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Prevalence and Antibiotic Resistance of *Salmonella* Species Isolated from Chicken Eggs by Standard Bacteriological Method

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Abstract

Salmonella have been found to be the major food borne disease in the world with a serious public health problem. The current study was carried out to detect and to determine the prevalence and antibiotic susceptibility of *Salmonella* isolated from fresh raw chicken eggs collected at Haramaya University Poultry Farm in Eastern Ethiopia. Among the total 384 chicken eggs, *Salmonella* spp. was detected from 2.9% (11/384) of egg samples using culture technique and was confirmed by biochemical test, nine *Salmonella* spp. (2.4%) were detected from egg shell and two (0.5%) from egg contents; predominantly occurred in floor house system. The prevalence of *Salmonella* in eggs on the bases of chicken breed sources was 2.9%, 3.8% and 2% for Bovans, Fayoumi and White leg horn, respectively. The prevalence difference did not show statistical significance (P>0.05) between the rate of detecting *Salmonella* spp. among the egg shell and egg contents, and similarly, non-significant analytical situation was observed in eggs sampled from different chicken breeds. Among the sample sources, egg samples examined from cage and floor house were found *Salmonella* positive with the prevalence of 2.3% and 3.3%, respectively. However, there was no statistically significant difference (P>0.05) in the prevalence of *Salmonella* among the two house systems. All identified isolates were tested for susceptibility to a six commonly used antimicrobials by disk diffusion technique. Out of the 11 isolates tested 8(72.7%) were resistant to one or more of the tested antimicrobials. The most common resistance observed was to tetracycline (72.7%), ampicillin (72.7%) and amoxicillin (63.6%). However, spectinomycin, kanamycin and chloramphenicol were effective against most of the *Salmonella* isolates.

Keywords: Antimicrobials; Eggs; Prevalence; Resistance; *Salmonella*

Introduction

Food borne diseases are among the most widespread global public health problems of recent times, and their implication for health and economy is increasingly recognized [1,2]. The majority of foodborne outbreaks are caused by *Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, and *Campylobacter* strains [3]. Among these pathogens, *Salmonella* are considered the most prevalent foodborne pathogens worldwide [4,5] and has long been recognized as an important zoonotic pathogen of economic significance in animals and humans, predominantly in the developing countries. The important route of transmission of *Salmonella* organism from animals to man is via food products of animal origin which may be contaminated at the source or during handling [6]. Epidemiological studies show that chicken eggs and meat are two of the most important sources for consumer ingestion and contact of pathogens [7]. Chicken eggs in particular continue to be identified as leading food sources for human Salmonellosis [5,8].

The true incidence of salmonellosis in both humans and animals is difficult to evaluate in developing countries because of the lack of epidemiological surveillance systems [9,10]. The ubiquity of *Salmonella* isolates makes them a persistent contamination hazard to all raw foods [11]. Those of animal origin food products are often implicated in sporadic cases and outbreaks of human salmonellosis [12,13]. The distribution of *Salmonella* serotypes from poultry sources is geographically variable and changes over time, although several serotypes are consistently detected at a high incidence throughout much of the world. Many of the *Salmonella* serotypes that are most prevalent in humans are also common in poultry [14], suggesting a possible epidemiologic connection between the poultry and human reservoirs of *Salmonella*. *Salmonella* infection in chickens has important implication on public health worldwide [15]. Infected chickens can deposit *Salmonella* in either the yolk or albumen of developing eggs because of the colonization of different regions of the reproductive tract [16,17]. It is not yet clear as to which route is most important for *Salmonella* to contaminate the egg contents, which may be contaminated with *Salmonellae* by vertical transmission and/or horizontal transmission [18]. Although some authors claim horizontal transmission to be the most important way to contaminate eggs. Barrow and Lovell, most authors claim that vertical transmission is the most important route of egg contamination [19].

In recent years, *Salmonella* related diseases have been documented by several food related studies conducted in different parts of Ethiopia [9,20]. An increased in the resistance of *Salmonella* to commonly used antimicrobials has been also noted in both public health and veterinary sectors in Ethiopia [20]. Antimicrobial resistance is a natural consequence of infectious agents' adaptation to exposure to antimicrobials used in medicine, food animals, crop production and use of disinfectants in farms and households [21-23]. However, scarcity of surveillance data on the incidences of *Salmonella* species associated with eggs and its antimicrobial resistance pattern in the poultry farm is a major epidemiological issue. Despite some attempts to study prevalence of *Salmonella* in Ethiopia, mainly in meat and meat products, the status of the problem in eggs is still very much unknown.

