Research Note: Characterization of antibiotic resistant phenotypes and linked genes of *Escherichia coli* and *Klebsiella pneumoniae* from healthy broiler chickens, Karnataka, India

Khyati Bhardwaj,[*] Suchitra Shenoy M,[*,†,1] Shrikala Baliga,[*,†] Unnikrishnan B,[‡] B. Shantharam Baliga,[§] and Vasanth Kumar Shetty[#]

[*]*Department of Microbiology, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, 576104, Karnataka, India;* [†]*Manipal McGill Centre for Infectious Diseases, PSPH, Manipal, India;* [‡]*Department of Community Medicine, Kasturba Medical College, Light House Hill Road, Hampankatta, Mangalore, 575001, Karnataka, India;* [§]*Department of Pediatrics, Kasturba Medical College, Light House Hill Road, Hampankatta, Mangalore, 575001, Karnataka, India; and* [#]*Animal Disease Diagnostic Laboratory, Government Veterinary Hospital, Jail Road, Karangalpady, Mangalore, 575002, Karnataka, India*

ABSTRACT The study was carried out to estimate the burden and pattern of antibiotic resistance and to identify antibiotic resistance genes with focus on ESBL producers, plasmid mediated quinolone resistance, and tetracycline efflux genes, in faecal bacterial isolates collected from poultry farms of coastal Southern Karnataka, India. High resistance to fluoroquinolones was observed with 94% *Escherichia coli* and 80% *Klebsiella pneumoniae* being resistant to both ciprofloxacin and levofloxacin. All the *Escherichia coli* strains were resistant to tetracycline (100%). qnrB (38%) was the most common gene detected followed by qnrS (27%) and qnrA (21.5%). All *Klebsiella pneumoniae* isolates resistant to tetracycline harbored tetA gene. Most of the isolates in our study had high MAR indices indicating rampant use of antibiotics.

Key words: poultry, antimicrobial resistance, plasmid mediated fluoroquinolone resistance, ESBLs, tetracycline resistance

2021 Poultry Science 100:101094
https://doi.org/10.1016/j.psj.2021.101094

INTRODUCTION

Antibiotic resistance is a 'One Health' issue with an impact on human health, animal health, and the environment. Antibiotics are frequently used for prophylaxis and growth promotion in livestock. Such indiscriminate use leads to the emergence of resistant bacterial strains. These resistant bacteria can spread from farm animals to handlers and the surrounding environment (Laube et al., 2014). In poultry farms, when treatment is required, often the antibiotics are mixed in the main water source or feed and the whole batch is treated because treating one bird alone is not practical.

India has one of the highest numbers of livestock in the world and the unrestricted use of antibiotics is rampant. Some previous studies done in North India have reported resistance to critically important antibiotics such as amoxiclav, cefotaxime, levofloxacin, and meropenem in *Escherichia coli* and *Klebsiella pneumoniae* (Bhushan et al., 2017).

Many studies suggest that healthy food-producing animals also harbor *Escherichia coli* and *Klebsiella spp* that are ESBL producers. Not only ESBLs but other plasmid-mediated antibiotic resistance-conferring genes such as qnr are increasingly being detected (Kar et al., 2015).

Karnataka, a state with intensive poultry farming, lacks data regarding antibiotic use and antibiotic resistance in the poultry farms. This study was therefore done to determine the antibiotic-resistant bacteria, study their susceptibility pattern, and identify the genes responsible for resistance to antibiotics in poultry in Southern Karnataka.

MATERIALS AND METHODS

Poultry Sample Collection, Bacterial Isolation, Identification, and Farmer Interview

By simple random sampling, 20 registered poultry farms were selected from Southern Karnataka. All the

Received October 15, 2020.
Accepted March 4, 2021.

[1]Corresponding author: suchitra.u@manipal.edu

Table 1. Poultry samples collected per age group, age-wise resistance pattern of isolates, and MAR indices.

