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Isolation and identification of *Escherichia coli*, *Salmonella* and *Pasteurella* from holding grounds of live-bird markets at Addis Ababa, Ethiopia

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A cross-sectional study was conducted to isolate and identify of *Escherchia coli, Pastuerella multocida*, *Salmonella gallinarum* and *Salmonella pullorum* from the holding grounds of five purposively selected Addis Ababa live bird markets from November 2016 to May 2017 using bacterial culture, grams staining and biochemical testing. A total of 90 pooled fecal samples were collected as both deep (35) (bottom layer of the feces) and surface (55) (top layer of the feces). The specimens were inoculated directly into blood agar and incubated at 37°C for 24 h. After subsequent subcultures of the colony on the blood agar, to get pure colonies, the isolates were cultured on MacConkey agar and *Salmonella shigella* agar to differentiate bacterial colonies. Gram staining and biochemical tests were carried out on the pure colonies and a loop of the isolates were inoculated into nutrient broth and kept for further investigation of the bacteria. The results of the study revealed that, out of the 90 total samples examined, 32 (35.55%) of the samples were found to be positive for *E. coli*. Based on the sample types the study indicated that 55 (61.10%) surface fecal samples, 32 (58.20%) samples were positive for *E. coli*, and in 35 (38.90%) deep fecal layers, there was no *E. coli* positive samples. There was a statistically significant difference between sample types and *E. coli* isolation rate (p< 0.05). *P. multocida*, *S. gallinarum* and *S. pullorum* were not found during this study. Live bird markets may serve as source of public health and economically important bacteria; however, further microbiological and epidemiological investigation is needed to determine the types of major bacteria predominating in the Addis Ababa live-bird markets and the roles and magnitudes played by these markets in the epidemiology of these pathogens.

Key words: Addis Ababa, live-bird markets, *Escherichia coli, Salmonella, Pastuerella.*

INTRODUCTION

In Ethiopia, poultry rearing is one of the economically important agricultural activities. The national chicken population is estimated to be 56.53 million (CSA, 2017), and poultry in this country is kept in traditional, small scale intensive and in large scale commercial system. Poultry production system in Ethiopia shows a clear

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distinction between traditional low input systems and modern production system using relatively advanced technology (Tadesse, 2015). The traditional backyard poultry production system which accounts for more than 98% of the poultry in the country is reared for two purposes; eggs and meat production (Asresie and Eshetu, 2015). The largest proportion of eggs and poultry meat consumed in the country comes from indigenous birds produced by rural growers. Hence, there is high movement of poultry and poultry products from rural producers to the urban consumers, which are favoring the spread of infectious agents all over the country (Duguma et al., 2017). The current live-bird marketing system represent a significant and potential hazard to both buyers and sellers in addition to causing a pronounced damage to chicken, yet implementation of bio-security and hygienic practices in the system is generally difficult (Heise et al., 2015).

The poultry population in Ethiopia is not growing as demanded; it is often under gradual decline and is highly constrained by many diseases among other factors (Heise et al., 2015). Viral and bacterial pathogens of poultry are therefore a major concern in both local and international scale, because they have represented a burden to human health and economics throughout times (Habte et al., 2017).

Avian colibacillosis which is caused by avian pathogenic *Escherichia coli* (APEC) is one of the most significant and wide spread infectious bacterial diseases occurring in poultry production. It is responsible for large financial losses for the poultry industry each year due to mortality and loses of production (Ronco et al., 2017).

Fowl typhoid and Pullorum diseases cause infectious enteritis leading to heavy mortality mostly in adult and growing chicks, respectively. These diseases are caused by the bacterium *Salmonella enterica* serovar *Gallinarum* biovar *Gallinarum* and *Salmonella enterica* serovar *Pullorum,* respectively. *Salmonella enterica* serovar *Gallinarum* is highly adapted and seldom causes significant problems in hosts other than chickens, turkeys and pheasants (Getinet et al*.,* 2017).

Pastuerella multocida, the causal agents of fowl cholera and it can cause a zoonotic infection in humans which typically is a result of bites or scratches from domestic pets (Qin et al., 2017). The disease occurs as a fulminating disease with massive bacteremia, and high morbidity and mortality. *P. multocida* is a fairly delicate organism which is easily inactivated by a common disinfectant, sun light, drying; heat and experiment suggest that the organism will survive for a maximum of thirty days in the environment (Saminathan et al., 2016).