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Very little information is available at this time for *Salmonella* infection of egg in the country. However, studies made elsewhere indicated that eggs are important sources of *Salmonella* particularly among those raw consumers [24]. Therefore, this study was aimed to determine the prevalence and distribution of *Salmonella* spp. on chicken eggs by conventional culture methods and biochemical assays, and also to assess the antimicrobial resistance of the isolates.

Materials and Methods

Study site

The study was performed on egg samples collected in Haramaya University Poultry Farm located at Haramaya, Ethiopia. It is approximately 500 kilometres away from Addis Ababa, capital city of Ethiopia. Geographically the study site located at 41° 59'58" North latitude and 90°24'10" South longitudes. The elevation of this area is about 2000 meters above sea level and its mean annual temperatures ranges from 10°Cto 18°C with the relative humidity of 65 percent respectively. The area receives an annual rain fall of 800 millimetres within a bimodal distribution of the season's pattern peaking in mid-April and mid-August of the year. The farm serves mainly for people residing in and the surrounding of East Ethiopia. In the farm, three breeds of chicken (Bovans, Fayoumi and White leg horn) are used for the purpose of egg production and production of day old chicks, which distributed for consumers and farmers of the surroundings and to different regions of the country.

Study design and sampling

A Cross-sectional study was conducted to determine the prevalence, distribution and antimicrobial susceptibility of *Salmonella* in chicken eggs from three breeds of layer chickens. Simple random sampling technique was used to collect egg samples from Fayoumi, Bovansand White leg horn breeds of layer chickens in cage and floor housing systems

Egg sample collection

The sample size was calculated according to Thrusfield, using 95% confidence interval and 0.05 absolute precision by assuming expected prevalence of 50% [25]. In total, 384 freshly laid and unwashed chicken eggs were aseptically collected from the farm. The collected egg samples were transported to the laboratory of Veterinary Microbiology, College of Veterinary Medicine, Haramaya University under cold chain and analysed using microbiological protocols for *Salmonella* isolation and identification. Information on breed, coded ID number of egg and house was registered during collection of egg samples.

Culture method

Standard cultivation method recommended by the International Organization for Standardization (ISO 6579, 2002) was carried out for isolation and identification of *Salmonella* [26]. Each chicken eggshell were dipped in sterile peptone broth and swabbed with sterile cotton swabs and then added in Buffered Peptone Water (BPW). In addition, surface sterilized eggs were cracked with a sterile knife and each egg's content was mixed thoroughly and 25 gm of the mixed egg content was inoculated into 225 ml of peptone broth. The mixture then homogenized using a laboratory blender (Stomacher 400R, Seward, England) for 30 seconds. The pre-enriched samples, both from egg shells and egg contents, were incubated for overnight at 37°C. After the overnight incubation, 1 ml of the pre-enrichment broths was transferred aseptically into a tube containing 10 ml of Muller-Kauffmann-tetrathionate(MK) broth and incubated at 37°C for overnight. Following incubation, a loopful of each enrichment broth culture streaked onto one plate of xylose lysine desoxycholate (XLD) agar and another plate on *Salmonella*-shigella (SS) agar and incubated at 37°C for 24 hr. The plates (XLD and SS agars) were examined for the presence of typical *Salmonella* colonies. Characteristic colony for *Salmonella* isolates were then transferred onto nutrient agar and incubated aerobically at 37°C for overnight.

Biochemical test

Each identified colonies with typical *Salmonella* morphology were confirmed biochemically by inoculating into lysine iron agar (LIA), triple sugar iron agar (TSI) slopes, urea agar base, tryptophan broth and methylered-vogesproskaur (MR-VP) medium with confirmation carried out following incubated at 37 °C for 18-48 hours, and interpreted with international organization for standardization (ISO 6579, 2002) [26].

Antimicrobial susceptibility test

Antibiotic sensitivity of the isolates was performed according to agar disc diffusion method on Mueller–Hinton Agar using National Committee for Clinical Laboratory Standard (NCCLS, 2002) guidelines [27]. The antibiotic discs (antibiotic concentration in mg) used were consisted of ampicillin (10 mg), tetracycline (30 mg), amoxicillin (20 mg), kanamycin (30 mg), chloramphenicol (30 mg) and spectinomycin (100 mg). Results were evaluated according to NCCLS of the reference zone diameter interpretive standards (millimeter) and minimal inhibitory concentration (MIC) breakpoints. Strains were evaluated as susceptible, intermediate and resistant. An isolate was defined as resistant if it was resistant to one or more of the antimicrobial drugs.

