PREVALENCE OF *Escherichia coli* and *Salmonella spp* FROM CHICKEN MEAT SAMPLES OF BHARATPUR, CHITWAN

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ABSTRACT

Bacterial contamination and high antibiotic resistance properties of broiler chicken meat are of major risk to public consumers and poultry farming. With the major objective to determine the prevalence rate of E. coli and Salmonella and their antibiotic resistance properties we conducted this cross-sectional study for the period of three months (April- July, 2018) at National Avian Disease Investigation Laboratory, Bharatpur, Chitwan. A total of 100 samples were collected in sterile/dry plastic bags from different shops located in the vicinity of Bharatpur, and transported to the laboratory for further processing following standard microbiological techniques. Samples were crushed in mortar pestle followed by streaking on EMB agar and XLD agar for isolation of E. coli and Salmonella. Isolates were identified by determining their colony characters, gram staining and biochemical results. Major isolates in our study was E. coli (n=56/100; 56%) and isolation rate was higher from the fresh meat sample (n=51/70; 72.8%) and which was washed by ground water (n=63/88; 71.5%). Isolation rate of E. coli and Salmonella was not significantly associated with the areas from where the samples were collected in urban areas (for E. coli; p=0.68 and for Salmonella; p=0.50 respectively) however higher rate of isolation was seen form shop located in urban area. Antibiotic sensitivity testing revealed that Ciprofloxacin was most effective against E. coli (n=45/56; 80.3%) and Azithromycin was effective against Salmonella, (n=9/10; 90.0%) however, both isolates showed maximum resistance over Nalidixic Acid. This study is strongly indicative of improper sanitary practices for chicken meat handling and suggests for performing antibiotics susceptibility testing against the bacteria before prescribing any antibiotics.

Keywords: *antibiotic, antibiotic resistance, bacteria, broiler chicken meat*

INTRODUCTION

Livestock sector is an important component of Nepalese economy in terms of income, employment and equality. Livestock sector contribute one-third of Agricultural Gross Domestic Product (AGDP) and 4% of the total export of the nation (MOAD, 2016). Livestock sector is an important component of Nepalese economy in terms of income, employment and quality.

Nepal have to go for One Health approach and it will demand Sustainable poultry production as it is ket to secure animal protein source. Nepal's self sufficiency declaration is boosting production which have potential for growth as Nepalese consumption is targeted to reach 25 kilogram of meat and 100 eggs per capita in few years (Acharya & Kaphle 2015).

"Chicken meat" which is a major meat product, principally refers either whole carcasses or parts of the carcass or boned out meat of the species *Gallus gallus*. However, regular report on zoonotic diseases outbreak that cost lives of both chicken and consumers which spreads via the chicken meat have raised increased concern over the health safety of consumers (Simmons et. al., 2003) and sustainable livestock farming among the farmers.

During the slaughter/processing of poultry birds, there can be fecal contamination of the carcasses from the gut of these birds which means bacteria present in the spilled gut content is passed on as contaminants. Additionally, there are chances of contamination from sub-standard water used for washing meat, chopping materials, etc. (Simmons et. al.,

2003). Of important, organisms of fecal origin like *E. coli* and *Salmonella* spp. are important indicators of contamination, which also presents significant public health threat to consumers, if infected during the handling of raw poultry carcasses and products, consumption of undercooked poultry meat (Panisello et. al., 2010).

Both *E. coli* and *Salmonella* are easily disseminated in poultry flocks due to the high density of birds rearing, which are distributed worldwide and may be found in soil, water, fruits, vegetables, grains, flowers, trees and animals (Holt,1994). Bacteria from the genus *Salmonella* are the most common enteric pathogens associated not only with food borne infections originated from poultry products consumed by humans but also responsible for severe economic losses (Hafez, 2005).

