Original Research Article

Microbial Load, Antibiotic Resistant Bacteria and Antibiotic Residues in Broiler Chicken

ABSTRACT

Chicken meat is highly preferred protein food worldwide. To meet the demand, huge poultry farms are established and using antibiotics as prophylaxis and treatment against the bacterial diseases. Uncontrolled usage of antibiotics has led to development of antibiotic resistance in poultry and antibiotic residues in poultry chicken. Fifty one chicken meat samples were collected from various retail outlets. Antibiotic residues were quantified by HPLC, total microbial load was measured by growth of bacteria on growth medium and antibiotic resistant profile of *Escherichia coli*, *Salmonella spp*, *Staphylococcus aureus* and *Campylobacter spp* was determined by well diffusion method. Except neomycin, all tested antibiotics were present in the range of 10-978 ppm, the average microbial load was in the range log 10 of 7.32 per gram of chicken sample, *E.coli*, *Salmonella spp*, *Staphylococcus aureus* and *Campylobacter spp* were resistant to several antibiotics studied. Hence there is a need of appropriate usage of antibiotics in poultry and proper handling of chicken during farming and slaughtering.

Key words: Poultry; Chicken meat; Antibiotic resistance; Antibiotic residues; Total microbial count.

1. INTRODUCTION

Poultry meat is among highest consumed protein source food worldwide due to its high nutrition level and relatively low cost (1). India got huge demand of poultry meat and eggs (2). To meet the huge demand inappropriate antibiotics are being used leading to antibiotic resistance among bacteria of poultry. The World Health Organization has warned that inappropriate use of antibiotics in poultry may lead to increased food insecurity and food hazard (3). Further inappropriate antibiotics usage has led to deposits of antibiotics residues xenobiotic in meat and eggs (4). Studies have shown that food from animal and poultry origin is implicated to be a crucial source of human infection and transmission mode has been through handling and meat consumption [5]

Due to antibiotic resistance, despite of antibiotics applied, many bacteria will remain live in poultry birds (6) and asystemic handling and slaughtering leads to presence of microbial load in chicken meat. Humans are not only affected of these microorganism but residual drugs also potentially disturb intestinal normal flora. Studies worldwide have shown

that *Campylobacter*, *Salmonella*, and *E. coli* are often present in fresh meat and poultry (7). Currently no official national statistics are available on the prevalence of antibiotic residues, type of microbial flora and antibiotic resistance patterns of chicken meat available for local consumer. The aim of this study was to screen for and quantitate antibiotic residues, microbial load, and antibiotic resistance patterns of *E.coli*, *Salmonella spp*, *S. aureus* and *Campylobacter spp* in broiler chicken meat collected at point of sale in Telangana and Andhra Pradesh states of India.

2. MATERIALS AND METHODS

2.1.Study site and sample collection

Fifty one samples of chicken meat were collected from chicken shops of different markets of Hyderabad, Medchal, Rangareddy districts of Telangana, Kurnool and Guntur districts of Andhra Pradesh, India during September 2022 rainy season, average temperatire being 27^{o}C. Using sterile bags, the samples were collected and kept in cool box and transported to the laboratory.

2.2.Total microbial count

One gram of meat sample was added to 9 ml of distilled water, vortexed and undergone up to 10^{-8} dilutions. 0.1ml of each dilutions of all meat samples were spread on nutrient agar plates. The plates were incubated at 37^{o}C for 24hr and CFU (colony forming units) were counted using colony counter (8).

2.3.Microbiological analysis of pathogens

- *E.coli*: *Escherichia coli* was isolated from meat samples by enriching each sample in lactose broth, incubated for 24 hrs and then streaked on MacConkey agar and incubated at 37^{o}C for 24hr. The *E.coli* colonies were examined by growth, gram staining and biochemical IMVIC tests (9).
- *Salmonella spp*: *Salmonella* species were isolated from meat samples by enriching each sample in peptone water made using buffer, incubated at 37^{o}C for 24hr, then 1ml of suspension samples were inoculated in 9ml of Tetrathionate broth and was incubated at 37^{o}C for 24hr and it was then streaked on Salmonella Shigella agar and incubated at 37^{o}C for 24hr. The *Salmonella spp* black colonies were examined by gram staining and biochemical tests like urease test, dulcitol and lactose fermentation test, lysine decarboxylase test (10).
- *Staphylococcus aureus*: *S.* aureus was isolated from meat samples by meat suspension in peptone water and streaking the samples onto mannitol salt agar and incubated at 37^{o}C for 24hr. The *S.* aureus colonies were examined by growth, gram staining and biochemical tests like catalase test, coagulase test, mannitol fermentation test (11).

