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Advances in Animal and Veterinary Sciences

Research Article

Prevalence of Extended Spectrum Beta-Lactamase Producing Enterobacteriaceae in Commercial Broilers and Backyard Chickens

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Abstract | The present study demonstrated the prevalence of extended spectrum beta-lactamase (ESBL) -producing enterobacteriaceae in liver samples of commercial broilers (commercial broilers) and backyard chickens (backyard chickens). Results demonstrated that *Escherichia coli* (*E. coli*) was the most common isolate recovered from both commercial broilers (44.58%) and backyard chickens (57.03%), that followed by salmonella (commercial broilers: 35.06%; backyard chickens: 21.09%), klebsiella (commercial broilers: 12.98%; backyard chickens: 13.28%), proteus (commercial broilers: 04.76%; backyard chickens: 06.25%), and enterobacter (commercial broilers: 02.59%; backyard chickens: 02.34%). The prevalence of *E. coli* and salmonella was found higher ($P < 0.05$) in commercial broilers as compared to backyard chickens. However the prevalence differences of klebsiella between commercial broilers and backyard chickens were found statistically non-significant ($P > 0.05$). Among these isolates, 7.76% and 10.95% *E. coli* isolates were recorded as ESBL -producing from commercial broilers and backyard chickens respectively. While 12.34% and 7.40% salmonella isolates were found positive for ESBL production from commercial broilers and backyard chickens respectively. However, 13.33% klebsiella isolates of commercial broilers were declared as ESBL -producers; whereas klebsiella isolates of backyard chickens and proteus and enterobacter of both commercial broilers and backyard chickens were found negative for ESBL production. These results indicates that microbiota of commercial broilers established a higher number of enterobacteriaceae as compared to backyard chickens, moreover, the prevalence of ESBL -producing enterobacteriaceae in liver of commercial broilers was also higher than backyard chickens.

Keywords | Broiler, Backyard, Enterobacteriaceae, ESBL, Prevalence

Editor | Kuldeep Dhama, Indian Veterinary Research Institute, Uttar Pradesh, India.
Received | April 06, 2016; **Accepted** | May 15, 2016; **Published** | April 24, 2016
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Citation | Shoaib M, Kamboh AA, Sajid A, Mughal GA, Leghari RA, Malhi KK, Bughio S, Ali A, Alam S, Khan S, Ali S (2016). Prevalence of extended spectrum beta-lactamase producing enterobacteriaceae in commercial broilers and backyard chickens. Adv. Anim. Vet. Sci. 4(4): 209-214.
DOI | http://dx.doi.org/10.14737/journal.aavs/2016/4.4.209.214
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Enterobacteriaceae are one of the most important groups of bacteria. It is a family of non-spore-forming, Gram-negative bacteria that normally inhabit the gastrointestinal tract, having 48 genera and 219 species (ILSI, 2011). Some members of this family have significant importance and are associated with food spoilage while many others are responsible for putrefaction of a variety of foods including poultry products, meats, milk, eggs, fish, sea foods and dairy products. Some genera (coliform bacteria) have the ability to ferment lactose which has long been

used as indicator organism by the water and food industry. Currently, both coliforms and enterobacteriaceae are isolated from foodstuffs for showing evidence of poor sanitation or inadequate processing (especially heat-treatment), post-process contamination of foods and process failure. Improper handling during evisceration and removal of intestinal tract may cause the rupture of intestine that results the contamination of meat and other visceral organs being contaminated (Khan et al., 2016; ILSI, 2011).

