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Original Research Article

Isolation and Characterization of Multidrug Resistant *Escherichia coli* Isolates from Contagion Syndrome Poultry Farm

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Abstract

Poultry production is an important agricultural activity in Ethiopia and the country is one of the few African countries with a large chicken population. However, the contribution of the poultry sector to the national economy and householders' livelihood is hampered by several interlinked factors of which disease is the most important. This study was conducted with the aim of identifying poultry disease causative agent at Ambo University poultry farm and to profile antibiotic resistant of the causative isolates. A total of 112 cloaca swabs and 144 organ samples were collected using stratified random sampling method and the samples were transported overnight to laboratory for isolation. Out of the 256 total collected samples 238 samples (92.17 %) were found to be positive for *E. coli* isolates. All the cloaca swabs, cecum and spleen samples were positive while 77.8 % liver and 75 % heart samples were found to be positive for *E. coli* isolates. The *E. coli* isolates of the current study were tested against 23 different antibiotics. All the 238 *E. coli* isolates were found to be resistant against 12 antibiotics. The complete resistant was observed against Ampicillin, Clindamycin, Ciprofloxacin, Erythromycin, Penicillin, Riphampicin, Tetracycline, Trimethoprim, Vancomycin, Cefoxitin, Doxycycline and Mecillinam antibiotics. Large numbers of isolates were also found to be resistant against Oxytetracycline (83.19 %), Streptomycin (37.39%), Ceftrioxone (36.55%), Amoxicillin (35.71%) and Kanamycin (26.89 %). On the other hand, all the current isolates were found to be susceptible against Chloramphenicol. High level of sensitivity was also observed against Sulphamethoxazole (80.25 %), Polymyxin (65.93%), Streptomycin (62.61%) and NalidixicAcid (61.76 %).

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Introduction

Poultry rearing is one of important agricultural activities in Ethiopia. It is carried out by 60% of Ethiopian householders (Birol et al., 2010) with substantial difference in distribution. In the highland part of the country most householders both in rural and urban areas keeping chickens whereas less households doing in lowland pastoral areas (Ayele et al., 2009; Wilson, 2010). With regard to poultry sector is one of the few African countries with a large population of chicken flock (Tadelle et al., 2003). The total poultry population of the country is estimated at 56.87 million (CSA, 2015) and it comprises 95.86% indigenous, 2.79% cross and 1.35% exotic breeds. Chicks, laying hens and cocks, respectively account for 37.68%, 33.1%, and 11% of the flock composition.

Poultry production in Ethiopia can be grouped into traditional backyards indigenous chicken, small scale intensive and semi intensive market oriented production systems (FAO, 2007). This classification was based on breed and flock size, feed resources, health programme and market. The traditional backyards indigenous chicken production system is the dominant poultry farming in the country whereas the modern poultry sub-sector which involves both small scale intensive and large scale commercial production systems are emerging at peri-urban and urban areas. The traditional backyards indigenous chicken production system is characterized by traditional production system which is categorized under low input production system.

Poultry production has a major role in economy of developing countries. It plays an important role in households' food security and poverty alleviation through income generation (Gondwe, 2004; Abderqader et al., 2007; Abubakar et al., 2007). In spite of its extensive production system, the traditional backyards indigenous chicken production system of Ethiopia is an income source of smallholder farmers and landless communities. It is also used for traditional ceremonies and festivals by all communities of the country and provides animals protein in the form of meat and eggs. Furthermore, the village poultry significantly contribute the livelihood of poor households economically as starter capital and as means of recovery from disaster (Emmanuel et al., 2015; Hunduma, 2010).