Poultry Age (Wk)	Number of samples	Number of farms sampled	Total number of isolates		% Resistant to two or more antibiotics		MAR index	
			E. coli	*Klebsiella*	*E. coli*	*Klebsiella*	*E. coli*	*Klebsiella*
1	34	1	10	3	50	100	0.13	0.15
1 wk	32	3	31	2	96	50	0.18	0.15
2 wk	40	4	30	4	75	75	0.32	0.13
3 wk	32	3	23	2	71	100	0.37	0.12
4 wk	38	3	30	2	85	100	0.28	0.17
5 wk	48	4	32	3	66	66	0.28	0.11
6 wk	32	2	31	4	96	100	0.29	0.17

farms sampled were comparable in size, housing on an average 10 flocks (flock size >50) of the same age group at a time (all-in all-out system). Clinically healthy live broiler chickens of different ages (1 day old to 6 weeks old) were sampled. One day old chicks had hatched on the previous night of hatchery visit. Swabs from 1-day-old chicks were collected from the main hatchery. 10 to 15 cloacal swabs (1−2 swabs from each flock) were randomly collected from each farm to a total of 256 samples (Table 1). The collection was done from January 2018 to June 2018.

The swabs were transported in saline and streaked on HiCrome agar (HiMedia, India) using aseptic precautions and incubated at 37°C for 24 hr. HiCrome agar facilitated the direct identification of *Escherichia coli* and *Klebsiella pneumoniae* based on different colors and morphology of the colony. For quality control, *E. coli* ATCC 25922 was used. Standard biochemical techniques were used to confirm the identity of the colonies. After swab collection, the head farmer of each farm was orally interviewed to collect information about the mode, duration, and reason of antibiotic use. A total of 20 farmers were interviewed.

Antibiotic Susceptibility Testing

All the *E. coli* and *K. pneumoniae* isolates were first subjected to phenotypic identification of ESBLs producers and fluoroquinolone resistance by culture on 2 media; cefotaxime (1 mg/L) incorporated MacConkey agar and ciprofloxacin incorporated (1 mg/L) MacConkey agar. The isolates that were found resistant in both the cultures were further subjected to routine antibiotic susceptibility testing to twenty antibiotics by the Kirby Bauer disk diffusion method. Clinical and Laboratory Standards Institute guidelines were used to interpret the results The tested antibiotics (Becton Dickinson, Sparks, MD) were ampicillin (AMP,10μg), amoxicillin/clavulanic acid (AMC,20/10 μg), ampicillin/sulbactam (SAM,10/10 μg), amikacin (AK,30 μg), aztreonam (AT,30 μg), ceftazidime (CAZ,30μg), ceftriaxone (CTR,30 μg), cefotaxime (CTX,30μg), cefoxitin (FOX,30 μg), chloramphenicol (C,30 μg), ciprofloxacin (CIP, 5μg), levofloxacin (LVX,5 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), ertapenem (ERT,10 μg), imipenem (IMP,10μg), meropenem (MEM,10 μg), piperacillin (PI,100 μg), piperacillin-tazobactam (TZP,100/10 μg), tetracycline (TE,30 μg), tigecycline (TGC,15 μg). Presumptive ESBL producers were further tested for ESBL production by the combination disc method. The following four discs were used − ceftazidime, ceftazidime/clavulanic acid, cefotaxime, and cefotaxime/clavulanic acid. An increase of ≥5 mm in the zone of inhibition of cephalosporin/clavulanic acid combination disc as opposed to cephalosporins alone was considered as phenotypic confirmation of ESBL production. *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive control and negative control for ESBL detection respectively.

Molecular Detection of Antimicrobial Resistance Genes

Bacterial isolates, across all the age groups, that were phenotypically confirmed as ESBL producers, fluoroquinolone-resistant, and tetracycline-resistant, were further analyzed by PCR. Pure bacterial colonies grown on blood agar (HiMedia, India) at 37°C for 24 hr were used for DNA extraction by QIAamp DNA mini kit (QIAGEN, Dusseldorf, Germany). ESBLs - bla_{TEM}, bla_{SHV} and bla_{CTX-M} (Sinha et al., 2015), $bla_{CTX-M-15}$ (Zowawi et al., 2014); plasmid-mediated quinolone resistance - qnrA, qnrB, qnrS (Huang et al., 2009) and tetracycline efflux - tet(A), tet(B) (Kurnia et al., 2018) genes were analyzed by PCR. The PCR reaction was as follows − 25 μL volume contained 12.5 μL KAPA Taq Hotstart 2X master mix (Kapa Biosystems, Wilmington, MA, USA), 1 μL each primer, 2μL DNA template, and 8.5 μL nuclease-free water. The PCR amplification was carried out in the Rotor-Gene Q 5plex HRM platform (QIAGEN, Hilden, Germany). The amplified products were run on 1.5% (w/v) agarose gel (HiMedia, India) containing ethidium-bromide (0.5 μg/mL). Gel documentation was done with the help of AlphaImager EC (ProteinSimple, San Jose, CA).