The overall poultry trade in Ethiopian is uncontrolled and involves collection of chicken from multiple households having few numbers of marketable ages, mixed together starting from the village markets moving to the big terminal markets of big cities. Daily introduction of new birds into live bird markets (LBMs) provides opportunities for replication and adaptation of different pathogens to a new environment and the infection to persist within the market system for extended periods of time (Haftom et al*.,* 2015).

Most bacteria pathogens of poultry especially *E. coli, P. multocida* and *Staphylococcus gallinarum* can be transmitted from one to others including humans in many ways and the most causal agents are excreted from chickens through excreta feces, nasal and oral secretion, and these pathogens can persist in the environmental litters, water, feed, holding and transporting cages, vehicles, clothing of animal attendants (Emery et al., 2017; Goldstone and Smith, 2016).

Fowl typhoid, pullorum disease, avian colibacillosis and fowl cholera are one of the most devastating poultry bacterial diseases (Nhung et al., 2017). Despite those facts, no any studies were conducted to isolate of etiological agents of these diseases particularly in holding grounds of live-bird markets of Addis Ababa and generally in Ethiopia. The objectives of this study were to isolate and identify *E. coli, P. multocida, S. gallinarum*, and *Salmonella Pullorum* from the feces accumulated on the holding grounds of live bird markets and quantity the role of live-bird market in the spread of avian pathogens.

MATERIALS AND METHODS

Study area

The study was conducted in Addis Ababa which is the capital city of Ethiopia. It covers an area of 530.14 km^2 and it lies at an altitude of 2,300 m above sea level. It is located between 9.03° north and 38.74° east, latitude and longitude, respectively. The city receives variable annual rainfall with lowest and the highest annual average temperature ranging between 9.89 and 24.46°C, respectively. This area of study was chosen because it has big poultry markets (thirteen live-bird markets with average of thirty traders per market) that host chicken originating from different parts of the country.

Study population

Study population were chicken's holding grounds in selected live-bird market of Addis Ababa, namely Akaki, Merkato, Mesalamia, Saris and Shola.

Study design

A cross-sectional study was undertaken from November 2016 to May 2017 with the objectives of isolation and identification of *E. coli, P. multocida, S. gallinarum*, and *Salmonella Pullorum* from the feces accumulated on the holding grounds of live bird markets and also to assess the role of live-bird market in the spread of avian pathogen.

Sampling method

There are about thirteen live-bird markets in Addis Ababa with average of thirty traders per markets from which five markets were purposively selected which are Akaki, Merkato, Mesalamia, Saris and Shola based on number of chicken. A total of 90 pooled fecal

samples out of which 35 were deep and 55 were surface sample type were collected randomly from chicken's holding grounds found in the selected market places (Akaki, Merkato, Mesalamia, Saris and Shola). Fecal samples were chosen to be collected from the chicken's holding grounds to know whether these areas were serving as sources of the selected bacteria for the chickens kept there and sold to different households either for consumption or further rearing.

Study methodology

Sample collection

Samples were collected using sterile cryovials, swabs, sterile water, water-proof markers, gloves, and scissors during the beginning of the two rounds of the biggest festival (Christmas and Easter) in Ethiopia. These time periods were chosen, because many chickens were brought to the live-bird markets of Addis Ababa from different areas of the country during these times (especially from Dire Dawa and Harar in the East, Gojjem and Gondar in the North, Asella, Bale, Borena, Hawas and Wolayta in the South, and Ambo and Jimma in the West). Collection of the fecal samples was done both from the deep and surface layer of accumulated feces in the chicken's holding grounds. The thickness of the feces in the holding cages varies from 2 to 5 cm; therefore fecal samples were designated as 'surface' as samples collected from the top layer and 'deep'as samples collected from the bottom layer of the feces accumulated in the chicken's holding cages. From 5 different sites (corner and center of cage), surface samples were swabbed with wet sterile swab and pooled into one sample, and similarly, the samples from the deep layer of the same cages were collected separately in a pool containing 5 samples. The collected samples were then labeled using markers and kept in an ice box containing ice packs and transported using a car and finally submitted to the National Animal Health Diagnostic and Investigation Center (NAHDIC) on the same date of collection. The samples were then stored in a refrigerator at +/-4°C until laboratory investigation were undertaken.

