Multi-drug Resistant and Extended Spectrum β-lactamase Producing Salmonella Species Isolated from Fresh Chicken Liver Samples

Adhikari S,[1] Khadka S,[1,2] Sapkota S,[1,3] Sharma BR,[1] Ghimire A,[1] Chalise M,[1] Gurung D,[1] Kunwar S[1]

[1]Department of Microbiology,
Birendra Multiple Campus,
Tribhuvan University, Bharatpur, Chitwan,
44200, Nepal.

[2]Department of Biochemistry and
Molecular Biology,
School of Life Sciences,
Central China Normal University,
Wuhan, Hubei, 430079, P.R. China.

[3]State Key Laboratory of Respiratory Disease,
Guangzhou Institutes of Biomedicine and Health,
Chinese Academy of Sciences,
Guangzhou 510530, P.R. China.

Corresponding Author

Sujan Khadka,

Department of Microbiology,

Birendra Multiple Campus,

Tribhuvan University, Bharatpur, Chitwan,

44200, Nepal.

E-mail: sukha11@yahoo.com

ABSTRACT

Background

Emergence of antibiotic resistance among microbes contaminating the fresh meat products is a global public health concern as they can be easily transmitted to humans through their consumption and contact.

Objective

The current study was conducted to determine the distribution of antimicrobial resistance among *Salmonella* species isolated from fresh chicken liver samples with special emphasis on extended spectrum beta-lactamase (ESBL) production.

Method

A total of 200 fresh chicken liver samples were cultivated for the isolation of *Salmonella* and further subcultivated to detect extended spectrum beta-lactamase production among them. Antimicrobial susceptibility testing (AST) was done by disk diffusion method using a panel of 7 antimicrobials.

Result

Out of 200 samples analyzed, 61 (30.5%) samples harbored *Salmonella* species out of which 15 (7.5%) samples showed the presence of *Salmonella* Typhi. A significant association was noted in the incidence of *Salmonella* with various factors pertaining to the butchers, such as age, sex, literacy rate, practices of washing knives and chopping board, wearing aprons and gloves and type of water used (p < 0.05). *Salmonella* isolates were highly sensitive to amikacin (82.0%) and least sensitive to tetracycline (3.3%). All the isolates were resistant to colistin. Sixty (98.4%) isolates were identified as multi-drug resistant (MDR). The total number of extended spectrum beta-lactamase producers reported among *Salmonella* isolates was 29 (47.5%).

Conclusion

The results indicate that the fresh chicken liver samples sold in Bharatpur Metropolis are reservoirs of multi-drug resistant *Salmonella*, including extended spectrum beta-lactamase producers, that could potentially be transmitted to the humans by direct contact or through inadequate cooking.

KEY WORDS

Antimicrobial susceptibility testing, Extended spectrum beta-lactamase, Fresh chicken liver samples, Multi-drug resistant, Salmonella

INTRODUCTION

Avian *Salmonella* infections are significant causes of clinical disease in poultry and a potential source of foodborne transmission of *Salmonella* in humans.[1] About 95.0% of salmonellosis cases were estimated to originate from food materials and the colonization of *Salmonella* covers humans and animals including livestock, poultry, rodents and birds.[2-4] Poor hygienic environment, improper storage or cooking, cross-contamination, infected stocks contribute to the development of *Salmonella* in poultry and poultry products leading to the major source of human foodborne illness and loss of product shelf-life.[5,6]

Poultry products have always topped the incidence of salmonellosis in many developing as well as developed countries.[7-9] The incidence of human salmonellosis has increased greatly over the past 20 years and this can mostly be attributed to epidemics of *S.* Enteritidis in poultry.[10,11] *Salmonella* serotypes differ significantly in their pathogenic potentials and a study suggested the confirmed cases of *Salmonella* sp. in the surveillance network Food Net from the period 1996-2006.[12]

Chicken liver is an important low-cost source of animal protein, phosphorus, others minerals, and B-complex vitamins; however, the presence of MDR resistant *Salmonella* sp. in chicken livers have become the solemn concern of food safety and one of the major public health problems.[13-16] The frequency of isolation of *Salmonella* strains resistant to several antimicrobial agents has increased in several countries worldwide including Nepal.[17-19] Thus, the purpose of the present study was to determine the prevalence of MDR and ESBL producing *Salmonella* sp. from chicken livers sold at different slaughterhouses in Bharatpur.