Statistical Analysis

Data analysis was carried out with SPSS, version 20.0. The data was reported as frequencies and percentages. Multiple antibiotic resistance (MAR) index was calculated for isolates from each age group as a/(b*c), where

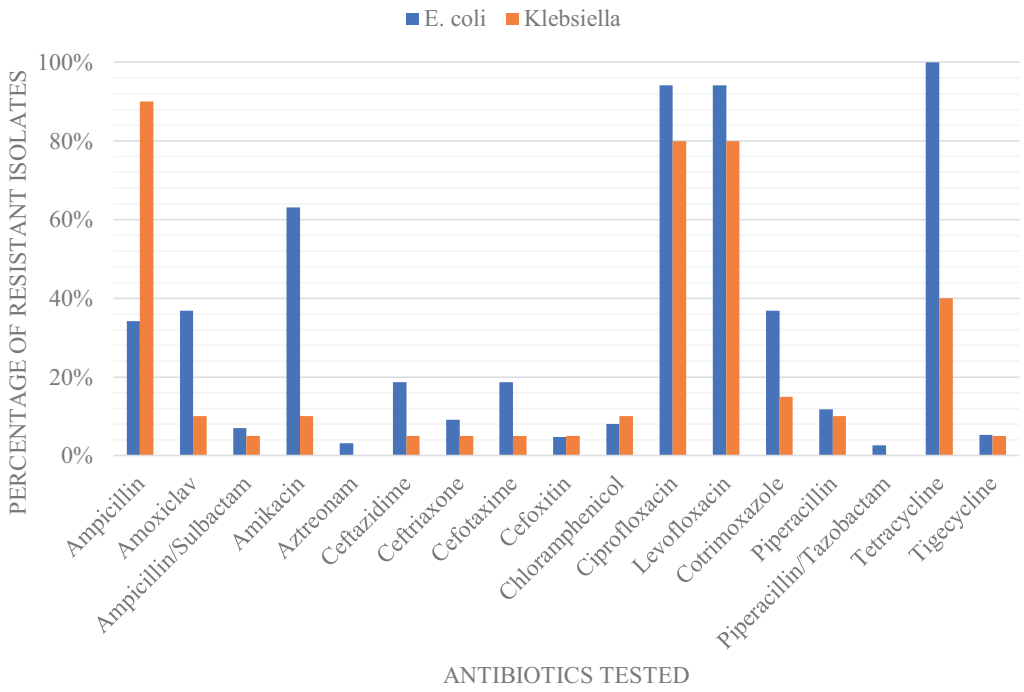

Figure. 1. Pattern of antibiotic resistance in *E. coli and Klebsiella* isolates against the tested antibiotics. The graph depicts the percentage of *E. coli* and *Klebsiella* isolates that were resistant against the tested antibiotics.

'a' represents aggregate antibiotic resistance score of all isolates from an age group, 'b' is the number of antibiotics tested and 'c' is the number of isolates obtained from each age group. An MAR index >0.2 indicated high-risk sources where bacteria were exposed to multiple antibiotics.

RESULTS AND DISCUSSION

Prevalence of Bacterial Isolates

Out of the 256 chicken cloacal swab samples, a total of 187 *E. coli* and 20 *K. pneumoniae* were isolated. The prevalence of *E. coli* was highest at 73% and of *Klebsiella* 7.8%.

Antibiotic Use in Poultry Farms

All the farmers who were interviewed, agreed to the use of antibiotics in feed and water as it was the standard practice across the district. The feed used in all poultry farms was supplied by a single manufacturer across the district. Tetracycline (250gm/ton of feed) was added as a growth promoter. Also, a water additive containing levofloxacin (10% w/w) was used in all farms as a prophylactic measure against *E. coli*. Antibiotic use started as soon as the chicks entered the farms. The primary reason for such antibiotic use was that a single infected bird would infect the entire flock leading to financial loss. Tetracyclines and fluoroquinolones are widely misused as growth promoters and for prophylaxis.

Antibiotic Susceptibility Testing

All 187 *Escherichia coli* isolates were found resistant to tetracycline. Total of 176 (94%) *Escherichia coli* isolates were resistant to both ciprofloxacin and levofloxacin. 6% isolates were intermediate to both ciprofloxacin and levofloxacin. None of the isolates were sensitive to either ciprofloxacin or levofloxacin. It was observed that out of the 10 isolates collected from 1d old chicks, 4 were intermediate and 6 were found resistant to both ciprofloxacin and levofloxacin. But as the age increased, only resistant strains were found.