- *Campylobacter spp*: *Campylobacter spp* was isolated from meat samples by enriching each sample in Bolton Broth (without blood) and incubated at 40^{o}C for 24hr micro aerobically. Streaked the enrichment onto Blood agar and incubated at 40^{o}C for 48hrs. The *Campylobacter spp* colonies were examined by growth, motility in dark field microscopy and biochemical tests like oxidase tests, resistance to cephalothin and resistance to nalidixic acid (12).

2.4.Antibiotic sensitivity test of the isolates

Antibiotic sensitivity of the isolates was determined using well diffusion techniques. The isolates of *E.coli*, *Salmonella spp*, *Staphylococci aureus*, and *Campylobacter spp* were spread on nutrient agar plates. Then 20µl of antibiotic suspensions prepared for 100µg concentration were placed in 10mm wells punched in nutrient agar plates. Kanamycin, ampicillin, neomycin, nitrofurantoin, doxycycline, tetracycline, ciprofloxacin, oxytetracycline, sulfamethoxazole, gentamycin, streptomycin and amoxicillin are the antibiotics used in this technique (13).

2.5.Antibiotic extraction and antimicrobial activity testing

Ten gram of chicken meat was soaked in 20ml of ethyl acetate and crushed in a mortar using a pistle. The suspension was centrifuged at 5000rpm for 10min and the supernatant obtained contains antibiotics. Fifty µl of supernatant was placed in 10mm wells, punched in spread plates of *E.coli*, *Salmonella spp*, *Staphylococci* aureus, *Campylobacter spp* and incubated at 37^{o}C for 24hr. Zones of inhibition were observed and measured (13).

2.6.Identification of antibiotics by HPLC

Ten gram of meat sample was placed in a centrifuge tube. The meat sample was covered with 50µl of 0.01µg of standard antibiotic solution for positive control. Twenty five ml of acetonitrile was added and centrifuged at 5000rpm for 10 minutes. The supernatant obtained was taken in another centrifuge tube and evaporated under nitrogen stream at 50^{o}C. Again the sample was reformed in 5ml of water and centrifuged at 5000rpm for 10 minutes. Then the supernatant was loaded in pre filter cartridge. The cartridge was constrained with 5ml of methanol and 5ml of deionized water. Fifty persent methanol was used to wash the column. The analytes elution was obtained using 5ml of 1% ammonia solution in methanol. Elute was evaporated under nitrogen stream at 50^{o}C. The residue was dissolved in 50% methanol solution and then transferred into a vial for HPLC analysis.

SHIMADZU GC 2010 HPLC system was used for chromatographic separation of antibiotics. The separation column was 100mm × 2.1mm Discovery analytical column. The mobile phase/component A was water and component B was methanol. Both contain 0.1% of formic acid. The flow rate was 300µl per minute. The mobile phase B was raised from 10% to 30% for

4min, and then carried on for 1min. The mobile phase B was raised to 95% from 5min to 10min and maintained until 10.5 min. Then mobile phase B percentage was reduced to 10% in 0.5min and was held constant at this point for 15min. The temperatures of the column and samples were 30^{o}C and 10^{o}C respectively.

2.7.Statistical analysis

Experiments were repeated thrice in triplicates (n = 9) and average values with standard deviation was provided.

