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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 04

Res. Jr. of Agril. Sci. (2022) 13: 1003–1006

CARAS

Res. Jr. of Agril. Sci. (Jul-Aug 2022)
13(4): 1003–1006

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

A Brief Study of Antibiotic Resistant Bacterial Population in Poultry Samples

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Received: 26 May 2022 | Revised accepted: 09 Jul 2022 | Published online: 13 July 2022

A B S T R A C T

Antibiotic usage is considered as the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine. Antibiotics are regularly used in poultry production as antimicrobial growth promoters. The presence of antibiotics may result in development of antibiotic resistant microbes in the chicken meat. Moreover, even if the microbes are killed by cooking the antibiotic resistant gene pool is not destroyed. Thus, the individuals handling raw poultry meat or consuming this chicken are exposed to antibiotic resistant bacteria &/or gene pool both of which may result in development of new antibiotic resistance in the exposed individuals. Here we report about the antibiotic resistant bacterial population in raw broiler chicken samples collected from different areas of Kolkata, West Bengal. Ciprofloxacin resistant faecal coliforms isolated from broiler chicken were found to be resistant to Neomycin, Oxytetracycline, Enrofloxacin and Doxycycline. Multidrug resistant strains were also quite abundant. Plasmids were detected in six of the multidrug resistant isolates. The presence of MDR strains carrying plasmids in chicken samples is quite detrimental as antibiotic resistance is often harboured on plasmids, which could facilitate horizontal gene transfer.

***Key words*:** Antibiotic resistance, MDR strains, Plasmids, Broiler chicken, Kolkata

Antibiotic resistance is a major global health problem mainly due to its excessive use in human and veterinary medicine, inappropriate prescribing, extensive agricultural use and unavailability of new drugs [1-2]. Antibiotics are used in food animals to prevent infection, to reduce food borne pathogen and to promote growth as antimicrobial growth promoters [3]. The maximum portion of antibiotics on this earth is used in raising food animals and often in bulk [4]. Such heavy exposure to antibiotics selects for antibiotic resistance not only in pathogenic but also in endogenous microorganisms of these animals. Many species of antibiotic resistant bacteria were shown to be transmitted to humans either by contact or by food chain [5-6].

Broiler chicken from poultry farms are a major source of animal food and presence of antibiotic resistant bacterial strains in their faecal flora is quite high [7]. These resistant strains are often transmitted to humans through direct contact while slaughtering or handing of raw meat under unhygienic conditions [8-9]. Resistant *E. coli* from animals were detected in human gut very early in the seventies [10-11]. However, the mode of transmission of antibiotic resistance from food animals to human is a major area of research. Some studies indicated the involvement of resistance plasmids in horizontal gene transfer [12-14]. Moreover, inappropriate cooking or insufficient cooking at low temperatures often cannot remove all the different types and forms of microbes present, as presence of antibiotic resistant bacteria in cooked meat and transferability of resistance genes have been reported in China [15].

India, along with China, represents the largest hotspots of antibiotic resistance and a marked increase in resistance in chickens have also been reported [16]. The scenario of antibiotic resistance in food animals and environment was found to be very grim in India [17]. The prevalence of antibiotic resistance *Salmonella sp.* have been reported in raw chicken liver and egg yolk in Mumbai [18], chicken meat in Bihar [19] and backyard birds in West Bengal [20]. Here we report the preliminary findings of antibiotic resistance in broiler chicken meat samples from two local markets of Kolkata, West Bengal. and its probable underlying molecular mechanism for the first time.

MATERIALS AND METHODS

Collection of poultry samples

Samples of raw broiler chicken meat were collected from two local markets one in North and another in South Kolkata area. Samples were collected in sterile container and transferred to the laboratory on ice.

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Enumeration of total viable count and antibiotic resistant bacterial population

1 gram of chicken samples were taken and homogenized with 2 ml of ice cold sterile 0.9% NaCl solution. The homogenate was centrifuged at 7000 rpm for 2 minutes and supernatant was collected in a fresh tube which was treated as the stock solution for each sample. The stock was serially diluted up to 10^{-3} dilutions. 0.1 ml of stock and all dilutions were plated on nutrient agar (NA) medium to determine the total viable count and on NA plates containing three different antibiotics separately. The antibiotics used were ciprofloxacin (4 mg/L), neomycin (16 mg/L) and oxytetracycline (16 mg/L). All plating's were done in duplicates.

Isolation of ciprofloxacin resistant faecal coliforms

Ciprofloxacin resistant colonies were grown on Eosin Methylene Blue (EMB) agar plates containing ciprofloxacin (4 mg/L) in case of Sample B, broiler chicken meat collected from North Kolkata market. However, for Sample BG, broiler chicken meat collected from South Kolkata market, 0.1 ml of stock and all dilution were plated directly on Eosin Methylene Blue (EMB) agar plates containing ciprofloxacin (4 mg/L). For both samples discrete colonies with greenish metallic sheen characteristic of *Escherichia coli* were selected and subsequently subcultured on EMB-ciprofloxacin plates till pure culture was obtained. All the isolates were gram stained. Those culture showing characteristic pink coloured short rod morphology of faecal coliforms were selected and IMViC tests were performed with the selected cultures to confirm the presence of faecal coliforms.