Despite of its large flock population, the contribution of poultry sector to national economy and householders' livelihood is hampered by several interlinked critical factors in Ethiopia. These factors include disease and predators, lack of proper health care, poor feeding and marketing information (Hunduma et al., 2010; Samson and Endalew, 2010; Emmanuel et al., 2015). These management problems may result in contamination and immunosuppression and cause infection diseases. A survey result conducted by Hailu and his colleagues indicated that 94% of the respondents stated disease as the most important constraints of chicken production in the northern part of Ethiopia (Hailu et al., 2012). The problem of disease is more critical in traditional backyards indigenous chicken production than both small scale intensive and semi intensive market oriented production systems (Emanuel et al., 2015).

Avian pathogenic *Escherichia coli* is one of the pathogenic bacterium that causes variety severe respiratory and systemic disease condition in poultry. Colisepticemia, coligranuloma and air sacculitis are some of the avian pathogenic *E. coli* caused disease condition in poultry and accounting for about 5-50% mortality in the flock (Roy et al., 2006; Ewers et al., 2009; Robineau and Moalic, 2010). Beside significant economic loss, avian pathogenic *E. coli* strains isolated from poultry are antibiotic resistant (Robineau and Moalic, 2010; Messai et al., 2013). However, in Ethiopia there are limit data with regard to colibacilosis epidemiology and antimicrobial resistance of avian pathogenic *E. coli* strains isolated from diseased chicken. Hence the aim of this study was to profile antibiotic resistance patterns among avian pathogenic *E. coli* isolated from diseased poultry farm in Ethiopia.

Materials and methods

Sample collection

In October 2014, lethal infectious poultry disease was reported from Ambo University poultry farm. Following this report, samples of this study were collected using stratified random sampling technique. The flocks were grouped into four categories on revealed disease symptoms and applied treatment actions. The four groups were: (i) healthy flock (where no observed disease symptoms), (ii) flock with improved sanitation and no daily death (where only few individuals revealed disease symptoms and consequently individuals with symptoms were isolated and the rest flock transferred to new disinfected house), (iii) flocks with disease symptoms and antibiotic treatment (where only one to three death recoded every day) and (iv) diseased and antibiotic treated flock with several individuals death (more than 15 individual death per day). For cloaca swab samples 21, 34, 36 and 21 individuals were selected randomly from group 1 to 4 respectively. To sample internal organs nine individuals were selected randomly from each group, and cecum, liver, spleen and heart samples were collected. All samples were collected in sterilized test tubes contained peptone water and transported to laboratory overnight for isolation and identification.

Isolation of the *E. coli*

Before the samples were cultured in the lab *Salmonella* was suspected as causative agent of the disease and the first culture was done on xylose lysine deoxycholateagar (XLD) media at 37^{o}C for 24 hrs.

Several bacterial colonies were grown with colony morphologies similar to *E. coli* than *Salmonella*. For further description all the grown colonies were cultured onto McConkeyagar, BioLog's *Salmonella* rainbow agar, BioLog's *E. coli* O157:H7 rainbow agar was used. Finally, all the isolated revealed colony morphologies analogous to *E. coli* than *Salmonella* and the isolates were grouped into seven categories with distinct colony characteristics.

BioLog identification

To identify the isolate of the seven distinct groups formed based on colony morphology, representative isolates weregrown on BUG (Bacteria Universal Growth) agar medium at 37°C for 24 hrs and then suspended in (IF-A) inoculating fluid at 98% turbidity cell density. Then for each isolate, 100 µl of the cell suspension was inoculated into each well of the Gen III MicroPlate, using a multichannel pipette and incubated at 32°C for 24 hr in automated BioLog.

Antibiotic resistance patterning

The antimicrobial resistance of the isolates was tested against antimicrobial agents usingdiscdiffusion method. A total of 23 antibiotic discs, Amoxycillin (30 µg), Ampicillin (10 µg), Ceftrioxone (30 µg), Cephalothin (30 µg), Chloramphenicol (30 µg), Clinomycin (10 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Kanamycin (20 µg), Penicillin (10 µg), Riphampicin (5µg), Streptomycin (25 µg), Sulphamethoxazole (25 µg), Tetracycline (30 µg), Trimethoprim (5 µg), Vancomycin (30 µg), Cefoxitin (30 µg), Nalidixic acid (30 µg), Polymyxin (30 µg), Oxytetracycline (30 µg), Doxycycline (30 µg), Mecillinam (10 µg) and Norflaxacine (10µg), were used.