In Nepal, poultry rearing and farming is one of the rapidly growing farming sectors, there is a tremendous growth of poultry farming in the last six decades (MoAD, 2013/14) and it creates income generation in urban and per urban area (Bhattarai, 2008). However, lack of uniform standard meat processing methods and rampant widespread use of antibiotics against the infection of poultry have risked the chances of infection to the consumers' and also the generation of multiple drugs resistant organisms (Simmons et. al., 2003). Since one of the major reasons for outbreak of multi-drug resistance isolates is via rampant use of the antibiotics. This particular issue is presenting a big challenge to the veterinary as well as public health workers, to find out the actual prevalence of contaminating organisms and determine the resistance property of the isolates. Thus study in this issue will ultimately help to determine the current burden of the *E. coli* and *Salmonella* spp. in broiler chicken meat sample from Bharatpur, Chitwan, which is a capital city for poultry farming (MoAD, 2013/14) and their antibiotic resistance properties, which will ultimately stimulate the concerned authorities to work for the reduction of contamination of chicken meat and rational use of antibiotics for the treatment of chicken to stop emergence of antibiotic resistance isolates.

Meat and meat products are prone to high microbial contamination owing to its high nutritional values (Frazier & Westhoff, 2003). Particularly, surfaces of raw meats which are exposed to external environment are contaminated heavily with variety of microbial genera ranging from normal flora to life threatening pathogenic species and strains. Consumption of such microbial contaminated meat and its products are considered as one of the major contributors to high incidence of food borne and zoonotic illnesses among human beings (Bryan, 1973). It has been reported that microbial pathogens which can be transmitted from animals to humans by food contains about 16 kinds of bacteria, three groups of viruses, 22 parasites and three protozoan (Singh et. al., 1995). The pathogenic bacteria like *E. coli, Salmonella* spp*., Clostridium* spp*., Staphylococcus* spp*., Campylobacter* spp*., etc.* not only spoil meat but also cause food poisoning and other illnesses to consumers. Sub-standard slaughtering facilities and meat handling practices contribute greatly to the spread of disease (Buxton et al., 1977). Further poor sanitary condition at herd of flock level, improper screening of disease animals, lairage of abattoirs with deteriorating condition are very much suitable for the growth of microorganisms and cross contamination of carcass. Bryan 1973 listed approximately 200 diseases that transmitted to man by foods.

Although, there are many organisms that are found to be associated with the poultry, bacterial organisms are primarily considered as indicators of contamination like *E. coli.* Similarly, another pathogen *Salmonella* species are frequently associated (CDC, 2013) with

the poultry disease and a human disease in Nepal (Simmons et. al., 2003). The issue is one of the major issues off poultry sector of Chitwan district.

Thus, the major objective of study was to assess the prevalence and to determine the antibiotic resistance property of the two bacterial pathogens *E. coli* and *Salmonella* species. Owing to the similar reports of high isolation rates of both genera by many previous researchers, the isolation rate was evaluated with the existing ones.

MATERIALS AND METHODS

Description of the study site

Chitwan district extend from 27°21' to 27°52' North latitude and 83°54' to 84°48' East longitude with a total land area of 218000 hectares, located at an altitude of 141 to 1943 meters. Chitwan is an inner Terai district possessing both hilly and plain valley. The district shares its boundaries with Makwanpur and Parsa district on east, Nawalparsai and Tanahu district on west, Gorkha and Dhading district on the north and Parsa and Bihar state of India on the south.

Sample collection

Broiler chicken meat samples either frozen or fresh, from meat shops of different locations were collected in clean and dry plastic bags. Various broiler chicken meat shops located in Bharatpur Metropolitan city, Chitwan was visited and samples were collected in a sterile process. All together 100 samples were collected by purposive random sampling method. Chicken meat samples were collected from the wholesale and retail chicken meat shops either fresh or frozen and from the shops that are willing to provide necessary information required for this study. A piece of (approximately 50 gm) chicken meat (randomly taken from outer exposed surface) sample was collected from the chicken meat shop and was packed in clean/dry and sterile plastic bag in ice pack box. Sample was then transported to the National Avian Disease Investigation Laboratory (NADIL), Bharatpur, Chitwan.