Isolation and identification of bacteria

Bacteriological culture: The specimens were directly inoculated into blood agar, and the plates were inverted and incubated aerobically at 37°C for 24 h, after which the plates were examined for growth. Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types on blood agar. The colonies were then examined with naked eye for their morphological properties and any change in the media. The isolates were then cultured on MacConkey agar and salmonella-shigella agar to differentiate colonies of bacteria and a loop of the isolates were inoculated into nutrient broth for further investigation.

Grams staining: This method used to differentiate bacteria into Gram-negative (pink) and Gram-positive bacteria (purple). It was done based on the ability of the bacteria to retain the color of stains used during gram reaction. Gram-negative bacteria were decolorized by alcohol, losing the purple color of the primary stains (crystal violet), but Gram-positive bacteria were not decolorized by alcohol and remain as purple. After decolorization step, a counter stain (carbol fuchsine) was used to impart a pink color to the decolorized Gram-negative bacteria.

Biochemical tests: These are a series of tests that were used to identify bacteria into its generic level based on the types of enzyme produced and metabolism type performed by bacteria with sugars. Different primary and secondary biochemical tests used were Catalase, Oxidase, Motility, Oxidative-Fermentative test (O-F) and the IMViC series that consisted of four definitive tests: indole production, the methyl red test, the Voges-Proskauer test, and the citrate utilization test.

For the isolation of *E. coli* like colonies (colonies with no hemolysis on blood agar and colonies with lactose fermenting on macConkey agar), Catalase (+), Oxidase (-), Motility (+), O-F (F) tests were used to make sure that the isolated colonies were suggestive for *E. coli* after which secondary biochemical tests, IMViC (+/+/-/-) were used to identify the isolated *E. coli* from other coliform bacteria.

The colonies which were suggestive for *P. multocida* (absence of hemolysis on blood agar and no growth on macConkey agar) and *Salmonella Gallinarum* and *Salmonella Pullorum* (absence of hemolysis on blood agar, non lactose fermanter on macConkey agar and ability to grow on Salmonella-shigella agar) were subjected to gram stain and different tests (Catalase, Oxidase, Motility, O-F, and IMViC tests.

Data management and analysis

Data collected from the study area were recorded, and stored in Microsoft® Excel for Windows 2010 and transferred to Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM SPSS, 2011). Data were coded and analysed using descriptive and analytical statistics (chi-square) as appropriate. Analyses were then made to determine if there were significant differences among markets, origins of chicken and between time of sampling and sample types for *E. coli*, *P. multocida*, *S. gallinarum* and *S. pullorum* isolation. For all the analyses, confidence level (CL) is at 95% and $P \leq 0.05$ were set for significance.

RESULTS

The holding grounds of Addis Ababa live bird markets were wooden cages which were not frequently cleaned and because of this the excreta from chicken held in them made layers thicker than 2-5 cm. In the study area, there were mixing of poultry originating from different parts of the country and the major sources of poultry were as far as Dire Dawa and Harar in East; Gojjem and Gondar in the North; Arbaminch, Assella, Bale, Borena, Hawasa and Wolayta in the South and Ambo and Jimma in the West and, most of them located at distance of greater than 300 km. Different live-bird markets sampled in Addis Ababa frequently consist of gathering of native chickens that satisfy local tastes and that are held in cages, baskets or tied-up for sale.

Out of the total 90 pooled fecal samples collected from Addis Ababa live-bird markets holding grounds, 32 (35.55%) of the samples were found to be positive for *E. coli*. The result revealed that, out of 90 fecal samples 55 (61.10%) were surface fecal samples, of which 32 (58.20%) was positive for *E. coli* and 35 (38.90%) were deep fecal samples, of which there was no *E. coli* positive sample (Table 1).

