Multi-drug Resistant (MDR) and Extended Spectrum β-lactamase (ESBL) Producing Salmonella species isolated from fresh chicken liver samples

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Research note

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Abstract

Objective: Emergence of antibiotic resistance among microbes contaminating the fresh meat and meat products is a global public health concern as they can be easily transmitted to humans through their consumption and contact. The current study aimed to isolate and identify Salmonella sp. from fresh chicken liver samples and determine their antibiotic susceptibility patterns with special emphasis on multidrug resistance (MDR) and extended spectrum beta-lactamase (ESBL) production.

Results: Out of 200 samples analyzed, 61 (30.5%) samples harbored Salmonella species among which 15 (7.5%) samples showed the presence of Salmonella Typhi isolates. A significant association was noted in the incidence of Salmonella with various factors pertaining to the butchers such as age, sex and literacy rate. Salmonella isolates were highly sensitive to amikacin (82.0%) and least sensitive to tetracycline (3.3%). All the isolates were resistant to colistin. Moreover, 56 isolates were identified as multidrug-resistant. The total number of ESBL producers reported among Salmonella isolates was 29 (47.5%). The study reported the presence of antibiotic-resistant Salmonella species in fresh chicken liver samples sold in Bharatpur metropolis suggesting a need of serious attention by the concerned authorities.

Keywords: Salmonella , Fresh chicken liver samples, Antibiotic Susceptibility Test, Multi-drug Resistance, Extended Spectrum β-lactamase

Introduction

Avian *Salmonella* infections are important causes of clinical disease in poultry and a potential source of foodborne transmission of *Salmonella* in human [1]. About 95.0% of salmonellosis cases were estimated to originate from food materials [2] and the colonization of *Salmonella* covers humans and animals including livestock, poultry, rodents and birds [3][4]. The adaptive ability of pathogen itself, the changing characteristics of the population, the increasing globalization of the food trade, and the changes in industrial structure, 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 India, Egypt, Brazil, Zimbabwe, Nepal and other developing countries [7][8] and are the most seriously perceived food risks in chicken meat, even in the developed countries [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 in numerous countries [10][11]. *Salmonella* serotypes differ significantly in their pathogenic potentials and a study suggested the confirmed cases of *Salmonella* sp. in the surveillance network FoodNet from the period 1996–2006 [12]. Chicken liver is an important low-cost source of animal protein, rich in nutrients, phosphorus, others minerals, and B-complex vitamins [13]; 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 [14][15][16]. Different food items have been documented as a reservoir of ESBL producing bacteria and such food items are probable sources for the acquirement of beta-lactamase-producing bacteria. The frequency of isolation of *Salmonella* strains resistant to several antimicrobial agents has increased in several countries worldwide including Nepal [17][18][8]. 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 slaughter houses in Bharatpur.

Main Text

A cross-sectional study was carried out among the slaughter houses of Bharatpur Metropolis, Nepal and laboratory analyses were performed at the Microbiology laboratory of Birendra Multiple Campus from February to June 2018. Random sampling was done to collect 200 non-repeated single meat samples from different slaughterhouses located at different places of Bharatpur (Baseni, Dipendra chowk, Hope chowk, Junhal road, Bel chowk, Malpot Chowk and Gitanagar). The sample size was determined in accordance with the incidence rate based on the previous study [8]. 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 brief interview using semi-structured questionnaire and through observations as well.

Methodology

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 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 pure culture of the isolates [19]. For further identification of *Salmonella* species, 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. 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 NA 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 MHA plate. The organism was classified as resistant, intermediate or sensitive according to the interpretative chart [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 p-value of the obtained results was calculated using SPSSv20 software. P-value ≤ 0.05 was considered to have a significant association.

Results

Total viable counts for the collected samples ranged from 7.8×10^{4}–1.9×10^{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* species, 15 (7.5%) were identified as *S.* Typhi.

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

Salmonella species 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 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 ($p \leq 0.05$) (Table 1).

Area-wise variation in the isolation of *Salmonella*

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%) (Table 2).