3. RESULTS

3.1.Total microbial count

The CFUs of 51 chicken meat samples from Hyderabad, Rangareddy, Medchal, Guntur and Kurnool were counted. Their Total Viable Count (TVC), Total *E.coli* Count (TEC), Total *Salmonella spp* Count (TSC), Total *S. aureus* Count (TSC), Total *Campylobacter spp* Count (TCC) was listed in table 1. Total microbial load was in the range of 6.5 to 8.2 log 10, total *E.coli* was in the range of 4.8 to 5.6 log 10, total *Salmonella spp* count was in the range of 2.1 to 3.5 log 10, total *S. aureus* count was in the range of 2.0 to 2.8 log 1 and total *Campylobacter spp* count was in the range of 0.8 to 1.6 log 10 values.

3.2.Microbiological analysis of pathogens

- *E.coli:*

Out of 51 samples of chicken meat collected, 44 samples (85%) were positive for *E.coli*. *E.coli* confirmed by pink colonies on MacConkey agar, gram negative short rods ,indole positive, Methyl red positive, vogaus-prausker negative and citrate negative.

- *Salmonella spp:*

Out of 51 samples of chicken meat collected, 7 samples (15%) were positive for *Salmonella spp*. *Salmonella spp* confirmed by black colonies on salmonella shigella agar, straight rods, urease negative, dulcitol fermentation test negative, lysine decarboxylase test positive, lactose fermentation test negative.

- *Staphylococcus aureus:*

Out of 51 samples of chicken meat collected, 40 samples (78%) were positive for *S. aureus*. *S. aureus* confirmed by yellow colonies on mannitol salt agar, purple coccus bacteria, catalase positive, coagulase positive and mannitol fermentation positive.

- *Campylobacter spp:*

Out of 51 samples of chicken meat collected, 10 samples (20%) were positive for *Campylobacter spp*. *Campylobacter spp* confirmed by greyish, smooth colonies on Blood agar, pink motile rods with a polar flagellum in dark field microscope, catalase positive and oxidase positive.

Fig.1 shows the percentage of meat samples positive for different pathogens.

3.3.Antibiotic sensitivity test of the isolates

E.coli, *Staphylococcus aureus* and *Campylobacter spp* were highly resistant to ampicillin, amoxicillin, doxycycline, tetracycline, oxytetracycline. They were intermediately susceptible to neomycin, nitrofurantoin, sulfamethoxazole. They were highly susceptible to kanamycin, gentamycin, streptomycin and ciprofloxacin. *Salmonella spp* was found to be multi-drug resistant as it was resistant to many antibiotics. Table 2 displays the antibiotic-sensitivity profile of *E.coli*, *Salmonella spp*, *Staphylococcus aureus* and *Campylobacter spp* as zone of inhibition (mm) with 100µg concentration.

3.4.Antibiotic extraction and antimicrobial activity testing

The zones of inhibition of antibiotics extracted were observed by well diffusion method. *E.coli*, *Salmonella spp*, *Campylobacter spp* and *S.aureus* were tested. Out of 51 samples extracted, 44 samples (86%) displayed inhibition zones on *Campylobacter spp*, 39 samples (76%) displayed inhibition zones on *S. aureus*, 35 samples (68%) displayed inhibition zones on *Salmonella spp* and 37 samples (72%) displayed inhibition zones on *E.coli*. This shows the presence of antibiotics in meat. The results are displayed in Fig.2.

3.5.Identification of antibiotics by HPLC

Ampicillin, kanamycin, gentamycin, doxycycline, ciprofloxacin, sulfamethoxazole, tetracycline, oxytetracycline and streptomycin were the antibiotics screened by HPLC. Concentration of all antibiotics except neomycin was more. The concentration of ampicillin, kanamycin, neomycin, gentamycin, doxycycline, ciprofloxacin, sulfamethoxazole, tetracycline, oxytetracycline and streptomycin were in the ranges of 10-19 ppm, 122-338 ppm, 0 ppm, 126-338 ppm, 97-386 ppm, 84-978 ppm, 97-919 ppm, 95-374 ppm, 94-369 ppm and 129-344 ppm respectively. Table 3 shows various antibiotics screened and verified by HPLC. Ciprofloxacin was present in highest amount, whereas ampicillin was present in lesser amount.