Study duration and design

This was conducted for the period of three months (April - July 2018). In this study, we attempted to find the prevalence of *E. coli* and *Salmonella* in chicken meat and the associated risks that might lead to contamination, thus we proceed through laboratory process for the isolation/identification of the target organisms and prepared a set of questions to identify the risks.

Sample processing

Laboratory processing of sample was done at National Avian Disease Investigation Laboratory (NADIL), Bharatpur, Chitwan. Sample thus collected were immediately processed in the same day following standard microbiological methods. Firstly, one gm sample was weighed and mixed with 9ml distilled water and minced properly in alcohol sterilized mortar and pestle. A loopfull of sample was then separately transferred in the suitable culture media viz. Eosin Methylene Blue Agar (EMBA) for the isolation of *E. coli* and to Xylose Lysine Deoxycholate Agar (XLDA) for the isolation of *Salmonella* species. Culture media thus inoculated were incubated overnight for the growth.

Isolation of *E. coli* and *Salmonella* species

For the isolation of *E. coli* colonies with greenish metallic sheen on EMB agar were selected and then gram staining was performed and further inoculation on nutrient agar media and incubation at 37°C for 24 hours was done for inoculation on biochemical media.

Similarly, for the isolation of *Salmonella* species, pink colonies with or without black center in XLD agar were selected and transferred on Nutrient Agar (NA) incubated overnight at 37°C, colony appeared was inoculated in different biochemical media.

Bio-chemical tests

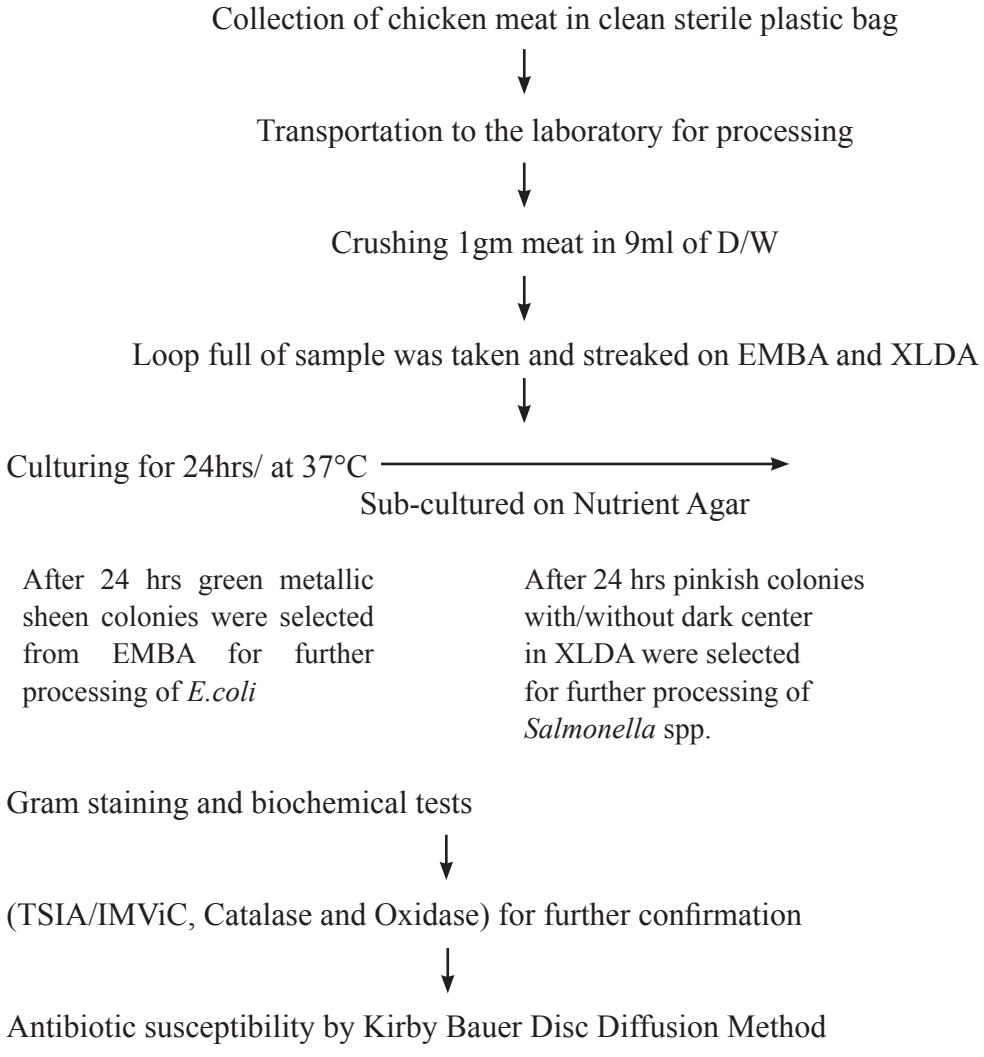
Biochemical tests were performed for further more confirmation of the isolates which was done by the using array of biochemical media which were as followes:

