence of *Salmonella* isolate was higher on animals living together with human (8.62%) as compared to animals living alone in separate room from human and other animal (3.64%). The statistical association between two living condition was not significant (p-value>0.05). On the other hand, higher *Salmonella* isolate was detected in male 3(7.69%) than female 4(5.41%) sheep. Even, there was no statistically significant association (p-value>0.05) between sexes (Table 1).

Discussion

Non-typhoidal *Salmonella* infection influences animal health and breeding negatively as they result in significant economic loss and important public health problem. The present study revealed that an overall prevalence rate of 6.19% *Salmonella* isolates in study area. This result is consistent with the range cited by D'Aoust [40] which indicated that the prevalence of *Salmonella* in sheep falls between 2 and 51.5%.

The current finding also relatively granted with the reports by Karim et al. [41] in Bangladesh who detected 5.7% Salmonella isolates from the fecal sample of sheep. However, it was lower than the previous findings by Zweifel et al. [42], Edrington et al. [43] and Teklu and Negussie [35] who reported 11%, 7% and 7.7% *Salmonella* isolates from the fecal samples of sheep slaughtered in Switzerland, USA and Export Abattoir of Modjo Ethiopia respectively. The differences in these results might be attributed to the difference in the stress condition and/or exposure of animal to the infected animal due to uncontrolled animal movement while held in the market and lairage which could leads to increased infection rate among the animal.

In Norway, Sandberg et al. [18] reported 0.8% *Salmonella* isolate in rectal swabs of sheep. In UK Davies et al. [44] reported 0.1% *Salmonella* isolates from the fecal samples of the sheep slaughtered in abattoirs. In Ethiopia Anbessa and katema [33] isolated 3.3% *Salmonella* from sheep faeces in Jimma, Woldemariam et al. [19] and Molla et al. [31] reported 2.1% and 4.8% isolates in the fecal samples of apparently healthy slaughtered sheep in Debrezeit abattoirs and sheep and goat in central Ethiopia respectively and Bedaso et al. [45] isolated 1.04% *Salmonella* from apparently healthy sheep and goats slaughtered at Addis Ababa Abattoir Enterprise. In contrast, the current study pointed out higher *Salmonella* isolates than these previous study reports. This result report variation might be due to the variation in the ecology of study area, sampling technique and sample origin, housing system of study site and research season. Studies from tropical Africa have consistently shown that NTS infection is more common during the rainy season. Grahams [17] documented that the seasonal pattern of diseases such as malnutrition may increase the risk of *Salmonella* infection.

The difference in the prevalence of *Salmonella* between sexes was not statistically significant. Eventhough, in the study observed the higher prevalence of *Salmonella* isolates from male (7.69%) than female sheep (5.41%). The proportion of *Salmonella* between two PAs was not statistically significant (p-value>0.05). Higher isolate was obtained from Yifa Bate as compared to Bacheke PAs. This variation could be because of the differences in the management system and sampling season. It is known that keeping animals overcrowded in small area could enhance the excretion and transmission of infection [25].

Siera, et al. [46] reported 10% isolates on freshly dressed lamb carcass in Spain. In contrast, even though, the difference was not statistically significant (p-value >0.05) the present study indicated that higher *Salmonella* isolates in older sheep (12.5%) than young and adult sheep. This variation might be because of the sample type as the carcass may be contaminated by personnel and slaughtering environment. In addition, adults and old sheep has more chance of acquiring the infection through repeated exposure to the different *Salmonella* strain due to communal pasture grazing and in close contact with different infected animal of the other herd. But, in young due to maternal immunity and less exposure to other herd as they kept mostly around homestead they have less chance of acquiring infection [47]. Animals living alone and together with human and other animals shows the prevalence of their *Salmonella* isolates insignificantly associated (p-value>0.05). However, infection rate was higher in animals living together with human and other animal in the same room (8.62%) than living alone (3.64%). This condition might arise from cross-contamination among animal and human.

Conclusion and Recommendations

Animals are the main reservoirs for non-typhoidal *Salmonella* and they shed the pathogen along with their feces. Studying the prevalence of non-typhoidal *Salmonella* from sheep is of paramount importance to reduce the possible transmission of *Salmonella* between sheep and humans. The current study revealed that high prevalence of non-typhoidal *Salmonella* isolates (6.19%) from sheep faeces in eastern Hararghe of Yifa Bate and Bacheke PAs. Non-typhoidal *Salmonella* has importance on zoonoses, revenue losses and animal health impact. However, scanty of studies were conducted on Non-typhoidal *Salmonella* and other *Salmonella* in the Ethiopia.

Therefore, the following points are forwarded as recommendations:

i. Further investigation on the isolation, identification, serotyping and antimicrobial susceptibility of *Salmonella* should have to done to monitor *Salmonella* infection rates and define the epidemiology of the disease.

ii. The animal and their owner should have to live in separate room to reduce cross-infection

iii. Prevention and control measures should be practiced against non-typhoidal *Salmonella* infection.

Acknowledgements and ethics approval and consent to participate

Not applicable.

Availability of data and materials

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Competing Interests

The authors declare that they have no competing interests.

Author's contribution

We author's contributions are equal.

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