Meat-type poultry chickens (broilers) have a complex population of bacteria, however, the digestive tract microflora of backyard chickens suggested a greater diversity (Saliu et al., 2012). Among the isolates, *Escherichia coli,* lactobacillus spp., *Enterobacter aerogenes*, *Bacillus cereus* and *Bacillus subtilis* were found colonized in both, backyard chickens and broilers. However, *Staphylococcus epidermis, Proteus vulgaris, Staphylococcus aureus, Enterococcus facium, Streptococcus pyogenes,* and *Bacillus megaterium* were habitating the free range (backyard) chickens only. Many isolates found with the capability of hydrolyzing cellulose and starch. So these microflora have important roles in the carbohydrates digestion especially cellulose (Saliu et al., 2012). Furthermore, the backyard chickens may be critical environmental indicators of multidrug resistance and they might take part as spreaders and long-term reservoirs of medically threatening pathogens with resistance genes, more actively than previously thought (Badrul et al., 2012).

There is a global rise in infections caused by Gram-negative bacteria of enterobacteriaceae family, producing extended spectrum beta-lactamases (ESBL). The incidence of ESBL-producing pathogens in the poultry gastrointestinal tract increased from 3% in 2003 to 15% in 2008. The poultry industry now has been considered a likely reservoir of ESBL-producing Gram-negative bacteria (Leverstein et al., 2011). The diseases caused by these pathogenic microbes (ESBL producers) are producing high economic losses all over the world, in terms of high morbidity, stress, mortality, decreased hatchability and egg production (Numan et al., 2005). The ESBL show resistance to most of the beta-lactam antibiotics, including third and fourth cephalosporin generation, causing major problem of treatment promises for infections produced by these pathogens (Serefhanoglu et al., 2009). Keeping in view the above facts, the present study was planned to investigate the prevalence of enterobacteriaceae in liver (an edible part) of commercial broilers and backyard chickens. Moreover, the results will also provide the first insights on prevalence of ESBL –producing enterobacteriaceae in both chicken types.

MATERIAL AND METHODS

Sample Collection

For present study apparently sick commercial broilers (n= 150) and backyard chickens (n=150) of adult age (body weight, commercial broilers: 1.6-2.0 kg, backyard chickens: 1.5-2.1 kg) were obtained from different farms of district Peshawar and brought to the post mortem room of Veterinary Research Institute (VRI) Peshawar. The birds were sacrificed, eviscerated and the fresh liver samples (about 25 g) were collected aseptically in sterilized sample bottles and stored immediately at -20°C until analyzed (Nzouankeu et al., 2010). All the experimental protocols were approved by the institutional Animal Care and Use Committee.

Isolation of *Enterobacteriaceae*

All the samples were processed for isolation of major enterobacteriaceae viz, *E. coli,* salmonella, klebsiella, proteus and enterobacter following the procedure adopted by Roy et al., (2012). The samples were aseptically treated with 225 ml of Buffered Peptone Water (Oxoid UK, CM0509) as per procedure of Mossel et al., (1963) and appropriate decimal dilutions were prepared that were streaked on to MacConkey agar, tryptose agar and Nutrient agar of (Hi-Media Laboratories Pvt. Ltd. India) and incubated at 37°C for 24 hours to get the primary bacterial growth. After 24 hours, the colony morphology was examined and each type of colony was picked and sub-cultured onto separate media plates (Roy et al., 2012). Pure cultures were achieved as per procedures described by OIE, (2000). The obtained pure bacterial growth was transferred to sterilize tryptose and nutrient slants, which were then incubated for 24 hours at 37°C. After 24 hours period, the growth were examined and the stock cultures of different purified organisms were kept in the refrigerator at 4°C for further investigation. The organisms were characterized as Gram-positive or Gram-negative by Gram's staining method according to the technique described by Merchant and Packer, (1967). Further confirmation of the isolates was made by API (Analytical profile Index) RapID-One strips (Remel Co, Lenexa, USA), which is a rapid detection method for the identification of important enterobacteriaceae members.