Data analysis

The raw data were entered and managed in Microsoft Excel work sheet; and descriptive statistic was utilized to summarize data. The prevalence was calculated for all data by dividing positive samples by total number of examined samples and multiplied by hundred. The association between the prevalence of *Salmonella* and associated factors (egg sample, breed and house) was assessed by Chi-square (χ^2). A statically significant association between variables is considered to exist if the computed p-value is less than 0.05.

Results

Prevalence

Salmonella spp. was isolated from 11 (2.9%) egg samples by conventional culture technique and all isolates were confirmed by biochemical test. Nine *Salmonella* spp. were identified from eggshells and 2 were recovered from egg content samples. Of the total egg samples examined, 3 (0.8%), 5 (1.3%) and 3 (0.8%) were found positive for *Salmonella* in eggs collected from Bovans, Fayoumi and White leg horn breeds of layer chickens, respectively (Table 2). Among the 11 chicken egg samples positive for *Salmonella*, 4 (1.0%) were collected from cage house system and 7 (1.9%) were received from floor house system (Table 2).

The specific prevalence of *Salmonella* detected from the total of 384

Samples	No positive	Prevalence (%)	χ^2	p-value
Eggshells	9	2.3	0.048	0.826
Egg contents	2	0.5		
Total	11	2.9		

Table 1: Prevalence of *Salmonella* by egg samples taken (n=384).

Source of Eggs	No of eggs examined	No of positive (%)	χ^2	p-value
Breeds				
Bovans	105	3 (2.9)	0.183	0.913
Fayoumi	132	5 (3.8)		
White leg horn	147	3 (2.0)		
House				
Cage	171	4 (2.3)	0.306	0.580
Floor	213	7 (3.3)		

Table 2: Association between Risk Factors.

egg samples examined for both egg shell and egg content were 2.3% and 0.5%, respectively (Table 1). Of the total egg samples examined, 9 egg shell samples found positive for *Salmonella*; 5 (2.3%) positive egg shell samples were obtained from floor house and 4 (2.3%) positive egg shell samples were obtained from cage house. From the total 384 egg contents examined only 2 (0.5%) were positive for *Salmonella*. There was slightly higher prevalence of *Salmonella* in egg shell than egg contents. On the other hand, the prevalence of *Salmonella* in the two houses of the farm is presented in Table 3. The prevalence of *Salmonella* in floor and cage houses was 3.3% and 2.3%, respectively. The result revealed that of 171 egg samples examined, 4 (2.3%) showed the presence of *Salmonella* while 7 out of 213 (3.3%) egg samples were found positive. The prevalence of *Salmonella* in cage house (2.3%) was slightly less than floor house (3.3%) in the studied farm.

Even though, there were different prevalence recorded in this study, the findings suggested that no statistically significant difference (χ^2=0.183, p-value=0.913) in the prevalence of *Salmonella* in eggs of Bovans (2.9%), Fayoumi (3.8%) and White leg horn (2%) breeds of layer chickens (Table 2). Similarly, there was no significant statistical variation (χ^2=0.306, p-value=0.580) in the prevalence of *Salmonella* in eggs among the sources from the two-house system, 3.3% in floor house and 2.3% in cage house of the farm.

Moreover, the study has also shown the prevalence of *Salmonella* between those egg shells and egg contents of the total sampled eggs, 2.9% in egg shell samples (2.3) and 0.5% in egg content samples. This result also indicated that there was no statistical significant difference (χ^2=0.048, p-value=0.826) in the prevalence of *Salmonella* in egg samples under classified egg shells and egg contents.