a. Triple Sugar Iron Agar (TSIA) - TSIA test was done by streaking the isolated organism in the TSIA slant and butt. After inoculation, it is incubated at 37°C for 18hrs and observed for the color change in butt and slant.
b. Indole- This test was done for both *E. coli* and *Salmonella* species. In this test, organism was inoculated in Sulfur Indole Motility Media and incubated at 37°C for 24 hrs and Kovac's reagent was added to determine positive or negative reaction.
c. Methyl Red and Voges Proskaur test- This test was performed by inoculating the isolated organism in the MRVP broth culture media and incubated at 37°C for 24 hrs and then after incubation reagents were added to determine the positivity.
d. Citrate utilization test - Citrate utilization test was done by streaking the organism in simmon's citrate agar medium and incubated for 24 hrs and observed the color change after incubation.
e. Catalase- This test was done by mixing organism with 3% H_2O_2 and observed for the gas effervescence.
f. Oxidase- This test was performed by mixing the organism in the oxidase paper and observed for the development of purple color for positive test.

Antibiotic susceptibility tests

Antibiotic susceptibility tests of the isolate were performed by Kirby Bauer Disc Diffusion Method, in which organism of 0.5 McFarland dilution was spread over Muller Hinton Agar (MHA) with the help of sterile cotton swab, left it for few minutes and then array of five antibiotic discs namely Ciprofloxacin, Amoxycillin, Cefixime, Nalidixic Acid and Azithromycin were added to the media swabbed with test organism which was then incubated at 37°C for 18 hrs and observed for the zone of inhibition.

Flow chart of the sample



Data analysis

Obtained data was statistically analyzed by using excel and SPSS 20. Correlations between various parameter were determined by using chi-square tests from SPSS and tabulations and simple other calculations were done via excel.

RESULTS AND DISCUSSION

Out of 100 chicken meat samples collected from 100 different meat shops of Bharatpur Metropolitan, Chitwan, for the purpose of evaluation of *E. coli* and *Salmonella* species, 66 of them contained either *E. coli* or *Salmonella* species or isolation rate of *E. coli* was highest (n=56) than *Salmonella* (n=10) and among those samples, six of chicken meat samples contained both *Salmonella* and *E. coli*. We found that *E. coli* isolation rate was highest from the respondent who has academic qualification of plus two and below, from the meat shop where chopping wood is infrequently washed fresh meat.

Table 1. Overall prevalence rate of E. coli and Salmonella in different conditions

Variables		Total number of samples n	Total positive samples *E. coli*	*Salmonella*	Total Positivity rate
Location of shop	Urban	50	27	6	33 (66.0%)
	Sub-urban	50	29	4	33 (66.0%)
Type of meat shop	Wholesaler	7	3	0	3 (42.8%)
	Retailer	93	53	10	63 (67.7%)
Type of meat	Fresh	70	44	7	51 (72.8%)
	Frozen	30	12	3	15 (30.0%)
Type of water used	Municipal	12	3	0	3 (25%)
	Ground water	88	53	10	63 (71.5%)

Similarly, the isolation rate of target organisms from meat samples collected from the market area and away from market area. Out of total 100 samples processed 50 were from market (urban) area and 54.0% (n=27/50) of them contained *E. coli*, whereas, among 50 samples from rural area, 68.0% (n=29/50) meat samples contained *E. coli.* There is no significant relation between isolation rates of *E. coli* and location of meat shop. Similarly, *Salmonella* isolation rate was 12% (n=12/50) in the market area than in sub-city area, which is 8% (n=4/50) (table-2).