ESBL Detection

For determining the ESBLs -producing species of family enterobacteriaceae, the Double Disk Synergy method was used as adopted by Sirot (1996). In brief, nutrient agar plates were inoculated with test organisms and after inoculation, the combination disc of amoxicillin (20µg) and clavulanic acid (10µg) were placed at centre of nutrient agar plate while discs of cefotaxime (30µg), ceftazidime (30µg) and ceftriaxone (30µg) of Liofilchem Pvt. Ltd. Company, Italy were placed 15mm apart from central disc. The plates were incubated at 37°C for 18 to 24 hrs. An expansion zone of inhibition between any of the cephalosporin and combination disc ≥5mm indicated as ESBL positive according to the guidelines recommended by CLSI (2006).

Table 1: Number and percentage of enterobacteriaceae isolates recovered from broilers and back-yard chickens

Enterobacteriaceae isolates	Chicken		Total No. (%)	*P- Value
	Broilers No. (%)	Backyard No. (%)		
E.coli	103 (44.58)	73 (57.03)	176 (49.02)	0.0237
Salmonella	81 (35.06)	27 (21.09)	108 (30.08)	0.0001
Klebsiella	30 (12.98)	17 (13.28)	47 (13.09)	0.0579
Proteus	11 (04.76)	08 (06.25)	19 (05.29)	0.4913
Enterobacter	06 (02.59)	03 (02.34)	09 (02.50)	0.3173
Total Isolates	**231**	**128**	**359**	-

*Results were considered significant when P < 0.05

Statistical Analysis

On completion of the study, the data obtained were stated in absolute values and percentages. For the determination of percentages and calculations, the Microsoft Office 2013 software package was used. The incidence of different enterobacteriaceae isolates and their extended spectrum beta-lactamase production difference between commercial broilers and backyard chickens were compared by the Chi-square test at a P < 0.05 probability level using Instat GraphPad software (San Diego, California).

RESULTS

Prevalence of Enterobacteriaceae in Commercial Broiler and Backyard Chickens

As shown in Table 1, the most prevalent specie among the overall isolated organisms in commercial broilers and backyard chickens was *E. coli* i.e., 176/359 (49.02%). It was followed by salmonella (108), klebsiella (47), proteus ssp., (19) enterobacter (09) with the prevalence of 30.09, 13.09, 05.29, and 02.50% respectively. The prevalence of *E. coli* was found higher (P < 0.05) in commercial broilers (103 isolates, 44.58%) as compared to backyard chickens (73 isolates, 57.03%). Similarly, 81 (35.06%) salmonella isolates were recovered from commercial broilers, whereas 27 (21.09%) from backyard chickens. The prevalence of salmonella isolates was observed higher (P < 0.05) in commercial broilers as compared to backyard chickens. The prevalence of klebsiella organism was also found higher (30 isolates, 12.98%) in commercial broilers than backyard chickens (17 isolates, 13.28%), however, their prevalence differences were found statistically non-significant (P > 0.05). Similarly, the organisms proteus isolated from commercial broilers were 11 (04.76%) and that from backyard chickens were 08 (06.25%), while, the prevalence rate of enterobacter in commercial broilers and backyard chickens were 06 (02.59%) and 03 (02.34%) respectively, the difference of which was not statistically significant (P > 0.05).

Prevalence of ESBL -producing Enterobacteriaceae in Commercial Broilers and Backyard Chickens

As shown in Table 2, there was great variation among the enterobacteriaceae members in term of ESBLs production, been isolated from commercial broilers and backyard chickens. The isolated ESBL positive *E. coli* were 08/103 (07.76%) and 08/73 (10.95%) in the commercial broilers and backyard chickens respectively, that showing no difference (P > 0.05) in microbiota in terms of ESBL-producing *E. coli*. However, the ESBL-producing salmonella detected from commercial broilers were 10/81 (12.34%) and that from backyard chickens were 02/27 (07.40%). The statistical analysis showed a significant differences (P < 0.05) in ESBL-producing population of salmonella as well as klebsiella organisms in commercial broilers and backyard chickens.