Antimicrobial susceptibility

Of the total 11 *Salmonella* isolates subjected to antimicrobial susceptibility test using six different antimicrobials, a total of 8 (72.7%) *Salmonella* isolates were found to be resistant to two or more (multidrug) antimicrobials tested. In relation to the total *Salmonella* isolates tested, 72.7%, 72.7% and 63.6% were found highly resistant to ampicillin, tetracycline and amoxacillin, respectively, while 36.4%, 27.3%, 18.2% and 9.0% were intermediate resistant to chloramphenicol, amoxicillin, ampicillin and kanamycin, respectively. Looking at individual antimicrobial drug, resistance to ampicillin and tetracycline was the most frequently observed, and followed by amoxacillin. In general, antimicrobial susceptibility test revealed that spectinomycin, kanamycin and chloramphenicol were the drugs indicated more active against *Salmonella* isolated from egg samples, while tetracycline, ampicillin and amoxacillin were less effective against *Salmonella* isolates.

Discussion

Eggs contaminated with micro-organisms play a significant role in poultry production pathology and in the spreading of diseases to humans [28]. The present study revealed an overall prevalence rate of 2.9% in the studied egg samples with the prevalence of *Salmonella* 2.3% from egg shells and 0.5% from egg contents, respectively. In this study, higher prevalence of *Salmonella* was obtained when compare with the prevalence reported by other studies, 0.8% from table eggs (EFSA) and 0.3% from poultry eggs in Dhaka [29,30]. The current finding is almost comparable with 3% prevalence observed in Belgium from egg shell and egg content samples examined in different housing system [31]. The prevalence of *Salmonella* in this study was however lower than 7.7% recorded in South India [32], 24.17% prevalence in Nigeria [33], 3.84% and 5.5% among the chicken eggs from poultry farm and marketing in North India [34], respectively and 8% *Salmonella* species isolated from chicken eggs of Dhaka city [35]. The variation in the prevalence of *Salmonella* in eggs may be due to lack of awareness of the status of *Salmonella* in chicken eggs and the unhygienic situation in the farm. Moreover, the management system in practice could also be the probable reason for the variation of the prevalence. Different authors reported that the presence of chickens of different ages in the farm, the presence of arthropod pests, wet and soiled litter in the farm [36], and the housing system and flock size could be important reasons for egg contamination with various micro-organisms. Chicken feeds and hatcheries also possible sources of *Salmonella* infections in the farm.

The result of this study showed a relatively higher prevalence of *Salmonella* in egg shells (2.3%) than 0.5% in egg contents. However, this difference was not statistically significant (p>0.05). The prevalence of *Salmonella* in egg shell (2.3%) in this study indicated relative agreement with 2.7% prevalence of *Salmonella* reported by Akhtar [37]. However, it is much lower than 10.5% prevalence reported by Loongyai et al. and 6.1% egg shell contamination [32,38]. Isolation of *Salmonella* from egg shell in this study may be due to contamination of eggshells at lay with faeces from intestinal carriers. De Buck et al. showed that infected birds produced the highest frequency of contaminated eggs in the first week post infection [39]. Chicken faeces, dust, litter and egg collector can also contaminate the egg shells. Smeltzer et al. indicated that eggs laid in wet, dirty nests or on the floor are more likely to be contaminated with microorganism [36]. Davies and Breslin also stated that farm environment, poor hygiene and disinfecting of materials are possible reasons of egg contamination in the farm. The findings of *Salmonella* prevalence in chicken egg contents was lower than the reports made by Suresh et al. [32] who reported 1.8% prevalence and also lower than Akhtar et al. who reports 8.33% prevalence [37]. The level of egg contents contamination in this study was slightly higher than the 0.017% prevalence reports (HKSAR) and no contamination of egg contents [38,40,41]. It is believed that the main source of egg content *Salmonella* contamination could be the infected ovary and/or oviduct. It is generally believed that the deposition of *Salmonella* inside eggs is thus most likely a consequence of reproductive tissue colonization in infected laying hens. Methner et al. also revealed that no correlation was found between the contamination of the eggshell and that of the egg contents [42]. In a study of naturally infected flocks, numerous

Antimicrobials	Number of antimicrobials tested isolates		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Ampicillin	1 (9.1)	2 (18.2)	8 (72.7)
Tetracycline	3 (27.3)	0.0	8 (72.7)
Amoxicillin	1 (9.1)	3 (27.3)	7 (63.6)
Kanamycin	10 (91.0)	1 (9.0)	0.0
Chloramphenicol	7 (63.6)	4 (36.4)	0.0
Spectinomycin	11 (100)	0.0	0.0

Table 3: Percentage of antimicrobial sensitivity test among *Salmonella* isolated from raw chicken eggs.