All isolates were grown on nutrient agar for 24 hr and inoculum suspension were prepared at turbidity equivalent to 0.5 McFarland standards and then 2ml of the suspensions were poured on the Muller-Hinton agars. The suspensions spread evenly over the entire surface of the platesand then allowed to dry for 5 minutes. Antibiotics impregnated discs were then applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. Within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C. After 18 hrs of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured.

Results and discussion

Positive samples for *E. coli* isolates

From 256 total collected samples 238 (92.97%) were found to be positive for *E. coli* isolates. Proportion of the positive samples varied with sample types. All cloaca swabs, cecum and spleen samples collected from all four flock groups were found to be positive for *E. coli* whereas, 77.8 and 75% of liver and heart samples were respectively found to be positive for *E. coli* isolates (Table 1).

Table 1.Total samples collected from different flock groups and percentage of positive samples.

Groups of flock	Samples collected per group and number of positive samples for *E. coli*	Sample type				
		Cloaca swab	Cecum	Spleen	Liver	Heart
Healthy flock (where no disease symptom was observed)	Number of samples	21	9	9	9	9
	Number of positive samples	21	9	8	1	0
	Percentage of positive samples	100	100	88.9	11.1	0
Flock with improved sanitation and no daily death but with observed disease symptoms	Number of samples	34	9	9	9	9
	Number of positive samples	34	9	9	9	9
	Percentage of positive samples	100	100	100	100	100
Flocks with disease symptoms and antibiotic treatment and one to three death per day	Number of samples	36	9	9	9	9
	Number of positive samples	36	9	9	9	9
	Percentage of positive samples	100	100	100	100	100
Diseased and antibiotic treated flock with several individuals death per day	Number of samples	21	9	9	9	9
	Number of positive samples	21	9	9	9	9
	Percentage of positive samples	100	100	100	100	100
Total samples and overall average of positive samples	Total sample	112	36	36	36	36
	Total positive samples	112	36	35	28	27

Percentage of positive samples	100	100	100	77.8	75

All heart samples collected from healthy flock were found to be free of any culture and only 11.1% of liver samples collected from health flock were found to be positive for *E. coli* isolates. Proportion of positive samples for *E. coli* of the present study was higher than reported in earlier studies (Akinlabi et al., 2008; Zinna et al., 2011; Geidam et al., 2012; Roy et al., 2012). In Malaysia, Syhada et al. (2016) examined various organs of broiler chickens with clinical signs and lesions of complicated chronic respiratory disease. Heart (26%) and spleen (26%) were the common samples positive for *E. coli* isolates followed by the liver (22%), air sacs (17%) and peritoneal swabs (9%). Such isolation of *E. coli* from multiple organs of chicken is an indicative for invasiveness of extra-intestinal *E. coli*. Arvind et al. (2004) conducted an experiment on *E. coli* challenged broiler chickens. Their result indicated that induced *E. coli* during the experiment produced perihepatitis and pericarditis in the liver and heart respectively.

Colony and cellular morphology of the isolates

All the 238 isolates were characterized for their colony and cellular morphology, Gram and lactase properties. For colony morphology blood agar, McConkey agar, BioLog's *Salmonella* Rainbow agar, BioLog's *E. coli* O157:H7Rainbow agar and XLD agar were used. Out of 238 isolates only 5 isolates (2.1%) were found to be lactose negative (Table 2).

Table 2. Cellular and colony morphology of the isolates grown on different media.