Table 2: Prevalence of Extended-Spectrum-β-Lactamase-Producing enterobacteriaceae in broilers and back-yard chickens

Enterobacteriaceae isolates	ESBLs producers No. (%)		*P- Value
	Broiler	Backyard	
E. coli	08 (07.76)	08 (10.95)	1.0000
Salmonella	10 (12.34)	02 (07.40)	0.0209
Klebsiella	04 (13.33)	0 (0)	0.0455
Proteus	0 (0)	0 (0)	-
Enterobacter	0 (0)	0 (0)	-

*Results were considered significant when P < 0.05

DISCUSSION

Enterobacteriaceae are a major part of gut microbiota that play a critical role in enteric diseases and competitive exclusion (ILSI, 2011). For present study we targeted five genera/species (viz., *E.coli*, salmonella, proteus, enterobacter and klebsiella) of enterobacteriaceae which is considered as the key part of gut ecosystem, and also play major role in host health. Our results revealed a significant difference in gut microbiota of both chicken types and showed a higher population of enterobacteriaceae in commercial broilers as compare to backyard chickens, however, there was no difference in quality/diversity of isolated organisms from the liver of both chicken types. The same findings have been reported by Saliu et al. (2012), who had found commercial broilers with high prevalence of enterobacteriaceae as

compared to backyard chickens. The variation in prevalence and diversity of enterobacteriaceae of commercial broilers and backyard chickens could be associated with mood of nutrition Saliu et al. (2012). The high prevalence of bacteria in broiler's intestine might be due to high nutrients ratio found there because commercial broilers are supplied with diet enriched with nutrients for the improved productivity and FCR. The gut microflora take advantage of these nutrients and thus multiply there rapidly as much as they found suitable conditions (Salah et al., 2015; Saliu et al., 2012). On the other hand, backyard chickens are omnivores, generally, they roam freely around the farmers' house at day time and pick food including wheat/maize grains, plant parts/leaves, and vegetable waste from the soil and/or in the area around the cooking place. These botanicals contains phytochemicals including flavonoids, stilbenes and polyphenols, which are known as strong anti-inflammatory and antimicrobial agents (Kamboh et al., 2015). The low microflora level (enterobacteriaceae) in backyard chickens could be associated with the antimicrobial activities of these phytochemicals.

Ojo et al. (2012) reported *Escherichia coli,* klebsiella, salmonella and enterobacter in free range chickens, which is somehow in line with our findings. Our results are also supported by the findings of Naldo et al. (1998), who had reported *Escherichia coli,* klebsiella, proteus and enterobacter in free ranging kori bustard chickens. Current finding of *E. coli* isolates is somewhat consistent with that of Roy et al. (2012) and Awad-Alla et al. (2010), who reported the prevalence of *E. coli* as 52% in commercial broilers. The high prevalence of *E. coli* in chicken's digestive tract are not unexpected as the coliforms are the main flora of farm animals as well as human beings (2, 7). Our further investigations showed that the overall prevalence of salmonella spp. was 30.08% in chicken, which is supported by Roy et al. (2012). However, a significant difference may be found in other findings like, Takehisa et al. (2013) who had examined 1,472 faecal samples of commercial broilers in Japan and found 93 isolates of salmonella spp. with 6.31%, which might be due to differences in geographical locations, feed ingredients and management conditions particularly those provided during embryonic life or early days of life (Ahmed et al., 2015; Nghonjuyi et al., 2015).

The prevalence of ESBL -producing gram negative enterobacteriaceae in poultry and poultry products and their subsequent transmission to human beings have been proposed by several studies. Some workers have reported the similar prevalence percentages of ESBL –producers in poultry as declared in our study. Like, Smet et al. (2008), reported 27.2% ESBLs positive samples out of 489 samples. Besides this, Blanc et al. (2006) examined 192 enterobacteriaceae positive samples for the screening of extended spectrum βeta-lactamases and found 51 (26.57%) isolates with ESBLs -producing enzymes.