The high incidence of resistance to tetracycline and fluoroquinolones may be attributed to the continuous exposure of chickens (1−6 weeks old) to levofloxacin (10% w/w) and tetracycline (250 gm/ton) in water and feed. All farm workers in our study confirmed the use of these 2 antibiotics in feed and water respectively.

Only 36 (19%) isolates were resistant to cefotaxime and ceftazidime (Figure 1). Out of these 36, only 10 (27.7%) were confirmed as ESBL producers. Similar results were obtained in a study done in Odisha, India (Kar et al., 2015).

All the *E. coli* isolates were susceptible to carbapenems. Resistance to 2 or more antibiotics was found in all *E. coli* isolates from 1-day-old to 6 weeks old chickens with isolates from 3 wk and 5 wk exhibiting the most resistance in 16 out of the 20 tested antibiotics. *E. coli* isolates from 2 weeks old to 6 weeks old chickens had MAR index >0.2 (Table 1). Resistance to fluoroquinolones can also lead to cross-resistance to other antibiotics (Gouvêa et al., 2015).

The pattern of multi drug resistance found in our study in most *E. coli* isolates was ciprofloxacin-

levofloxacin-tetracycline in combination with other antibiotics. Prolonged exposure to antibiotics with increasing age results in the emergence of multidrug resistance. This was evidenced in the MAR (Multiple antibiotic resistance) indices of *E. coli* isolates from 2 wk onward (>0.2) indicating their origin from high-risk sources with rampant use of antibiotics. Presence of fluoroquinolone resistant isolates in 1-day-old chicks is an indication of pre-existence of resistance at the source of eggs. As the fluoroquinolone use increases in chickens, resistant isolates are selected over sensitive isolates.

Among the *Klebsiella* isolates, 18 (90%) out of 20 showed resistance to ampicillin, followed by ciprofloxacin, levofloxacin (80% each), and tetracycline (40%). All the 20 *Klebsiella* isolates were resistant to multiple antibiotics (Figure 1). Only one of the *Klebsiella* isolates was ESBL producer. None were resistant to the tested carbapenems. *Klebsiella spp* across the age groups had MAR index <0.2 (Table 1). Similar results were found in some previous studies (Bhushan et al., 2017). Though most of the *Klebsiella* isolates were resistant to 2 or more antibiotics across all the age groups, their MAR indices were <0.2. This may possibly be because of the low numbers of *Klebsiella* (n = 20) isolated from all the age groups. Also, some isolates being resistant to some antibiotics but sensitive to others may be because of possible admixing of strains of different origins.

Molecular Detection of Antimicrobial Resistance Genes

Out of the 176 (94%) *E. coli* isolates resistant to both ciprofloxacin and levofloxacin, 38 (21.5%) harbored qnrA, 67 (38%) harbored qnrB, and 48 (27%) were positive for qnrS genes. 23 (13%) carried both qnrA and qnrS.

Out of the 16 fluoroquinolone-resistant *Klebsiella* isolates, 15 (94%) carried qnrB while one carried qnrS. qnrA was not detected in any of the isolates. Heavy use of fluoroquinolones may be responsible for the prevalence of plasmid-mediated quinolone resistance, thus playing a role in horizontal gene transfer.

All the 187 *E. coli* isolates were resistant to tetracycline. 139 (74%) carried tetA gene while 25 (13%) carried tetB gene. 23 (12%) were positive for both tetA and tetB. All the 8 tetracycline-resistant *Klebsiella* carried tetA gene.

Of the 10 ESBL producing *E. coli,* 4 carried the SHV gene only, 1 had TEM along with SHV. 4 carried CTX-M15 in addition to SHV and 1 carried CTX-M1 in addition to SHV. Among the 20 *Klebsiella* isolates, the only ESBL producer harbored SHV, CTX-M15, and TEM. Not only do the CTX-M15 provide resistance to extended spectrum beta-lactams but mutations in SHV and TEM alone can also confer resistance to broad spectrum beta-lactams.