Media used	Colony Color	Gram Test	Cellular morphology	Motility
Blood agar	Smooth, dull gray	Negative	Rod cocci	+
McConkey agar	Lactose positives-pink Lactose negative- colorless			
BioLog's *Salmonella* rainbow agar	White			
BioLog's *E. coli* OH157:H7 rainbow agar	Blue to blue black			
XLD agar	Yellow			

Antimicrobial susceptibility pattern

The current study showed that *E. coli*strains isolated from diseased poultry farm were multi-drug resistant (Table 3 and Fig. 1). All the 238 *E. coli* isolates tested in this study were resistant against 12 (52.17%) out of 23 antibiotics used. The complete (100%) resistant was observed against Ampicillin, Clindamycin, Ciprofloxacin, Erythromycin, Penicillin, Riphampicin, Tetracycline, Trimethoprim, Vancomycin, Cefoxitin, Doxycycline and Mecillinam antibiotics. Large numbers of isolates were also found to be resistant against Oxytetracycline (83.19%), Streptomycin (37.39%), Ceftrioxone (36.55%), Amoxicillin (35.71%) and Kanamycin (26.89%). Similar to the current finding, multi drug resistant *E. coli*isolates were reported by a number of previous studies (Magiorakos, 2011; Reza et al., 2015). Kazemnia et al. (2014) evaluated antibiotic resistance pattern of different *E. coli* phylogenetic groups isolated from human urinary tract infection and avian colibacillosis. The result revealed that the avian pathogenic isolates were clustered separately and the most drugs resistant isolates. High level of *E. coli* isolates resistant to Ampicillin, Erythromycin and Amoxicillin were also reported by former study Zinnah et al. (2008). Unlike the current finding Zinnah et al. (2008) reported high resistant *E. coli* isolates against Ciprofloxacin. Similarly, an experiment conducted to determine comparative efficacy of doxycycline and flumequine for the treatment of colibacillosis Akbar et al. (2009). Their data indicated that doxycycline is highly effective for the treatment of experimental *E. coli* in chicken whereas our current isolates totally resistance to doxycycline.This indicated isolates respond differently against different antibiotics. On the other hand, all the 238 tested *E. coli* isolates were sensitive to Chloramphenicol and Norflaxacine. The complete (100%) isolates susceptibility against Chloramphenicol in current study is not in conformity with the earlier report of complete resistance (Hansen et al., 2005). High level of sensitivity was also observed against Sulphamethoxazole (80.25%), Polymyxin (65.93%), Streptomycin (62.61%) and Nalidixic acid (61.76%). Moreover, some isolates were sensitive to Amoxycillin (16.65%) and Kanamycin (35.71%).

BioLog System identification of the isolates

The BioLog System identified all the seven groups made based on their colony morphology. Except group five all the suspected *E. coli* cultures isolated were identified as *E. coli* species. These groups comprised all cultures

isolated from cloaca swabs, cecum and organs samples of flock with improved sanitation and no daily death but with observed disease symptoms, flocks with disease symptoms and antibiotic treatment and one to three death per day and diseased and antibiotic treated flock with several individuals' death per day. It involved few cultures isolated from cloaca swab, cecum and spleen as well as very few cultures isolated from liver and heart samples of healthy flock (where no disease symptom was observed). On the other hand, majority of suspected *E. coli* cultures isolated from liver and heart samples of healthy flock were identified as inactive *E. coli*. Very few cultures isolated from cloaca swab, cecum and spleen samples of healthy flocks were also clustered in this group as revealed similar colony morphology on different agar media. The similarity, distance and probability of each group have been indicated in Table 4.

Table 3. Susceptibility pattern of 238 *E. coli* isolates against different antibiotics.