Table 2. Isolation rate of E. coli and Salmonella from different locations of meat shops

Location of meat shop	*E. coli* isolation			P value	*Salmonella* isolation			P value
	-ve	+ve	Total		-ve	+ve	Total	
Urban area	23 (46.0)	27 (54.0)	50 (100)	0.68	44 (88.0%)	6 (12.0%)	50	0.50
Sub-urban area	21 (42.0)	29 (68.0)	50		46 (92.0%)	4 (8.0%)	50	
Total	44	56	100		90	10	100	

Note: -ve=negative, +ve =positive and figure in the parenthesis is percentage

Among 100 the meat samples collected 93 were retailer meat shop whereas seven were from wholesaler and our study found that 53 out of 93 samples from retail meat shop and three out of seven meat samples from wholesale meat shop contained *E. coli.* Isolation rate was statistically insignificant with the type of meat shop at 95% confidence interval (p=0.47). However, *Salmonella* was isolated only from retail meat shop (i.e. n=10).

Table 3. Isolation rate from types of meat shops

Type of meat shop	*E. coli* isolation			P value	*Salmonella* isolation			P value
	-ve	+ve	Total		-ve	+ve	Total	
Wholesaler	4 (57.2)	3 (42.8)	7	0.47	7 (100.0)	0	7	0.21
Retailer	40 (43.1)	53 (56.9)	93		83 (89.3)	10 (10.7)	93	
Total	44	56	100		90	10	100	

Note: -ve=negative, +ve =positive and figure in the parenthesis is percentage

Of total 100 meat samples collected, 70 were freshly cut and processed samples and rest 30 were cut frozen meat samples. In our study fresh 62.8% (n=44/70) of fresh meat samples and 40.0% (n=12/30) of chilled meat contained *E. coli.* Isolation rate was not associated with the type of meat since p value is greater than 0.05. However, rate of isolation of *Salmonella* species is same i.e. 10.0% from both fresh meat (n=7/70) and frozen meat (n=3/30).

Table 4. Isolation rate in different types of meat samples

Type of meat samples	*E. coli* isolation			P value	*Salmonella* isolation			P value
	-ve	+ve	Total		-ve	+ve	Total	
Fresh	26 (37.2)	44 (62.8)	70	0.35	63 (90.0)	7 (10.0)	70	0.1
Frozen	18 (60)	12 (40)	30		27 (90.0)	3 (10.0)	30	
Total	44	56	100		90	10	100	

Note: -ve=negative, +ve =positive and figure in the parenthesis is percentage

Similarly, the isolation rate from the meat based on the type of water used for washing the carcass. Most of the meat shops uses ground water for carcass washing (n=88) rather than using municipal water (n=12).We found 60.2% (n=53/88) of meat samples washed with ground water and 25% (n=3/12) of meat sample washed with municipal water, contained *E. coli* and there is no significant association between isolation rate of *E. coli* with type of water used (p=0.21) at 95% confidence interval. However, all 10 *Salmonella* species were isolated from meat samples washed with ground water and none of the municipal washed carcass contains contaminating bacteria.