The result of laboratory investigation also indicated that, out of 90 pooled fecal samples, 13 (14.44%) and 5 (5.56%) were revealed the morphological properties of *Pastuerella multocida* (absence of hemolysis on blood agar and no growth on macConkey agar), *Salmonella*

Table 1. Frequency (RF) and isolation rate of *E. coli, P. multocida*, *S. Gallinarum* and *S. Pullorum* from pooled fecal samples from live-bird markets at Addis Ababa.

Variable	Category	No. (%) tested	RF (%) of pooled samples positive for		
			E. coli	S. Gallinarum and S. Pullorum	*P. multocida*
Type of sample	Deep	35 (38.90)	0(0)	0(0)	0 (0)
	Surface	55(61.10)	32 (58.20)	0 (0)	0 (0)
Origin of birds	East	19 (21.12)	6 (31.60)	0 (0)	0 (0)
	North	13 (14.44)	5(38.50)	0 (0)	0 (0)
	South	45 (50)	17 (37.78)	0(0)	0(0)
	West	13 (14.44)	4(30.80)	0 (0)	0 (0)
Market places	Akaki	16 (17.78)	5 (31.25)	0 (0)	0 (0)
	Merkato	21 (23.33)	6 (28.60)	0 (0)	0 (0)
	Mesalamia	14 (15.56)	5 (35.71)	0 (0)	0 (0)
	Saris	23 (25.55)	10 (43.50)	0 (0)	0 (0)
	Shola	16 (17.78)	6 (37.50)	0 (0)	0 (0)
Time of sampling	Christmas	39 (43.33)	15(38.46)	0 (0)	0 (0)
	Easter	51 (56.67)	17 (33.33)	0 (0)	0 (0)

Gallinarum and *Salmonella Pullorum* (absence of hemolysis on blood agar, non-lactose fermanter on MacConkey agar and ability to grow on salmonella-shigella agar), respectively (Figure 1) however, no specific simultaneous laboratory results (gram-negative, catalase (+), oxidase (+), motility (-), O-F (F) and Indole (+) for Pastuerella multocida; and gram-negative, catalase (+), oxidase (-), motility (-), and IMViC (-/+/-+)) for *Salmonella Gallinarum* and *Salmonella Pullorum*) were recovered revealing that there was no positive samples for these organisms (Table 1).

According to chi-square analyses of the data, there was a statistically significant difference between *E. coli* isolation rate in sample types collected from surface 32 (58.20%) and those collected from deeper layer 0 (0%)($p < 0.05$). On the other hand, there was no significant difference ($p > 0.05$) among the origins of birds (East, North, South and West), market places (Akaki, Merkato, Mesalamia, Saris and Shola) and between time of sample collection (Christmas and Easter) for the fecal *E. coli* isolation rate (Table 2).

DISCUSSIONS

Out of 90 pooled fecal samples, the overall bacterial isolation rate was, 32 (35.55%) for *E. coli* and zero percent isolation rate for *Pastuerella multocida, Salmonella Gallinarum* and *Salmonella Pullorum*. The results of this study indicated that, 32 (58.20%) of 55 pooled fecal samples collected from top surface were positive for *E. coli* as compared to zero percent isolation rate from 35 pooled fecal samples collected from deeper layers. There is statistically significant ($P < 0.05$) association between *E. coli* isolation rate and sample types. This might be due to once the layer of the feces accumulated in the holding cages become deeper and deeper, it might creates anaerobic and acidic condition in which the organisms might not survived. In addition, the presence of a greater number of *E. coli* on the surface layer of the feces might be due to wind action and daily contact with external environment of the chicken.

Previous investigation indicated that some strains of *E. coli,* the one marked as Avian Pathogenic *Escherichia coli,* share a significant genetic similarities with the strains isolated from humans (Kołsut et al., 2017) and that numerous new genetically modified strains of *E. coli* can be found every day with potential pathogenic effect on humans and birds (Hussain et al., 2017). In this investigation although serotyping or genotyping of *E. coli* was not conducted, the abundant occurrence (58.2%) of *E.coli* positive surface fecal samples has both public health and economic implications. This study was the first and original report; it may serve as a basis for further researches.