Antibiotic susceptibility assay

The ciprofloxacin resistant isolates were streaked on EMB-neomycin and EMB-oxytetacycline plates to assess their sensitivity to these antibiotics. Moreover, the isolates were individually plated on LA plates and discs of the following antibiotics enrofloxacin (5µg/disc) and doxycycline (30µg/disc) were placed aseptically and the plates were incubated at 37°C for 18 hrs. Diameters of zone of inhibition were measured.

Plasmid isolation

Plasmid isolation was performed from all the ciprofloxacin isolates. LB broth was inoculated with individual isolate and incubated at 37°C for 18 hrs. 1 ml of the culture was centrifuged at 6000 rpm for 8 minutes and supernatant was discarded. The pellet was resuspended in 100 ul of solution I (25mM Tris.HCl, 10 mM EDTA, 50 mM glucose pH 8). Then 200 ul of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and mixed properly. To it 150 ul of ice-cold solution III (3M KOAc pH 4.8) was added, mixed properly and incubated on ice for 10 minutes. The tubes were centrifuged at 12000 rpm for 15 minutes. The supernatant was collected in a fresh tube and to it 0.7 volume isopropanol was added, mixed and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12000 rpm for 20 minutes and supernatant discarded. The pellet was washed with 70% ethanol and then air dried. Finally, the pellet was dissolved in 20 ul TE buffer. From it 10 ul of sample was loaded in 0.8% agarose gel. The gel was run at 100 V for 40 minutes and observed on a uv-transilluminator.

RESULTS AND DISCUSSION

The broiler chicken meat collected from North Kolkata local market, sample B showed a higher total viable count as compared to Sample BG, collected from South Kolkata local market (Table 1). Three groups of antibiotics - fluoroquinolones (ciprofloxacin and enrofloxacin), tetracyclines (oxytetracycline and doxycycline) and aminoglycosides (neomycin), that are routinely used in poultry were used to study the resistance profile [21]. In spite of having lower viable count sample BG showed greater load of antibiotic resistant bacterial population (Table 1).

Table 1 Comparative chart of antibiotic resistant population of the samples

Sample	Total viable count (cfu/ml)	Antibiotic resistant bacterial count (cfu/ml)		
		Ciprofloxacin	Neomycin	Oxytetracycline
B	1.6×10^5cfu/ml	5.4×10^3	5.4×10^3	4.9×10^4
BG	6.8×10^4cfu/ml	2.4×10^4	1.14×10^4	4.2×10^4

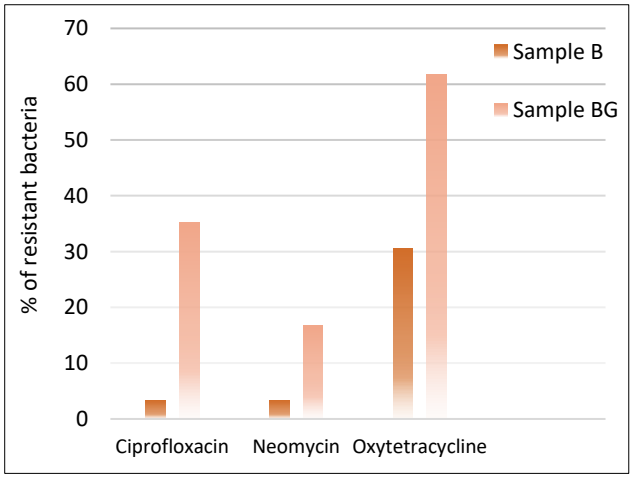

Fig 1 Plot showing proportion of total bacteria population resistant to antibiotics

In general proportion of bacterial population resistant to oxytetracycline is much more in both meat samples -30.6% for sample B and 61.7% for sample BG, compared to other two antibiotics (Fig 1). Moreover, for sample BG, 35.2% and 16.7% of total bacterial population was resistant to ciprofloxacin and neomycin respectively which is quite high (Fig 1).

Ciprofloxacin resistant colonies showing greenish metallic sheen on EMB plates, characteristic of *Escherichia coli*, were selected. For sample B eight such colonies and for sample BG five such colonies, that is, total thirteen were isolated and pure culture were obtained for all the isolates by repeated subculturing. All the thirteen isolates were gram negative short rods and gave positive Indole and Methyl red tests and negative VP and citrate test for IMViC assay. So, all the ciprofloxacin resistant isolates were confirmed to be faecal coliforms. Antibiotic susceptibility test revealed that many of the isolated strains were multidrug resistant (Table 2).

