

# Antimicrobial drug resistant non-typhoidal *Salmonella enterica* in commercial poultry value chain in Chitwan, Nepal

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## Research

**Keywords:** Poultry, non-typhoidal *Salmonella*, Nepal, antimicrobial resistance, chicken, one health, environment

**Posted Date:** July 2nd, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-16336/v2>

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**Version of Record:** A version of this preprint was published on October 1st, 2020. See the published version at <https://doi.org/10.1186/s42522-020-00025-4>.

# Abstract

Background Antimicrobial resistance (AMR) among bacterial pathogens is a fast-growing public health concern. AMR in non-typhoidal *Salmonella* serovars (NTS) among food animals is of special concern as this may transmit resistant pathogens to humans during handling or consumption of animal products. In Nepal, the possibility of AMR *Salmonella* serovars among food animals is an important area of research, particularly in light of the rapidly growing poultry industry, lack of surveillance and proper biosecurity measures; and paucity of relevant data. This study was conducted with the aim to estimate the burden of NTS and associated antimicrobial resistance in the environments of commercial poultry farms and the poultry carcasses in slaughter house. This study also intends to find some basic knowledge of the poultry farmers and their practice relating to the use of antimicrobials, vaccination and biosecurity measures..

Methods Taking one health approach, a cross-sectional study was carried out in Chitwan district of Nepal between May and October 2017. Various environmental samples viz. farm litter, feed, water, poultry faeces, vehicle swabs, farm swabs from 12 broiler poultry farms and various sections of poultry carcasses from 21 slaughter houses were aseptically collected. These were microbiologically assessed for the presence of NTS serovars and their phenotypic and genotypic indicators of antimicrobial resistance. The poultry farmers were also briefly interviewed regarding basic their basic biosecurity related knowledge and practices before collecting the environmental samples.

Results Overall, of total environmental samples collected, 50% (31/62) tested positive for NTS serovars with environmental swabs (70%, 8/12) being the most culture positive sample types. Similarly, of 159 tissue samples collected from 24 carcasses, 79% (126/159) were culture positive for NTS serovars. Nearly 97% (153/157) of isolates showed antimicrobial resistance to tetracycline, while 11% (17/157) to ciprofloxacin and 5% (8/157) of isolates were resistant against azithromycin. All 157 isolates were sensitive to meropenem. In terms of AMR genes, tetA (83%, 131/157), QrnS (40%,64/157), mefA (8%, 13/157) and VIM-1 (0.6%, 1/157) were detected in the isolates that corresponded to the AMR to tetracycline, ciprofloxacin, azithromycin and meropenem respectively. In farmers interview, only 42% (5/12) of farmers mentioned of using basic biosecurity measures such as applying lime powder around the farm; 84% (10/12) of farmers reported vaccinating their birds with some vaccine and 75% (9/12) of farmers used various antimicrobials prophylactically such as neomycin (33%, 4/12), colistin (33%, 4/12), furaltadone (33%, 4/12), doxycycline (25%, 3/12), sulfatrimethoprim (25%, 3/12) and tylosin (16%, 2/12).

Conclusions This study revealed gross contamination of farm environment and subsequent poultry meat samples with NTS serovars that were resistant to several clinically important antimicrobials. Further, inadequacy of even basic biosecurity measures and frequent prophylactic use of antimicrobials in the commercial poultry farms was observed. This reinforces an urgent need to raise awareness and implement proper biosecurity approaches from farms slaughter houses in order to reduce the burden of NTS contamination of surrounding environment and poultry products. Further, high prevalence AMR among NTS isolates also underscores the need to strengthen the policies to prevent the rampant use of clinically used human antimicrobials in poultry sector.

## Background

In 2010, non-typhoidal *Salmonella enterica* (NTS) caused over 153 million illnesses and 122,000 deaths around the world, with children under 5 bearing a disproportionate burden(1,2). According to the World Health Organization (WHO), about 50% of those illnesses were caused by the consumption of contaminated food; however, animal contact, consumption of contaminated water are also potential vehicles (1,2). Of the food products linked to *Salmonella* contamination, poultry is among the most frequent, resulting in international efforts to control and reduce the burden of *Salmonella* among poultry(3,4).

In addition to the significant public health impact due to large burden of NTS illnesses, the increase in antimicrobial resistance (AMR) is a great concern(5). Infections caused by drug resistant *Salmonella* tend to be more severe and lead to higher hospitalizations and deaths(6). While global efforts are being targeted to monitor and track the development of drug resistant *Salmonella* globally(7,8), systematic surveillance of illnesses and AMR can be extremely challenging in low resource countries and therefore the detection of pathogens and associated AMR may go undetected.