4. DISCUSSION

Poultry production is one of the largest and most widespread industries in India, using large quantities of various antimicrobials as prophylactic and therapeutic making critical for human health (3, 14). Unregulated antibiotics usage as growth promoters caused selective pressure for

multidrug resistant (MDR) bacteria (15, 16, 17). In the present study, found high prevalence of antibiotic residues in meat samples (38 of 51), verified by antimicrobial activity measured by well diffusion, as reported by Pugajeva *et al.* (18) 37 of 40 samples contained antibiotic residues. The strains of *Salmonella spp*, *E.coli*, *Campylobacter spp* and *S. aureus* are known for majority of diseases in poultry. Hence their antibiotic resistance profiling is carried out. All the studied strains were reported to have multidrug resistance as reported by Suresh et al. (19).

This study which looks into chicken meat contamination by pathogens showed positive results for *E.coli* (85%), *Salmonella spp* (15%), *S. aureus* (78%), *and Campylobacter spp* (20%). Especially, the prevalence of *S. aureus* (78%) in chicken meat from different markets of Telangana and Andhra Pradesh was more than that found in raw chicken meat sold in Indonesian markets (58.3%) (20). Fifteen percent of the samples (8 out of 51) were positive for *Salmonella spp*, this was higher than the studies conducted in other countries, which have reported a prevalence of 10.8% for the chicken meat sold in Ghana (21) and 9.5% for the chicken meat sold in Chongqing, China (22). In this study, 85% of the samples (44 out of 51) contained *E.coli*, whereas, 65% of chicken meat samples from Bangladesh contained *E.coli* (23), no *E.coli* was detected in chicken meat sample from a market in Ibadan, Nigeria (24) and 96.7% of the chicken meat samples from markets in Surabaya, East Java (Indonesia) were positive for *E.coli* (25). In this study, 10 samples out of 51 (20%) were positive for *Campylobacter*, whereas 141 of 429 (32.9%) broiler chicken meat samples from Estonia were positive for *Campylobacter* (26). This high percentage of pathogen content in chicken meat from the markets is high-risk for human consumption (9). Campylobacteriosis incidence has been rising in both developed and developing countries (27). *Campylobacter* species have many unique growth requirements that can limit but not eliminate their prevalence outside warm-blooded hosts. Most *Campylobacter* bacteria grow optimally at either 42°C (chicken body temperature) or 37°C (human body temperature), but none of them can grow below 30°C (28)

In this study, the average microbial load was in the range log 10 of 7.32 per gram of chicken samples. Microbial load reported by Bhandari et al. (29) in Nepal poultry samples is very high in the range of log10 of 6-12 and also high levels of *Salmonella spp*, *E.coli*, *Campylobacter* spp and *S. aureus*, indicating unhygienic practices in farming and sales outlets. Microbial load in 60% sample collected is more than 10^6 CFU per gram hence comes under rejected grade (30). Bacterial contamination can also be built on processing equipment and cross contaminate to other birds, workers and air as well (31). Slaughtering is the main site of pathogen contamination (31) hence hygienic practices at slaughter house should be practiced. In our study microbial load in chicken meat was in the range of log10 of 6.5±0.7 to 8.2±0.5. Birds slaughtering and processing causes the spread of gut microbes and environmental flora is contaminated during defeathering and evisceration process (32, 33). It is essential to control fecal and ceacal pathogens such as *Salmonella spp*, *E.coli* and *Campylobacter spp* (34, 35).

In our study, various antibiotics were verified and screened by HPLC; they were ampicillin, kanamycin, gentamycin, doxycycline, ciprofloxacin, sulfamethoxazole, tetracycline,

oxytetracycline and streptomycin. D. Baazize-Ammi et al. (36) reported amoxicillin, ampicillin, penicillin G, oxacillin, erythromycin, sulfisoxazole antibiotics in chicken meat by HPLC. India has not yet set any limits of antibiotics residues in chicken.

Based on the findings it is advised to use rational and required antibiotics as a treatment only to prevent the antibiotic residues and antibiotics resistance in poultry. It is also required to avoid contamination of chicken meat during slaughtering with vigorous washing.