Table 5. Isolation rate from different types of water used for washing

Type of water used	*E. coli* isolation			P value	*Salmonella* isolation			P value
	-ve	+ve	Total		-ve	+ve	Total	
Municipal	9	3	12	0.21	12	0	12	0.1
Ground	35	53	88		78	10	88	
Total	44	56	100		90	10	100	

Note:-ve=negative, +ve

Antibiotic susceptibility test of all 66 isolates of both *E. coli* and *Salmonella* using array of five antibiotic discs viz: Ciprofloxacin, Cefexime, Azithromycin, Nalidixic Acid and Amoxycillin as per the given concentration. On performing the antibiotic susceptibility testing, ciprofloxacin was found to be most effective against *E. coli* (80.3%), followed by Azithromycin (60.7%), Cefexime (55.3%), Amoxycillin (46.4%) and least effective was Nalidixic Acid (30.3%) (table-6).

Similarly, based on antibiotic susceptibility pattern of the *Salmonella* species against the five antibiotics (same antibiotics used against *E. coli*), we found that Azithromycin was most effective antibiotic (nine out of 10 isolates were sensitive) against *Salmonella* species and least effective antibiotic was Nalidixic acid (only three out of nine isolates were sensitive). Second most effective antibiotic against *Salmonella* species was Amoxycillin (five out of 10 isolates were sensitive), followed by Cefexime and Ciprofloxacin (four out of 10 isolates were sensitive to both isolates) (table-7).

Table 6. Antibiotic susceptibility pattern of E. coli

Organisms	Number of isolates showing response to different antibiotics n														
	Azithromycin			Ciprofloxacin			Cefexime			Amoxycillin			Nalidixic Acid		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
E. coli	34	18	4	45	8	3	31	15	10	26	20	10	17	27	12

Note: *S- Sensitive, I- Intermediate, R- Resistant*

Table 7. Antibiotic susceptibility pattern of *Salmonella* species

Organisms	Number of isolates showing response to different antibiotics n														
	Azithromycin			Ciprofloxacin			Cefexime			Amoxycillin			Nalidixic Acid		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Salmonella	9	1	0	4	3	3	4	5	1	5	3	2	3	3	4

Note: S- Sensitive, I- Intermediate, R- Resistant

This study attempted to determine the prevalence rate and associated risk factors for *E. coli* and *Salmonella* species contamination in broiler Chicken meat of Bhratpur, Chitwan. These are not only contaminating microbes but also pathogens causing loss of both poultry (Kumari et.al. 2013) and human beings; thus, additionally we also determined the antibiotic susceptibility pattern of the isolates.

This study found more than half (66.0%) of total chicken meat sample processed is contaminated by *E. coli* and *Salmonella,* which is ready to sell for consumption in Bharatpur, Chitwan. *E. coli* only attributed more than half of the total isolate (56%), whereas *Salmonella* species isolation rate is comparatively lower (10%). *E. coli* being more common intestinal bacteria easily transmitted from the meat handlers but *Salmonella* is strict pathogen might be the reason for higher isolation rate of *E. coli* than *Salmonella*. Isolation rate of *Salmonella* was less than the previous research done by Mukhopadyay et. al*.,* 2004 who reported 40.2% and 46.2% of the *Salmonella* isolation rate respectively. However, our result nearly coincides with the prevalence rate with the finding of Joshi et al*.,* 2005, who reported 11.3% prevalence rate. Discrepancies in our findings might be because of sample size, sample collection site/ area and duration of the study.

Similarly, we tried to compare between the isolation rate of *E. coli* and *Salmonella* from wholesale and retail broiler chicken meat shops, we found that isolation rate of the bacteria is not influenced by the type of meat shop. However, *E. coli* isolation rate is comparatively higher from retail meat shop (56.9%) than from wholesale (42.8%). Our result coincides with the previous similar studies done by Kumari et. al., 2013 in Nepal. Similar type of result was obtained in case of *Salmonella* isolation rate as well, since all *Salmonella* were isolated from chicken meat samples obtained from retail meat shops. Such findings might be due to long exposure duration of chicken meat that happened when transported from the wholesaler to retailer meat shop. Moreover, increased exposure in the external environment, regular washings of carcass and frequent handling of chicken meat with improperly washed hands, tools etc. at retail meat shop might have led to increased contamination by *Salmonella* and *E. coli.*

Comparatively, we found fresh chicken meat samples harbored more *E. coli* than in frozen meats. More than half (62.8%) of the meat samples processed gave *E. coli* isolation, whereas less than half (40.0%) of the total frozen meat contained *E. coli.* However, there was same rate of Salmonella isolation in both fresh and frozen meat samples (10.0% each).