As high as 69% of the faecal coliform isolates were resistant to four of the five drugs tested whereas 15.3% of the isolates were resistant to all the five antibiotics. Thus, alarming prevalence of MDR faecal coliforms were observed in broiler chicken meat. *Salmonella* isolated from chicken meat in Bihar were 100% resistant to ampicillin whereas moderately sensitive

to ciprofloxacin [19]. *Salmonella* species isolated from backyard birds and their environment were 100% resistant to ciprofloxacin, gentamicin and tetracyclines [20].

The transmissibility of the antibiotic resistance from animal and human strains of *Escherichia* to resident *Escherichia coli* in GI tract of human have long been reported [8]. Many a times different plasmids like R plasmids [22], IncH plasmids [23] were detected in antibiotic resistant bacterial strains isolated from chicken samples and these plasmids are often responsible for horizontal gene transfer of antibiotic resistance from chicken to humans [12]. Out of the thirteen isolates plasmid DNA could be isolated from six strains (Fig 2).

Table 2 Antibiotic Susceptibility Profile of the eight isolates

Isolates	Ciprofloxacin	Neomycin	Oxytetracycline	Enrofloxacin	Doxycycline
B_1	Resistant	Resistant	Resistant	Sensitive	Resistant
B_3	Resistant	Sensitive	Resistant	Sensitive	Sensitive
B_5	Resistant	Resistant	Resistant	Sensitive	Resistant
B_6	Resistant	Resistant	Resistant	Sensitive	Resistant
B_7	Resistant	Resistant	Resistant	Sensitive	Resistant
B_8	Resistant	Resistant	Resistant	Sensitive	Resistant
B_9	Resistant	Resistant	Resistant	Sensitive	Resistant
B_{10}	Resistant	Resistant	Resistant	Sensitive	Resistant
BG_1	Resistant	Resistant	Resistant	Resistant	Resistant
BG_2	Resistant	Resistant	Resistant	Resistant	Resistant
BG_3	Resistant	Sensitive	Resistant	Resistant	Resistant
BG_4	Resistant	Sensitive	Resistant	Resistant	Resistant
BG_5	Resistant	Sensitive	Sensitive	Sensitive	Sensitive

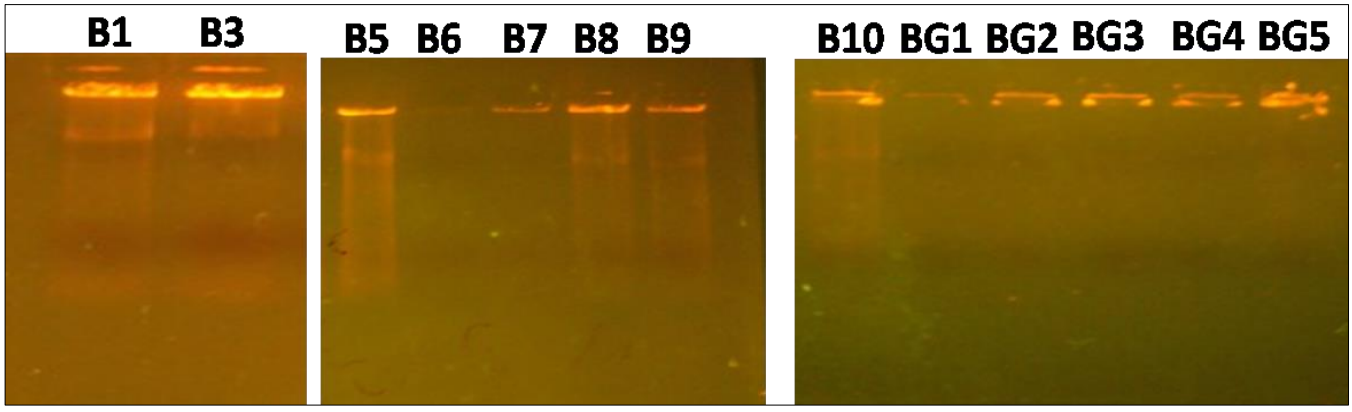

Fig 2 Agarose gel electrophoresis of plasmids isolated from the six individual bacterial isolates B_1, B_3, B_5, B_8, B_9 and B10. B_1, B_3, B_5, B_6-B_{10} are strains isolated from North kolkata broiler samples and BG_1- BG_5 are strains isolated from south Kolkata market

CONCLUSION

In this report 92% of the ciprofloxacin resistant isolates were resistant to oxytetracycline, 84% were resistant to doxycycline, 69% to neomycin and 30% to enrofloxacin. A high proportion of the isolates were resistant to four of the five drugs tested. Above 15% of the isolates were resistant to all the tested drugs. Moreover, multidrug resistant strains carrying plasmids were detected in chicken samples in this initial report. These findings are really alarming. Further work should be done to assess if antibiotic resistance genes are plasmid borne as that could facilitate horizontal gene transfer which is a driving force for propagation of antibiotic resistance.

Acknowledgement

The authors gratefully acknowledge DST-FIST, Govt. of India, for funding the development of infrastructural facilities used for this work.

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