METHODS

A descriptive cross-sectional study was conducted for 5 months from February to June 2018 in Bharatpur Metropolitan City and laboratory analyses were performed at the Microbiology laboratory of Birendra Multiple Campus, Nepal. Random sampling was done to collect 200 non-repeated single whole liver samples from different slaughterhouses located at different places of Bharatpur (Baseni, Dipendra Chowk, Hope Chowk, Junhal Road, Bel Chowk, Malpot Chowk and Gitanagar). An approval was initially sought from the Research Committee of Birendra Multiple Campus to conduct this work. Animal samples were processed according to the animal research ethical guidelines. The sample size was determined in accordance with the incidence rate based on the previous study.[8] Properly labeled fresh chicken liver samples were included in this study whereas refrigerated and improperly labeled samples were excluded. Each butcher was briefed of the purpose of sample collection and verbal informed consent was taken assuring them of total confidentiality.

Slaughterhouse's sanitary and salubrious status was studied by a brief interview using semi-structured questionnaires and through observations as well.

Whole fresh chicken liver samples were collected separately in sterile zip-locked plastic bags with the help of sterile forceps and scissors, stored in cold box and transported aseptically to the laboratory for further processing within an hour. The samples were ground in sterile mortar and pestle to make fine particles and 1 g of them was inoculated into 9 ml of distilled water and dilutions up to 10^{-5} were made. From each of 10^{-3}, 10^{-4} and 10^{-5} dilutions, 0.1 ml of inoculum was spread in nutrient agar plates in triplicate and incubated at 37°C for 24 h to obtain a viable count of the bacteria. For the isolation of *Salmonella*, 1 ml of the inoculum was enriched in Selenite F-broth (Himedia, M025S) and incubated at 37°C for 24 h. A loopful of culture in Selenite F-broth was directly streaked on XLD agar (Himedia, MH031) and incubated at 37°C for 24 h. Black-centered red colonies on XLD agar were sub-cultured on NA plates at 37°C for 24 h to obtain a pure culture of the isolates.[19] For further identification of *Salmonella* sp., Gram staining and various biochemical tests (SIM, MR-VP, citrate, catalase, oxidase, urease and TSI) were performed. A slide agglutination test using antisera (Statens serum institute, Copenhagen) was used to detect *S.* Typhi O9, poly O and H antigens.

All the isolates were tested for susceptibility to antimicrobial agents on MHA by Modified Kirby-Bauer disc diffusion method as recommended by the Clinical Laboratory Standards Institute.[20] The antibiotic discs used were amikacin (30 µg), cotrimoxazole (25 µg), ciprofloxacin (5 µg), colistin (10 µg), tetracycline (30 µg), gentamicin (10 µg) and azithromycin (15 µg). The turbidity of inoculums from a pure culture of *Salmonella* isolates on nutrient agar plates incubated at 37°C for 24 h were adjusted to the equivalent turbidity of 0.5 McFarland standards before spreading uniformly over the surface of Mueller-Hinton agar (MHA) (Titan Biotech Ltd. Bhiwadi-301019, Rajasthan, India) plates. Using sterile tweezers, the antibiotic discs were placed widely spaced aseptically on the surface of the MHA plate. The organism was classified as resistant, intermediate or sensitive according to CLSI performance standards.[20] Resistance to more than three structural classes of the antimicrobials tested was considered as MDR.[21] *Salmonella* Typhimurium ATCC 14028 was used as a reference strain for quality control purposes.

Primary screening test for ESBL production was done by using ceftazidime and cefotaxime discs against which the organisms showing the zone of inhibition < 22 mm for ceftazidime (CAZ) (30 µg) and < 27 mm for cefotaxime (CTX) (30 µg) were considered to be probable ESBL producers. The phenotypic confirmatory test was done for suspected ESBL producing isolates for which antibiotics combinations of ceftazidime + clavulanic acid (CAZ/CAC) (30/10 µg) and cefotaxime + clavulanic acid (CTX/CTC) (30/10 µg) were

used according to the protocols recommended by CLSI.[22] An increase in the zone of inhibition by ≥ 5 mm around the discs containing cephalosporin with clavulanate over the discs containing cephalosporin alone were ESBL producers.[22]

The data obtained from laboratory investigation were tabulated and presented in defined tables and chi-square test (χ^2) was performed using SPSS V. 20 software. P value ≤ 0.05 was assigned to have a significant association.

RESULTS

Total viable counts for the collected samples ranged from $7.8 \times 10^4 - 1.9 \times 10^7$. Among 200 fresh chicken liver samples, 61 (30.5%) showed the growth of *Salmonella* sp. while 139 (69.5%) didn't. Of 61 *Salmonella*, 15 (7.5%) were identified as *S.* Typhi.