In Pakistan, the frequent use of antibiotics in commercial poultry farming may also play a major role in the occurrence of resistance to the bacterial organisms by producing the enzyme ESBLs. Many bacteria attain resistance by exposure to antibiotics. The exposure may in shape of use of growth promoting antibiotics in poultry feed and/or non-judicial use of antibiotics for treatment/prevention of bacterial diseases (Ansari et al., 2014). There are two groups of resistance. In the first group: the bacteria have natural ability to resist against antibiotics by enzymatic inactivation while, in the 2nd group, the bacteria have the ability to survive in the antibiotic environment by gene action without the interaction/alteration of antibiotics (Apata, 2009).

Our study have reported first time the prevalence of ESBL-producing enterobacteriaceae in backyard (free rangers) and commercial chickens. It is generally hypothesized that backyard chicken farming system results the less or no dissemination of antibiotic resistance (Mirandaa et al., 2008). Because, in rural areas there is no high scale of farming of backyard chickens, usually the birds are kept in small cages in houses where they have less chances of getting antibiotics as they pay no significant attention for vaccines or treatment purposes. Our present findings were also in agreement with the above theory, as we have find less distribution of ESBL –producing enterobacteriaceae isolates in backyard chickens. Moreover, our results have also indicated the difference in diversity of ESBL-producers between both chicken types (as no klebsiella isolate was recorded as ESBL-producer in backyard chickens). This could be associated with the host-response mechanisms for stimulation of ESBL and non-ESBL strains (Demirel et al., 2013). Furthermore, all the isolates of proteus and enterobacter were declared as non-ESBL –producers regardless of chicken type. proteus are usually dispersed in nature everywhere as saprophytes and are mostly found in manure, soil, and human and animal faeces (Senior et al., 1997), while enterobacter are prototrophic in nature and are commonly found on a number of different plants and seeds (Francine and Patrick, 2006). Hence, these pathogens could enter frequently in commercial broilers as well as backyard chickens. The common infections of backyard poultry that were treated by antibiotics could be associated with these pathogens.

It is obvious that backyard poultry hardly receive antibiotics but they may be interconnected through a numerous paths with other organisms like humans and exotic poultry that had been formerly wide-open to a number of antibacterial agents, e.g., in many rural communities the people commonly defecate and urinate around surroundings where the backyard chicken move freely for picking feed. Such type of unhygienic human excretes disposal leads to

exposure of the human harbour resistant bacterial agents via faeces. So the observed high ESBL isolates almost certainly reflect the high exposure/usage of antibacterial agents in the country. Hence, on the basis of high occurrence of ESBL organisms in both commercial and backyard chickens, banned on the use of antibiotics in animal production and strict hygiene measures should be advised.

From the results, it would be concluded that ESBL –producing enterobacteriaceae are more prevalent in liver of commercial broilers than backyard chickens that probably due to indiscriminate use of antibiotics in commercial broilers farming.

CONFLICT OF INTERESTS

The authors have no conflict of interest regarding the publication of this article.

FINANCIAL DISCLOSURE

This work was supported by the Sindh Agriculture University, Tandojam.

ACKNOWLEDGEMENT

This research work was financially supported in part by the Veterinary Research Institute (VRI) directorate, Peshawar. We are thankful to the staff of VRI, Peshawar for giving us the opportunity to conduct the research work there.

AUTHORS' CONTRIBUTION

Muhammad Shoaib is the main author of the study, Asghar Ali Kamboh and Abdul Sajid were the potential and co-supervisors, respectively. Gulfam Ali Mughal and Raiz Ahmad Leghari have revised the Manuscript. Shamas-U-Din Bughio, Kanwar Kumar Malhi and Akhtar Ali, carried out the technical work. Shafiq Alam, Sajid Khan and Sardar Ali helped in statistics.

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