In Nepal, poultry production is a significant economic activity and represented 4% of the country's gross domestic product in 2017(9). According to the Food and Agriculture Organization (FAO), in Nepal from 2007 to 2017, the production of poultry meat increased by 398% from 19.4 million chickens in 2007 to 75.4 million in 2017 (10). And this number is expected to continue growing in the next coming years(11). Various antimicrobials are commonly used in poultry production in Nepal to promote growth and prevent disease. This unregulated consumption of antimicrobials is highly expected to increase in Nepal(12). A 2012 survey conducted in Nepal found that the volume of sales of veterinary antimicrobials rose by over 50% between 2008 and 2012, and that more than 70% were obtained without a prescription(12). The significant increase in production coupled with the likely misuse and overuse of antimicrobials in poultry production in Nepal might be accelerating the development of drug resistant *Salmonella* in the region.

Most surveillance efforts conducted in Nepal have focused on *Salmonella enterica* Typhi isolated from febrile humans(13,14), and little knowledge exists concerning the burden and antimicrobial resistance of non-typhoidal *Salmonella enterica* in poultry sector of Nepal. Still, the limited data available highlight the data gaps that must be addressed. Therefore, we used a one-health approach to conduct a preliminary study on prevalence of non-typhoidal *Salmonella* and associated AMR in poultry sector in Chitwan region of Nepal using both phenotypic and molecular characterization methods.

## Methods

The study was conducted in the Chitwan district in southern Nepal between May and October 2017; this time period coincides with summer monsoons and typically expected to have the highest rates of human

salmonellosis. Chitwan is one of the largest poultry egg and broiler producing districts in the country(15), with a population of 580,000 in the 2011 census.

## **Sample collection**

### **Broiler farms**

Twelve commercial poultry farms of Chitwan district registered with National Poultry Federation were selected to represent a range of production volume, housing types, and levels of biosecurity and hygiene. They were further categorized as “satisfactory”, “average”, or “unsatisfactory” as per Poultry Federation guidelines. Categorizations were based on characteristics such as quality of poultry housing, food and water storage practices, availability of technical personnel, and use of personal protective equipment such as boots and gloves. Upon receiving the consent from the farm owner, an assessment (not formal interview) was conducted by the research team with the farm owner to obtain information on volume of production and practices employed, such as the use of vaccines, antimicrobials, and hygiene and biosecurity measures. The GPS location of each farm was recorded using mobile app. Environmental samples were collected by taking the following from each farm: approximately 20 grams of farm litter; 25 grams of poultry feed collected directly from the feed sack in use; and a spatula full of moist poultry feces from a chicken house. Each sample was collected into a sterile container. Approximately 100 ml of drinking water used for poultry was collected in a sterile screw capped container by direct decanting. Further, a sterile cotton swab moistened with sterile skim milk was dragged along the inner walls of the poultry farm and immersed in vial containing 10 ml of sterile skimmed milk. A similar strategy was used with a sterile cotton swab to sample the wheels, side bars, and bird cages of the vehicle used for transporting birds from the farm to the slaughter facility. All samples were stored on ice and transported to the laboratory within 6 hours.

### **Poultry tissue**

Twenty-one slaughter houses and retail meat shops in the Chitwan District were selected. The GPS points for each establishment were recorded using smart phone app. In each establishment, a chicken carcass ready for sale was selected. From each carcass, roughly 50 grams of each seven different types of meat samples were collected using a sterile scalpel blade and aseptically transferred into a sterile container. The sample types were gizzard, liver, heart, intestine, skin, spleen and muscle. These were stored and transported on ice to the laboratory within six hours of sample collection. All the slaughter and retail establishments selected in this study received the birds that were reared at the farms that were included and sampled in this study.

### **Microbiological analysis**

All samples except swabs were pre-enriched in Buffered peptone water (Himedia laboratories, India) and incubated at  $35 \pm 2$  °C for 18 hours. Swab samples in skimmed milk were directly incubated at  $35 \pm 2$  °C for 18 hours. Samples were then selectively enriched by transferring 100 µl of pre-enriched content into

9.9 ml of Rappaport Vassiliadis Broth (Himedia laboratories, India) and incubated at  $40 \pm 2$  °C for 18 hours. A loop full of content was sub-cultured on Xylose Lysine Deoxycholate (XLD) agar (Himedia laboratories, India) and incubated at  $35 \pm 2$  °C for 18 hours.