Salmonella sp. were isolated from 51 (28.2%) male butchers and 10 (52.6%) female butchers. The highest proportion of the samples contaminated by *Salmonella* sp. was obtained in the butchers of the age group 36-45 (55.7%) followed by the age group 46-55 (23.0%). Samples collected from the butchers of the age group 25-35 showed a lower prevalence of *Salmonella* sp. (9.8%). Forty-five (26.2%) samples collected from illiterate and 16 (57.1%) samples collected from literate butchers were *Salmonella* positive. The higher contamination of *Salmonella* sp. was recovered from the butchers who seldom-washed knives and chopping boards (48.9%), from those who used groundwater (52.6%) and from the butchers without apron

Table 1. Association of different attributes of butchers with the contamination of meat by *Salmonella* species

Attributes		Frequency (%)	*Salmonella* isolates (%)	P-value
Butcher's gender	Male	181 (90.5)	51 (28.2)	0.0276*
	Female	19 (9.5)	10 (52.6)	
Butcher's age	25-35	46 (23.0)	6 (9.8)	0.004*
	36-45	78 (39.0)	34 (55.7)	
	46-55	53 (26.5)	14 (23.0)	
	>55	23 (11.5)	7 (11.5)	
Butcher's literacy rate	Literate	172 (86.0)	45 (26.2)	0.001*
	Illiterate	28 (14.0)	16 (57.1)	
Washing of knives and chopping board	Washed frequently	157 (78.5)	40 (25.5)	0.003*
	Seldom washed	43 (21.5)	21 (48.9)	
Type of water used	Municipal	181 (90.5)	51 (28.2)	0.0276*
	Ground water	19 (9.5)	10 (52.6)	
Use of apron/ gloves	Yes	147 (73.5)	33 (22.4%)	
	No	53 (26.5)	28 (52.8%)	0.0001*

*Significant at 5% level of significance

and gloves (52.8%). A significant association was noted between the contamination of liver samples with the age, gender and literacy rate of butchers, type of water used, practices of washing knives and chopping board and wearing aprons and gloves (p < 0.05) (Table 1).

Out of seven different locations the samples were collected from, the highest proportion of *Salmonella* sp. was recovered from Junhal Road (53.3%) followed by Baseni (40.9%) and Malpot Chowk (37.0%). Samples collected from Hope Chowk showed the lowest prevalence of *Salmonella* (12.9%). Moreover, the highest incidence of *S.* Typhi (20.0%) was reported from samples collected from Junhal Road (Table 2).

Table 2. Area-wise variation in the isolation of *Salmonella*

Location	No. of samples examined	*Salmonella* positive samples (%)	*S.* Typhi positive samples (%)
Baseni	22	9 (40.9)	2 (9.1)
Dipendra Chowk	35	9 (25.7)	2 (5.7)
Hope Chowk	31	4 (12.9)	2 (6.4)
Bel Chowk	37	11 (29.7)	3 (8.1)
Malpot Chowk	27	10 (37.0)	2 (7.4)
Gitanagar	33	10 (30.3)	1 (3.0)
Junhal Road	15	8 (53.3)	3 (20.0)
Total	200	61	15

Amikacin was found to be the most effective antibiotic inhibiting the growth of 82.0% of the bacterial isolates followed by gentamicin. All of the isolates were resistant to colistin. A large proportion of the isolates showed resistance to tetracycline (96.7%) and azithromycin (77.0%). Out of 61 isolates, 60 (98.8%) isolates were found to be multidrug-resistant. The frequency of *Salmonella* isolates that gave ESBL screening test positive was found to be 33 (54.1%). The confirmed ESBL production was reported in 29 (47.5%) isolates (Table 3).

DISCUSSION

In the present study, out of 200 samples, 61 (30.5%) were *Salmonella* positive and 139 (69.5%) were negative. Within the positive samples, 15 (7.5%) were identified as *S.* Typhi and the remaining 46 (23.0%) were other *Salmonella* species. This result showed a higher incidence of *Salmonella* than a study by Gupta which reported that the presence of *Salmonella* in layer chicken was 9.3%.[23] Similarly, in another study in Yangzhou city, China, between April 2011 and March 2012, a total of 240 chicken carcasses were tested, and the overall contamination rate for *Salmonella* was 33.8%.[24] However, the incidence of *Salmonella* in the present study is higher than a study by Shrestha et al. who isolated 26.2% *Salmonella* in poultry meat in Chitwan district of Nepal.[8] In another study in the same district, 26.1% occurrence of