Chicken meat kept in frozen condition slows down microbial load that came from exogenous source and adhered in external surface and this might have attributed to low isolation rate of *E. coli* from frozen meat samples than from fresh meat.

Municipal water seems safer to use for washing of carcass, since only 1/4th (25%) of *E. coli* was isolated from the chicken meat sample which is washed with municipal water as compared to carcass washed by ground water (60.3%). Similar, result was obtained in case of *Salmonella* isolation as well, since none of the municipal water washed carcass contained *Salmonella* (Joshi et. al., 2003). This finding also suggests that water might be one of the major sources of contamination. This well correlates with the finding of previous research done by Rai et al., in 2013 for bacterial contamination of drinking water in Bharatpur metropolitan city that reported more than least fecal *coliforms* in municipal water used for drinking. The reason for higher isolation of organisms from ground water washed samples might be due to the reason that municipal water is well treated and there is good pipelines system that prevents the entry of contaminated water to pipeline.

In this study we also attempted to determine if the contamination of chicken meat is associated with the level of academic qualification of the respondents. Although, we found that statistically there is no significant relation between academic qualification and isolation rate of target organisms, we found higher rate of *E. coli* and *Salmonella* isolation rate; 60.9% and 12.5% from the respondents whose academic qualification is equal to and below plus two, compared to those having higher qualification above plus two. Academic qualification is also associated with the level of understanding of hygiene and safety of the meat and meat products and this might have attributed to low isolation rate of target organisms from meat obtained from high qualified respondents. Academic parameters for the rate of isolation of bacterial isolates from chicken meat were rarely studied.

Isolation of pathogens and potent pathogens has importance regarding public health concern of the consumers and these pathogens are of concern to poultry as well since it is associated with poultry disease. However, it may not be possible to completely check the cross contamination, hence, in addition to this, important part that is to be addressed is about the disease management. Use of antibiotic is one of the parts of disease management and in both poultry and human beings same class of antibiotics are used. Thus, it is important to know the susceptibility pattern of the isolates to early determine whether or not the commonly used drugs work against if the pathogens infect the consumers or poultry. In this context we also attempted for determining antibiotic susceptibility pattern of the isolates using five different groups of antibiotics. Our finding showed that *E. coli* were most sensitive to the Ciprofloxacin and least to Nalidixic acid. Although, both are from the same group of antibiotics ciprofloxacin seems more effective.

Our finding on antibiotic susceptibility test on Nalidixic Acid closely coincides with that of Kumari et. al. (2013) to both *Salmonella* and *E. coli* but unlike amoxycillin which was most effective in their result we found Azithromycin as most effective against *Salmonella* and Ciprofloxacin as most effective against *E. coli.* Discrepancies in antibiotic susceptibility tests seem common in every study since individual organism response to the antibiotic response is different in different locations.

CONCLUSION

Sustainable poultry production is the only way out for Nepal as this livestock component lead in terms of revenue and employment generation for Nepal. Self sufficiency declaration by the government of Nepal is forcing the industry to produce more as Nepalese consumption is targeted to consume 25 kilogram of meat and 100 eggs per capita in few years. Emerging and remerging diseases, poor management practices, lack of awareness among producers, processors and consumers have been the biggest hurdle. Following conclusions have been drawn based on our findings. We conclude that broiler chicken meat is highly contaminated by bacteria of fecal origin contaminated through water and via unhygienic practices for meat handling and processing. Our finding suggested that *E. coli* is the major isolate than *Salmonella* from chicken meat sample. Hygienic practices and water contamination are major doubtful factors for the high prevalence of bacterial contamination of chicken meat and each organism responded differently to different antibiotics.

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