Salmonella serotypes, such as *Salmonella enteritidis, S. typhimurium* and *S. hadar*, were isolated from eggshells, whereas only *S. enteritidis* was isolated from egg contents [43]. Other study also reported that one-day-old chicks orally infected with *S. pullorum* produced contaminated eggs frequently during the period of sexual maturity as a consequence of reproductive tract colonization [44].

In this study, the prevalence of *Salmonella* is not much house dependent even though there was a slight increase in the prevalence of *Salmonella* from egg sample in the floor housing system (3.3%) than cage housing system (2.3%). The difference was not statistically significant. This finding was supported by the prevalence result of 1.8% in floor house and 1.4% in cage house [31]. The slight increase of prevalence may be because of hygienic status, air quality and confinement of birds. In addition, dust originated from feed and faeces may contain large number of microorganisms. The study also indicated that *Salmonella* was isolated from egg shells collected from both floor and cage house systems, whereas *Salmonella* was detected in egg contents sampled from only floor house system. The positive egg contents in the floor house system may be due to cross contamination of eggs at time of laying. Contamination of eggs with *Salmonella* was believed to occur when the organism passed from the shells into its inner contents. Spark and Board, (1985) showed that the moisture content in newly laid eggs diminishes the ability of cuticle to protect the egg contents. With so-called bed wet eggs, drops of water penetrate the cuticle, change its structure and enable micro-organisms to enter the egg contents immediately after laying. According to Humphrey increased stress could play a role to induce some changes in the chemistry of the oviduct, which might create an environment that is more susceptible for *Salmonella* in floor house system [45]. Occurrence of *Salmonella* in eggs collected from Bovans, Fayoumi, and White leg horn chicken breeds were 2.9%, 3.8% and 2.0%, respectively. There was no statistical significant difference (p>0.05) in the recovery rate of *Salmonella* from the eggs of the three breeds of layer chickens. This is presumably due to unequal exposure to the risk factors as the breeds were housed in different house system. Slightly higher prevalence was observed in eggs collected from Fayoumi chicken breed (3.8%). This difference might be due to Fayoumi breed was kept in the floor house system in which there is lower hygienic and high cross contamination between the flock eggs at laying than the cage house system.

Many of the isolates are resistant to multiple antimicrobial agents tested. The antimicrobial resistant recorded in this study is in consistent with 81% of tetracycline and 73% of ampicillin reported by Miko et al. [13]. High level of antimicrobial resistant *Salmonella* occurred in this study is probably an indication of their frequent usage both in the animal and public health sectors. The finding of this study shows slightly lower resistant than the study reported 93.1% for tetracycline and amoxicillin in Nigeria from *Salmonella* isolates in chicken eggs [33]. Alemayehu et al. also showed 52% of the *Salmonella* isolates from beef were resistant to at least three antibiotics from beef in Ethiopia, *Salmonella* isolates sensitivity to spectinomycin (100%) and kanamycin (91%) indicates the most active antimicrobial against *Salmonella* in poultry farms, which agrees with the report of an overall 2.9% spectinomycin resistance for *Salmonella* isolates from swine slaughtered in Addis Ababa abattoir [9,46]. Since the 1990s the frequency of antimicrobial drug resistance in zoonotic *Salmonella* and number of drugs to which the strains are resistant have increased, primarily as a consequence of antimicrobial use in food production may be associated with adverse consequences in several ways including treatment failures [47,48].

In the present study, the antimicrobial-resistant strains were found up to 72% of the total 11 *Salmonella* isolates tested, which is greater than those in previous studies of nonclinical isolates from dairy cattle. Most of these isolates are resistant to multiple antimicrobial agents tested, particularly for ampicillin, amoxicillin and tetracycline. Resistance to ampicillin, amoxicillin and tetracycline were widespread. This may be due to the widespread use of antibiotics included in feeds and in chickens. When compared to the resistant *Salmonella* isolates obtained from chickens in other studies, the prevalence of antimicrobial resistance in this study is much lower. 92% resistant to tetracycline *Salmonella* isolated from meat products in Ireland. The possible explanation could be the increased antimicrobial use in poultry farm and an association between resistance and virulence factors. Resistance rates to ampicillin, chloramphenicol, gentamycin, and trimethoprim of the isolates in the present study were low. However, it is important to note that these antibiotics are commonly used in veterinary medicine, and infections with these resistant *Salmonella* isolates could lower the efficiency of antibiotic treatment.

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