Table 3. Antibiotic susceptibility pattern, MDR and ESBL Producers

Antibiotics	Antibiotic	Susceptibility	Pattern	MDR isolates (%)	ESBL producers	
	S (%)	R (%)	I (%)		Screening test positive (%)	Confirmed test positive (%)
Gentamicin	46 (75.4)	12 (19.7)	3 (4.9)			
Cotrimoxazole	26 (42.6)	35 (57.4)	0 (0)			
Ciprofloxacin	31 (50.8)	14 (23.0)	16 (26.8)			
Colistin	0 (0)	61 (100.0)	0 (0)	60 (98.4)	33 (54.1)	29 (47.5)
Tetracycline	2 (3.3)	59 (96.7)	0 (0)			
Azithromycin	14 (23.0)	47 (77.0)	0 (0)			
Amikacin	50 (82.0)	0 (0)	11 (18)			

S=sensitive R=resistant I=intermediate

Salmonella was reported from the poultry meat samples which is lesser than the prevalence of *Salmonella* reported in the current study.[25] These differences might be due to differences in geography, time and season of study.

A large number of *Salmonella* sp. (52.6%) was isolated from the meat collected from female butchers compared to male butchers (28.2%). There was a significant association between the gender of the butchers with the number of *Salmonella* isolates (p < 0.05). Female butchers usually involve in household activities, children caring and cleanliness which increase their exposures to microbes as compared to the male. This may possibly lead them to be the carrier of bacteria and cause more contamination in the food products they handle.[26,27] The occurrence of *Salmonella* in the fresh chicken liver sample was significantly affected by age of butchers (p < 0.05). In the current study, maximum contamination was found in the age group 36-45 years probably due to the lack of sanitation and personal hygiene as the people of this age group are mostly involved in children caring and cleaning which might make them more likely to be contaminated with bacteria. The presence of *Salmonella* in liver samples was significantly affected by the literacy rate of butchers (p < 0.05). This might be due to the lack of knowledge about the importance of sanitation in illiterate ones. In contrast, the literate butchers might know the importance of sanitation and so they use clean water and clean the slaughter area frequently.[28] The highest number of the sample (37) was collected from Bel Chowk area in which 11 (29.7%) samples showed the presence of *Salmonella* including 3 *S.* Typhi. The lowest number of samples (15) was collected from Junhal Road area in which 8 (53.3%) were positive to *Salmonella* including 3 *S.* Typhi. Most of the sample collected areas were densely populated and the butchers weren't aware of good hygienic practices while handling the poultry. In every location, most of them used bare dirty hands to slaughter the chicken which might be the reason for the contamination. A significant association was noted between the contamination of liver samples with water sources, practices of washing knives and chopping board and wearing aprons and gloves (p < 0.05). Therefore, the use of municipal water, gloves,

aprons, good hygienic environment of the slaughterhouse as well as proper personal hygiene of the butchers should be prioritized.

Amikacin was found to be the most effective antibiotic inhibiting the growth of 50 (82.0%) bacteria followed by gentamicin which was able to inhibit 46 (75.4%) isolates. Thus, amikacin and gentamicin can be used for the treatment against *Salmonella* species causing various diseases in humans due to the consumption of contaminated poultry products. Colistin was found to be 100.0% resisted followed by tetracycline (96.7%) and azithromycin (77.0%) which means they are not appropriate antibiotics for *Salmonella*. Moreover, the use of colistin has been banned due to their side effects of nephrotoxicity and neurotoxicity and has been replaced by other antibiotics.[29] In the present study, out of 61 isolates, only one *Salmonella* isolate was single drug-resistant whereas 60 (98.4%) others were identified as multidrug-resistant. In a similar study by Halawa et al. in 2016, out of 23 *Salmonella* isolates, 87.0% were reported to be MDR.[30] The frequency for ESBL producing *Salmonella* isolates was 29 (47.5%) in the current study. A similar study performed by Shrestha et al. detected 55.2% of the total *Salmonella* isolates as ESBL producers.[8] Moreover, similar research conducted by Wu et al. in China detected 8.6% of *Salmonella* sp. as ESBL producer which is very low compared to our study.[31] Extreme and haphazard use of broad-spectrum antibiotics might be associated with a higher rate of ESBL production in *Salmonella*.

This study only determines the prevalence of *Salmonella* species and their MDR pattern with special focus on ESBL production. The source of contamination of meat was not assessed though. We were unable to perform the molecular characterization of bacteria due to the financial and laboratory scarcity. Future studies should address these limitations.

CONCLUSION

The present study shows that chicken meat sold at Bharatpur Metropolis are contaminated with MDR and

ESBL producing *Salmonella* sp. This suggests a dire need of taking initiatives to control the dissemination of such pathogens. Haphazard use of antibiotics, poor personal hygiene, illiteracy rate of the butchers, improper handling and storage practices are some of the factors concerned authorities should address.

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