Antibiotics disc	Sensitivity groups of *Escherichia coli* isolates					
	Resistant		Intermediate		Sensitive	
	No. of isolates	Positive isolates (%)	No. of isolates	Positive isolates (%)	No. of isolates	Positive isolates (%)
Amoxycillin (30 µg)	85	35.71	111	46.64	42	17.65
Ampicillin (10 µg)	238	100	0	0	0	0
Ceftrioxone (30 µg)	87	36.55	151	63.45	0	0
Cephalothin (30 µg)	238	100	0	0	0	0
Chloramphenicol (30 µg)	0	0	0	0	238	100
Clindamycin (10 µg)	238	100	0	0	0	0
Ciprofloxacin (5 µg)	238	100	0	0	0	0
Erythromycin (15 µg)	238	100	0	0	0	0
Kanamycin (20 µg)	64	26.89	89	37.39	85	35.71
Penicillin (10 µg)	238	100	0	0	0	0
Riphampicin (5µg)	238	100	0	0	0	0
Streptomycin (25 µg)	89	37.39	0	0	149	62.61
Sulphamethoxazole (25 µg)	47	19.75	0	0	191	80.25
Tetracycline (30 µg)	238	100	0	0	0	0
Trimethoprim (5 µg)	238	100	0	0	0	0
Vancomycin (30 µg)	238	100	0	0	0	0
Cefoxitin (30 µg)	238	100	0	0	0	0
NalidixicAcid (30 µg)	45	18.91	47	19.75	147	61.76
Polymyxin (30 µg)	0	0	81	34.03	157	65.93
Oxytetracycline (30 µg)	198	83.19	40	16.81	0	0
Doxycycline (30 µg)	238	100	0	0	0	0
Mecillinam (10 µg)	238	100	0	0	0	0
Norflaxacine (10µg)	0	0	0	0	238	100

Table 4. Colony featuresof the *E. coli* isolates representing seven morphological categories grown on McConkey agar and species identified.

Categories of the isolates and colony morphology on McConkey agar	OmniLog data output				
	PROB.	SIM	DIST	Organism type	Species ID
Small, pink to red, entire, circular and raised	0.895	0.803	2.755	GN-Ent	*E. coli*
Medium, pink, entire, circular and raised	0.891	0.803	2.825	GN-Ent	*E. coli*
Small, pink, entire, circular and raised	0.937	0.850	2.616	GN-Ent	*E. coli*
Medium, pink to red, entire, circular and raised at center	0.941	0.870	2.606	GN-Ent	*E. coli*
Medium, pink to red, irregular and flat	0.971	0.89	2.00	GN-Ent	*E. coli* inactive
Large, pink to red, irregular and flat	0.902	0.75	2.60	GN-Ent	*E. coli*
Large, pink to red, irregular, flat raised at center	0.854	0.762	3.49	GN-Ent	*E. coli*

PROB. Probability; SIM, similarity; DIST, distance and species ID, species identified.