5. CONCLUSION

Chicken meat samples were analyzed for antibiotic residues, microbial load, antibiotic resistance patrons of isolated microbes. Antibiotic residues were quantified by HPLC, total microbial load was measured by growth of bacteria on growth medium and antibiotic resistant profile of *E.coli*, *Salmonella spp*, *S. aureus* and *Campylobacter spp* was determined by well diffusion method. Except neomycin, all tested antibiotics were present in the range of 10-978 ppm, the average microbial load was in the range log 10 of 7.32 per gram of chicken sample, *E.coli*, *Salmonella spp*, *S. aureus* and *Campylobacter spp* were resistant to several antibiotics studied.

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Table 1: Log 10 values of CFUs of meat samples collected from different markets of Telangana and Andhra Pradesh.

		Total samples	Log Total viable Count	Log Total *E.coli* count	Log Total *Salmonella* count	Log Total *Staphylococcus* count	Log Total *Campylobacter* count
T.S	Hyderabad	11	8.2 ± 0.5	5.6 ± 0.3	3.4 ± 0.7	2.8 ± 0.8	1.6 ± 0.4
	Rangareddy	10	7.8 ± 0.8	5.1 ± 0.4	2.8 ± 0.5	2.4 ± 0.7	1.2 ± 0.8
	Medchal	10	6.9 ± 0.7	4.8 ± 0.2	2.1 ± 0.9	2.0 ± 0.6	0.8 ± 0.1
A.P	Guntur	10	7.2 ± 0.9	5.5 ± 0.7	2.9 ± 0.8	2.5 ± 0.5	1.3 ± 0.2
	Kurnool	10	6.5 ± 0.7	5.2 ± 0.1	3.5 ± 0.4	2.6 ± 0.4	1.1 ± 0.5

Table 2: The Antibiotic-sensitivity profile of Isolated *E.coli, Salmonella, Staphylococcus* and *Campylobacter* as zone of inhibition (mm) with 100µg antibiotics.

Bacteria	**Antibiotics**											
	Kanamycin	**Ampicillin**	**Neomycin**	**Nitrofurantoin**	**Doxycycline**	**Tetracycline**	**Ciprofloxacin**	**Oxytetracycline**	**Sulfamethoxazole**	**Gentamycin**	**Streptomycin**	**Amoxixillin**
E.coli	25 ± 0.5	0	12 ± 0.4	14 ± 1.1	9 ± 1.1	8 ± 1.2	28 ± 0.3	9 ± 1.1	9 ± 0.2	27 ± 0.1	22 ± 0.5	5 ± 0.8
Salmonella	2 ± 1.2	2 ± 0.4	5 ± 0.8	6 ± 0.9	5 ± 0.8	5 ± 0.8	25 ± 0.4	5 ± 1.2	6 ± 0.4	5 ± 0.3	5 ± 0.4	2 ± 1.2
Staphylococcus	24 ± 1.1	0	9 ± 1.1	8 ± 0.8	8 ± 0.5	8 ± 0.7	29 ± 0.3	7 ± 0.9	11 ± 0.5	24 ± 0.1	21 ± 0.4	3 ± 1.1
Campylobacter	22 ± 0.8	3 ± 0.2	15 ± 1.2	15 ± 0.7	5 ± 0.6	6 ± 0.4	30 ± 0.6	8 ± 0.9	14 ± 0.2	29 ± 0.3	25 ± 0.5	4 ± 0.7

Table 3: Antibiotics Residues in Chicken meat in (PPM)