The inoculated XLD plates were examined for transparent red colonies with black centers, the characteristic feature of *Salmonella* species. In biochemical tests, the colonies giving indole negative, methyl red positive, voges-proskauer negative, citrate utilization positive, alkaline slant by acidic butt with or without H<sub>2</sub>S in TSI (triple sugar iron) test, motile and urease negative results were confirmed as *Salmonella enterica* isolates. The typhoidal *Salmonella enterica* serovars viz., *Salmonella enterica* Typhi, Paratyphi A, B and C are highly adapted to humans and have no known animal or environmental reservoirs(16). Thus, all *Salmonella enterica* serovars isolated from poultry and environmental samples in this study were assumed to be non-typhoidal *Salmonella enterica* serovars.

The antimicrobial susceptibility test was performed by modified Kirby Bauer disk diffusion method for azithromycin (15 µg), tetracycline (35 µg), ciprofloxacin (10 µg) and meropenem (10 µg). The disc diffusion breakpoint interpretation was carried out as per the CLSI (Clinical and Laboratory Standards Institute) 2017 guidelines (17). The isolates were stored in glycerol stock at -80 °C.

## **Molecular tests of antimicrobial resistance marker genes**

The bacterial DNA was extracted from few isolated colonies of *Salmonella enterica* using Quick-DNA™ Fungal Bacterial Miniprep Kit (Zymo research, USA) as per the protocol of the manufacturer.

The real time PCR based detection of antimicrobial resistance genes was carried out using Qiagen Microbial DNA qPCR Array kit (Qiagen, Germany) in a 25 µl final volume using the protocol of the manufacturer in Sa Cyler-96 thermocycler (Sacace Biotechnologies, Italy). The following resistance associated genes for specific antimicrobials were tested: QnrS for fluoroquinolones; OXA-62, NDM, IMP-1group, VIM-1group for beta-lactams; aacC1 for aminoglycosides; tetA for tetracyclines; ermA and mefA for macrolides/lincosamides.

## **Analysis**

Descriptive statistics and regressions were carried out using STATA software. For the purposes of analysis, specimens determined to have intermediate antibiotic sensitivity were classified as sensitive. Logistic regression was used to assess the relationship between phenotypic and genotypic outcomes.

## **Results**

Interviews were conducted with the farm owners from each of the 12 poultry farms visited. Broiler operations ranged in size from 70 birds to 1,000 birds (median, 400; inter quartile range 200, 425). All farms used commercial pellet feed and raised birds of the same age in deep litter. At the end of each harvest, most of the farms (75%, N = 9) disposed of litter by having it removed by a contractor to be used as a fertilizer, while the rest disposed it themselves in their own farms. When farmers were informally

asked about measures of biosecurity, 41.6% (n = 5) of respondents reported using lime powder around the farm, 16.6% (2/12) didn't maintain any biosecurity measures, 8.3% (1/12) had no knowledge about biosecurity, and rest did not respond to the question. Ten (83.3%) of 12 farmers reported vaccinating their birds with some vaccine, while the remaining two refused to answer the question. Of these ten, nine also reported using some medication (no further definition given). Nine (75%) farmers also used antimicrobials; classes of antimicrobials employed varied, including neomycin, colistin, or furaltadone (33.33% each, 4/12); doxycycline or sulfatrimethoprim (25% each, 3/12); and tylosin (16%, 2/12). All eight farmers responding to the question reported that they obtained antibiotics from the feed supplier. Seven farmers did not know about Salmonella.

## Prevalence of Salmonella enterica in the poultry setting

Twelve broiler farms were interviewed, but environmental samples were collected from 11. Out of the 11 operations, three were classified as "satisfactory," five were "average," and three were "unsatisfactory" regarding their overall management practices. Table 1 shows the samples collected and their culture positivity rate for Salmonella enterica. Of 62 environmental samples collected, 31 (50%) tested positive for Salmonella enterica serovars. Almost 70% (8/12) of the farm and vehicle swab samples and 50% of the fecal samples were culture positive for Salmonella enterica. With exception of one farm, which was categorized as "average", all farms had at least one Salmonella enterica culture positive sample. The poultry drinking water samples were culture negative.