During the course of this study, *P. multocida, Salmonella Gallinarum* and *Salmonellas Pullorum* were not recovered from all the fecal samples. This might be either due to limitation of the study period and absence of out-breaks at their origins or according to Milton (2015) it might be due to susceptibility of these organisms to external environment (heat, sun light, drying, a common disinfectants). In addition, this could be due to fact that most birds in the markets are of adult age and Antonio et

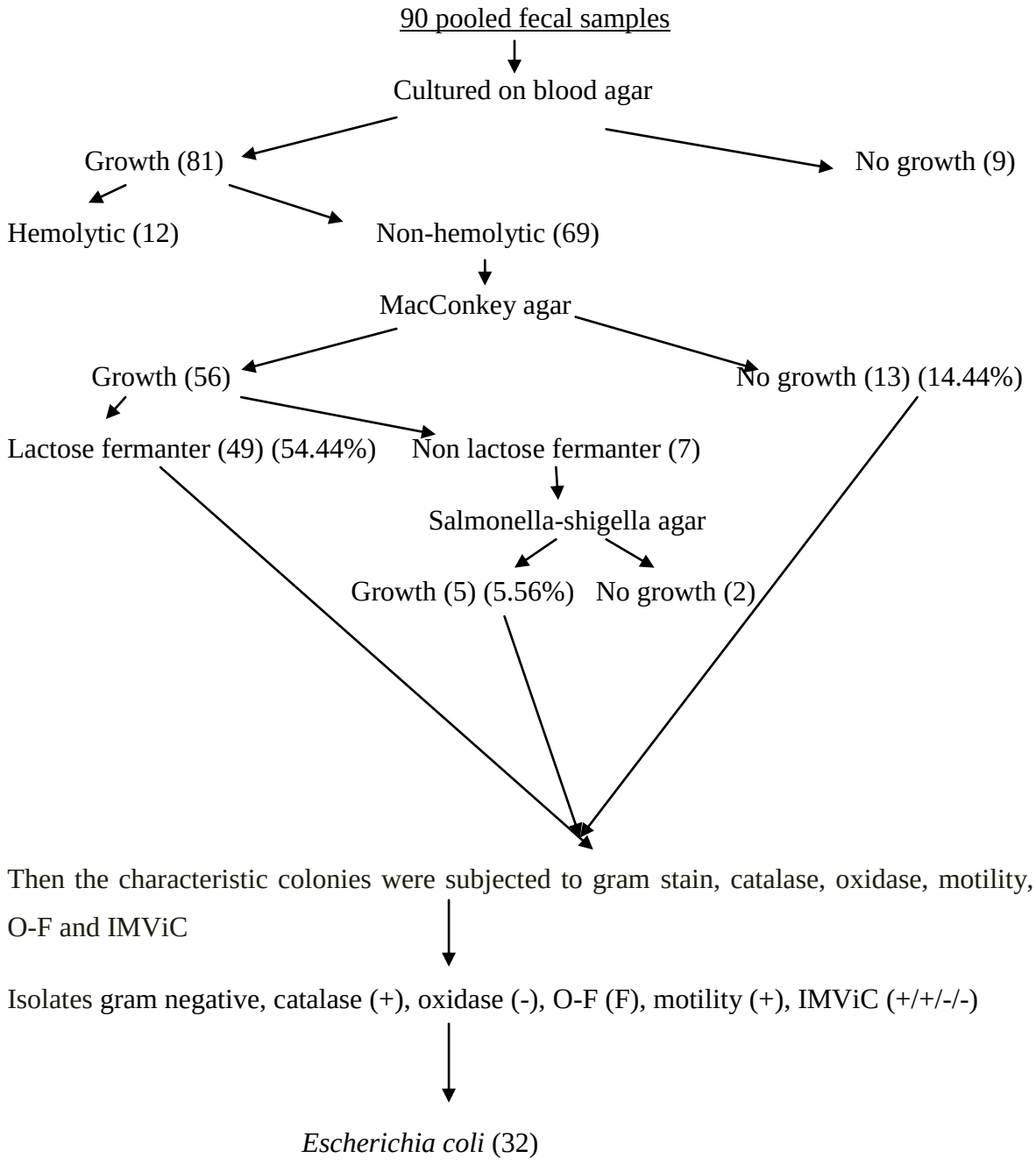

Figure 1. Flow diagram showing the schematic representation of laboratory works.

al. (2017) reported that *Salmonella Pullorum* affect only chicks less than three weeks of age.