Many studies suggest the possibility of transfer of antibiotic resistance genes from poultry to humans in close contact with it. A study in Netherlands showed similar ESBL producing isolates were present in poultry meat samples and handlers (Leverstein-van Hall et al., 2011) A study conducted in six European countries showed that *E. coli* isolates from animals, environment and humans carrying $bla_{\text{CTX-M-1}}$ and qnrS and qnrB genes had identical RFLP patterns (Dolejska et al., 2013)

Fluoroquinolones, third generation cephalosporins, beta-lactams, and tetracyclines are important antimicrobials for human and animal treatment. High prevalence of multiple drug resistant isolates in broilers starting from a day-old chick is highly indicative of antibiotic abuse in poultry. This needs to be addressed to prevent critical antibiotics from becoming ineffective for treatment. The presence of plasmid-mediated resistance mechanisms in broilers could lead to the dissemination of these determinants in the food chain and environment also. One Health involves the interaction between humans, animals and, the environment. To realize One Health, it is imperative that antibiotic use should be strictly limited to treatment purpose in both animals and humans.

ACKNOWLEDGMENTS

We express our sincere gratitude to all the poultry farmers for taking part in the study. The study was financially supported by Department of Biotechnology, Ministry of Science and Technology, Government of India in the form of DBT scholarship for PhD scholars and Manipal Academy of Higher Education, Manipal, Karnataka, India in the form of contingency fund for PhD scholars to cover equipment, consumables, and publication costs.

DISCLOSURES

The authors declare that they have no conflicts of interest.

REFERENCES

Bhushan, C., A. Khurana, R. Sinha, and M. Nagaraju. 2017. Antibiotic Resistance in Poultry Environment: Spread of Resistance from Poultry Farm to Agricultural Field. Centre for Science and Environment, New Delhi. Accessed Dec 1, 2018. https://cdn.cseindia.org/userfiles/report-antibiotic-resistance-poultry-environment.pdf.

Dolejska, M., L. Villa, H. Hasman, L. Hansen, and A. Carattoli. 2013. Characterization of IncN plasmids carrying $bla_{\text{CTX-M-1}}$ and *qnr* genes in *Escherichia coli* and *Salmonella* from animals, the environment and humans. J. Antimicrob. Chemother. 68:333–339.

Gouvêa, R., F. F. dos Santos, M. H. C. de Aquino, and V. L. de Pereira. 2015. Fluoroquinolones in industrial poultry production, bacterial resistance and food residues: a review. Braz. J. Poult. Sci. 17:1–10.

Huang, S., L. Dai, L. Xia, X. Du, Y. Qi, and H. Liu. 2009. Increased prevalence of plasmid-mediated quinolone resistance determinants in chicken Escherichia coli isolates from 2001 to 2007. FOODBORNE Pathog. Dis. 6:1203–1209.

Kar, D., S. Bandyopadhyay, D. Bhattacharyya, I. Samanta, A. Mahanti, and P. K. Nanda. 2015. Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-

lactamase producing *Escherichia coli* isolated from poultry and cattle in Odisha, India. Infect. Genet. Evol. 29:82–90.

Kurnia, R. S., A. Indrawati, N. Luh, P. Ika, and A. Priadi. 2018. Molecular detection of genes encoding resistance to tetracycline and determination of plasmid-mediated resistance to quinolones in avian pathogenic Escherichia coli in Sukabumi, Indonesia. Vet. World 11:1581–1586.

Laube, H., A. Friese, C. von Salviati, B. Guerra, and U. Rösler. 2014. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. Vet. Microbiol. 172:519–527.

Leverstein-van Hall, M. A., C. M. Dierikx, J. Cohen Stuart, G. M. Voets, M. P. van den Munckhof, A. van Essen-Zandbergen, T. Platteel, A. C. Fluit, N. van de Sande-Bruinsma, J. Scharinga, M. J. M. Bonten, and D. J. Mevius. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin. Microbiol. Infect. 17:873–880.

Sinha, R., S. Kamath, and M. Suchitra Shenoy. 2015. Association of risk factors, antimicrobial resistance trends and occurrence of bla-TEM, bla SHV and bla CTX M in Escherichia coli causing bacteremia. Infect. Disord. Drug Targets 16:95–100.

Zowawi, H. M., A. L. Sartor, and H. H. Balkhy. 2014. Molecular characterization of carbapenemase-producing Escherichia coli and Klebsiella pneumoniae in the countries of the Gulf cooperation council: dominance of OXA-48 and NDM producers. Antimicrob. Agents Chemother. 58:3085–3090.