Antibiotic susceptibility pattern, MDR and ESBL Producers

Amikacin was found to be the most effective antibiotic inhibiting the growth of 82.0% of the bacterial isolates followed by gentamicin which was sensitive to 75.4% of the isolates. 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).

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

Attributes		Frequency (%)	*Salmonella* isolates (%)	P-value
1. Butcher's gender	Male	181 (90.5)	51 (28.2)	≤ 0.05*
	Female	19 (9.5)	10 (52.6)	
2. Butcher's age	25-35	46 (23.0)	6 (9.8)	≤ 0.05*
	36-45	78 (39.0)	34 (55.7)	
	46-55	53 (26.5)	14 (23.0)	
	>55	23 (11.5)	7 (11.5)	
3. Butcher's literacy rate	Literate	172 (86.0)	45 (26.2)	≤ 0.05*
	Illiterate	28 (14.0)	16 (57.1)	
4. Washing of knives and chopping board	Washed frequently	157 (78.5)	40 (25.5)	≤ 0.05*
	Seldom washed	43 (21.5)	21 (48.9%)	
5. Water type used	Municipal water	181 (90.5)	51 (28.2%)	≤ 0.05*
	Ground water	19 (9.5)	10 (52.6%)	
6. Use of apron/gloves	Yes	147 (73.5)	33 (22.4%)	≤ 0.05*
	No	53 (26.5)	28 (52.8%)	

*Significant at 5% level of significance

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

Table 3 Antibiotic susceptibility pattern, MDR and ESBL Producers

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

S=sensitive R=resistant I=intermediate

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 the study of

Guptain which reported that the presence of *Salmonella* in layer chicken was 9.3% [23]. Similarly, in a study in Yangzhou city, China, between April 2011 and March 2012, total 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 *Salmonella* was reported from the poultry meat samples [25] which is lesser than the presence of *Salmonella* reported in the current study. These differences might be due to differences in geography, time and season of study among the researchers.

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 ($p \leq 0.05$). Females usually involve in household activities, children caring and cleanliness and mainly for various physiological reasons chances of microbes present in female might be comparatively more as compared to the male which 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 ($p \leq 0.05$). 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 because the people of this age group are mostly involved in children caring, raring 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 ($p \leq 0.05$). 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, they 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 ($p \leq 0.05$). Therefore, the use of municipal water, gloves, aprons, good hygienic environment of the slaughter house 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 human originated from the consumption of contaminated poultry products. Colistin was found to be 100.0% resistant which means it is 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 others were identified as multidrug-resistant. In a similar study by Dahal in 2007, out

of 52 *Salmonella* isolates, 13.5% were reported to be MDR [30]. The frequency for ESBL producing *Salmonella* isolates was 29 (47.5%). A similar study performed by Shrestha et al. detected 55.2% of the total *Salmonella* isolates were ESBL producers [8]. Moreover, similar research conducted by Wu et al. in China detected 8.6% of *Salmonella* sp. as ESBL producer which was 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.*

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.

Limitations

This study only determines the prevalence of *Salmonella* species and their MDR pattern with 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.

Abbreviations

SIM: sulfide indole motility; TSI: triple sugar iron; MR-VP: methyl-red Voges Proskauer; CLSI: Clinical and Laboratory Standards Institute; MHA: Mueller Hinton agar; XLD: Xylose lysine deoxycholate; ATCC: American type culture collection

Declarations

Authors’ contributions

SA and SK conceived the concept and design of the study. BRS, AG, MC, DG, SS and SK performed experimental work. SA, SK and SS analyzed the data and prepared the final draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

All authors declare that they have no competing interests.

Availability of data and materials

All data obtained during this study are available within the article.

Consent for publication

Not applicable.

Ethical approval and consent to participate

Research Committee of Birendra Multiple Campus, Tribhuvan University, Nepal gave the approval to conduct this work. Animal samples were processed according to the animal research ethical guidelines. Verbal informed consent was obtained from all the butchers included in the study.

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