In the study area, there were mixing of poultry originating from different parts of the country and the major sources of poultry were as far as Dire Dawa and Harar in the East; Gojjem and Gondar in the North; Arbaminch, Assella, Bale, Borena, Hawasa and Wolayta in South and Ambo and Jimma in the West and, most of them located at distance of greater than 300 km. Haftom et al. (2015) reported that live-bird markets are essential for marketing poultry in developing countries and they are preferred places for many people to purchase poultry for consumption and /or for further rearing throughout the world. Such mixing of chicken from different origins with their own health problems creates fertile grounds for exchanging and dispatching pathogens (Siraju et al., 2016).

Different live-bird markets sampled in Addis Ababa frequently consist of gathering of native chickens that satisfy local tastes and that are held in cages, baskets or

Table 2. Chi-square (X^2) tests for association between different variables (factors) and fecal *E. coli* isolation or positivity.

Variables	Category	No. of sample tested (%)	No. positive (%)	X^2	*P*-value
Origin of birds	East	19 (21.12)	6 (31.60)	0.406	0.939
	North	13 (14.44)	5 (38.50)		
	South	45 (50)	17 (37.78)		
	West	13 (14.44)	4 (30.80)		
Sample type	Deep	35 (38.90)	0(0)	31.599	0.00
	Surface	55 (61.10)	32 (58.20)		
Markets	Akaki	16 (17.78)	5 (31.25)	1.233	0.873
	Merkato	21 (23.33)	6 (28.60)		
	Mesalamia	14 (15.56)	5 (35.71)		
	Saris	23 (25.55)	10 (43.50)		
	Shola	16 (17.78)	6 (37.50)		
Time of sampling	Christmas	39 (43.33)	15 (38.46)	0.254	0.615
	Easter	51 (56.67)	17 (33.33)		

tied-up for sale. It was found that the high customers' demand for local breed chickens that might led to the transportation of birds over very long distances during holiday seasons.

Live-bird markets in Addis Ababa involves collection of chicken from multiple households who own few numbers of marketable ages, then mixed together at various density starting from the village markets to the big terminal markets in Addis Ababa that leads to spread of infection across the long distance. In addition, a high bird density in holding ground creates stressful condition and cross-infection and increased surface contamination. Thus, the isolation rate of *E. coli* from feces of holding grounds of birds from different origins found in the study area can also indicates the role of live bird markets in the biosecurity of avian pathogens of economic and public health importance and spread of pathogens along their long course of distance to market and after redistribution from markets.

CONCLUSION AND RECOMMENDATIONS

The existing customary practices of uncontrolled bird movement from long distance of origin is inhumane, stressor and spread diseases and can bring new infection. More badly mixing densely birds favors cross transmission and ground contamination. Therefore the worst scenario birds move to home or farms in public transport which can risk both farmers and public. In the study area, the holding grounds of live-bird were wooden cages which were not frequently cleaned and because of this the excreta from chicken play a great role as source for spread of avian pathogens. The significantly higher proportion of *E. coli* on surface type fecal samples indicates the lack of biosecurity measures revealing that, there may be potentially pathogenic micro-organisms present in the droppings of Addis Ababa live bird markets, thus the fact cannot be over emphasized as live bird market pose a public health hazard to humans and environment if their droppings accumulated in one place for a long period of time. During this study, *P. multocida, Salmonella Gallinarum* and *Salmonella Pullorum* were not isolated. Based on the above conclusion, the following recommendations are given:

(i) Further microbiological and epidemiological investigation is needed to correctly determine the types of major bacteria predominating in the Addis Ababa live-bird markets and the roles and magnitudes played by these markets in the epidemiology of these pathogens
(ii) Personnel should be educated on the significances of infectious diseases agents and the needs to apply biosecurity practices to prevent dissemination of these agents
(iii) Live bird that leaves the markets should be housed separately from other birds for periods of time to minimize the potential dissemination of infectious agents of poultry
(iv) The surface layer pathogens should be reduced by training merchants to exercise all in- all out put principle with proper cleaning and disinfection
(v) Further study should be done on tracking the spread of out-breaks through live-bird markets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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