Antibiotic	Residues in ppm									
	Hyderabad		Rangareddy		Medchal		Guntur		Kurnool	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Ampicillin	11.4 ± 0.1	18.5 ± 0.3	12.8 ± 0.1	19.6 ± 0.2	10.2 ± 0.1	17.9 ± 0.1	12.4 ± 0.2	19.6 ± 0.3	11.8 ± 0.1	18.9 ± 0.2
Kanamycin	126.2 ± 0.01	333.6 ± 0.02	125.9 ± 0.05	330.2 ± 0.04	124.5 ± 0.01	325.2 ± 0.03	128.3 ± 0.02	336.5 ± 0.02	122 ± 0.06	338.2 ± 0.07
Neomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gentamycin	129.1 ± 0.05	337.1 ± 0.07	127.5 ± 0.08	335.8 ± 0.01	126.4 ± 0.03	336.5 ± 0.06	129.5 ± 0.05	335.4 ± 0.04	128.2 ± 0.02	338.2 ± 0.07
Doxycycline	97.4 ± 0.03	360.2 ± 0.09	99.2 ± 0.02	365.8 ± 0.01	98.5 ± 0.08	371.2 ± 0.07	97.2 ± 0.06	386 ± 0.05	99.01 ± 0.04	364.8 ± 0.08
Ciprofloxacin	85.2 ± 0.05	954.2 ± 0.08	87.2 ± 0.07	958.1± 0.09	88.2 ± 0.03	948.2 ± 0.04	89.2 ± 0.07	968.2 ± 0.08	84.2 ± 0.05	978.2 ± 0.08
Sulfamethoxazole	99.2 ± 0.1	914.2 ± 0.8	97.2 ± 0.2	910.6 ± 0.5	99.1 ± 0.7	917.2 ± 0.3	98.2 ± 0.7	915.2 ± 0.6	100.1 ± 0.8	919.2 ± 0.7
Tetracycline	98.2 ± 0.2	370.2 ± 0.3	97.2 ± 0.2	368.2 ± 0.7	95.0 ± 0.8	367.2 ± 0.7	99.2 ± 0.9	372.2 ± 0.8	97.2 ± 0.5	374.2 ± 0.8
Oxytetracycline	97.2 ± 0.03	369.2 ± 0.07	96.4 ± 0.05	368.2 ± 0.04	99.3 ± 0.03	369.2 ± 0.09	95.2 ± 0.08	367.2 ± 0.07	94.2 ± 0.06	365.2 ± 0.05
Streptomycin	130.2 ± 0.04	340.2 ± 0.08	129.2 ± 0.06	342.1 ± 0.04	132.8 ± 0.08	341 ± 0.09	131.4 ± 0.07	343.2 ± 0.08	133.5 ± 0.06	344.1 ± 0.02

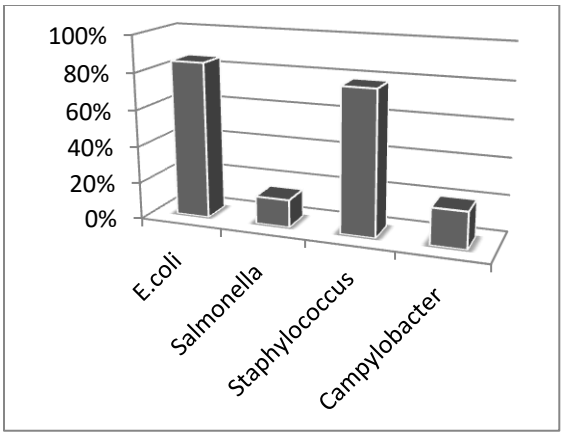

Fig.1 Percentage of meat samples positive for different pathogens.

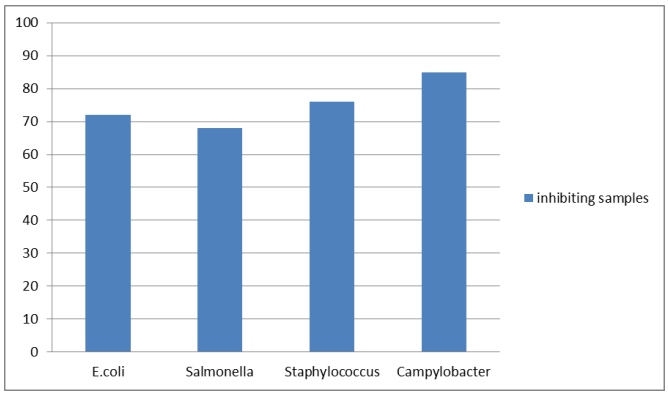

Fig 2: Percentage of samples with antibiotics inhibiting pathogens tested.