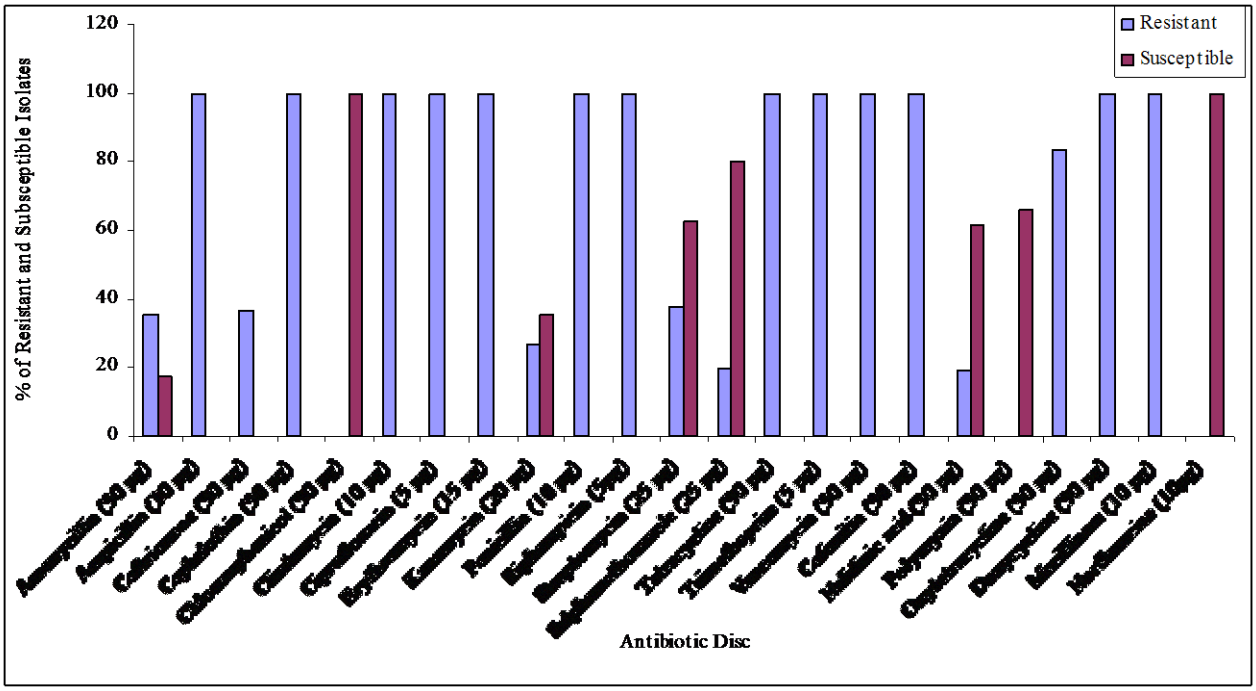

Fig. 1: Drug susceptibility pattern of the isolates against 23 antibiotics.

Carbon source utilization and chemical sensitivity pattern of the *E. coli* isolates

Almost all the *E. coli* isolates identified by BioLog system revealed similar profile of carbon utilization and chemical sensitivity assay. The isolates were positive to D-sorbitol, D-maltose, Dextrin, D-galactonic acid, methyl pyruvate, D-melibiose, D-fructose, L-alanine, L-galactonic acid lactone, D-trehalose, B-methyl-D-glucoside, D-galactose, D-gluconic acid, citric acid, glycerol, L-asparitic acid, D-glucuronic acid, N-acethyl-D-glucosamine, D-glucose-6PO_4, Glucuronamide, L-factose D-fructose6PO_4, mulic acid, D-mslic acid, N-acetyl D-galactose amine, L-Rhamnose, L-malic acid, acetic acid, N-acethylneuramic acid, inosine, D-serine, and L-serine carbon source utilization assays. On the other hand the isolates were negative D-raffinose, gelatin, tween 40, γ-amino-butric acid, myo-inositol, D-arobitol,β-hydroxy-D-L-butricacid, D-cellubose, D-salicin, 3-methyl glucose, gentibiose, α-Keto-Butyric Acid, sucrose, L-histidine, D-Turanose, L-pyroglutancic acid and starchyose carbon source assays.

The chemical sensitivity assay of the *E. coli* strains isolated in the current study were showed positive to 1% NaCl, 4% NaCl, 1% sodium lactate, Troleandomycin, licomycine, vancomycine, nalidixic acid, aztreonam, pH 4, fusidic acid, rifanycin 5ug, guandine, HCL, tetrazolium, violate, lithium chloride, sodium butrate, pH5, 8% NaCl, D-serinetetrazolium blue and potassium tellurite. The assay was negative to sodium bromate.

Conclusion

This study revealed that multidrug resistance *Escherichia coli* is emerging and causing high flock mortality in poultry farm. Such occurrence of antibiotic resistant pathogenic microorganism in poultry sector is an alarming issue for both public health and poultry rearing. As this study focused on semi-intensive poultry farm, further study is required on the occurrence and pathogenicity of such drug resistant *E. coli* isolates in all poultry farming systems of the country.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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