Table 1  
Percentage of Salmonella enterica culture positivity by sample types

| <b>Poultry tissue samples</b>           | <b>N</b>   | <b>Culture positive, n (%)</b> | <b>Environmental samples</b> | <b>N</b>   | <b>Culture positive, n (%)</b> |
|---|------------|--------------------------------|------------------------------|------------|--------------------------------|
| Muscle                                  | 24         | 20 (83%)                       | Farm swab                    | 12         | 8 (67%)                        |
| Skin                                    | 24         | 19 (79%)                       | Feces                        | 12         | 6 (50%)                        |
| Intestine                               | 24         | 21 (88%)                       | Litter                       | 12         | 4 (33%)                        |
| Gizzard                                 | 24         | 18 (75%)                       | Vehicle swab                 | 12         | 8 (67%)                        |
| Heart                                   | 23         | 16 (70%)                       | Feed                         | 12         | 5 (42%)                        |
| Liver                                   | 24         | 18 (75%)                       | Water                        | 2          | 0 (0%)                         |
| Spleen                                  | 16         | 14 (88%)                       |                              |            |                                |
| <b>Total</b>                            | <b>159</b> | <b>126 (79%)</b>               | <b>Total</b>                 | <b>62</b>  | <b>31 (50%)</b>                |
| <b>Total (tissue and environmental)</b> |            |                                |                              | <b>221</b> | <b>157 (71%)</b>               |

In total, 159 tissue samples, including skin, muscle, heart, intestine, liver, gizzard, and spleen were collected from 24 chicken carcasses obtained from slaughter houses and retail shops. Of these, 126 (79%) were culture positive for *Salmonella enterica*. At least 70% of each tissue type was culture positive, and all chicken carcasses had at least four samples that were culture positive for *Salmonella enterica* (Table 1).

## **Antimicrobial resistance profile**

The antimicrobial susceptibility test results for 157 *Salmonella enterica* isolates from poultry tissue and environmental samples are shown in Table 2. Almost all isolates from environmental and tissue samples were resistant to tetracycline. At least 10% and 5% of the isolates from both the sample types were resistant to ciprofloxacin and azithromycin, respectively. Resistance to meropenem was not detected in any of the isolates. Overall, the percentage of resistance to each antimicrobial were comparable between environmental and poultry tissue samples. Sample sizes are too small to measure the statistical difference in prevalence of AMR among various sample types.

Table 2

Phenotypic antimicrobial resistance results of *Salmonella enterica* isolates by sample types

| Sample types                                  | N   | Tetracycline, n (%) | Ciprofloxacin, n (%) | Azithromycin, n (%) |
|---|-----|---------------------|----------------------|---------------------|
| Farm environmental samples                    | 31  | 29 (94%)            | 3 (10%)              | 2 (6%)              |
| Farm swabs                                    | 8   | 6 (75%)             | 0                    | 0                   |
| Feces   | 6   | 6 (100%)            | 0                    | 2 (33%)             |
| Litter  | 4   | 4 (100%)            | 0                    | 0                   |
| Vehicle swabs                                 | 8   | 8 (100%)            | 1(13%)               | 0                   |
| Feed  | 5   | 5 (100%)            | 2(40%)               | 0                   |
| Poultry tissue samples                        | 126 | 124 (98%)           | 14 (11%)             | 6 (5%)              |
| Muscle  | 20  | 20 (100%)           | 3(15%)               | 1 (5%)              |
| Skin  | 19  | 19 (100%)           | 3(16%)               | 1 (5%)              |
| Intestine                                     | 21  | 21 (100%)           | 0                    | 1 (5%)              |
| Gizzard                                       | 18  | 18 (100%)           | 4(22%)               | 1 (6%)              |
| Heart   | 16  | 16 (100%)           | 4(25%)               | 0                   |
| Liver   | 18  | 16 (89%)            | 0                    | 1 (6%)              |
| Spleen  | 14  | 14 (100%)           | 0                    | 1 (7%)              |
| Grand total                                   | 157 | 153 (97%)           | 17 (9%)              | 8 (4%)              |
| * All isolates were susceptible to meropenem. |     |                     |                      |                     |

The PCR results for the markers associated with resistance to specific antimicrobials are shown in Table 3. The tetA gene, a marker for resistance against tetracycline, was the most prevalent, with over 80% of isolates from both the samples types testing positive. Out of the 10 farms, four had one or more isolates with only tetA detected, and no other markers. The isolates from five farms tested positive for co-existence of QnrS or mefA along with tetA. One of the farms had the isolates that were positive for all three markers: tetA, QnrS and mefA. Among poultry, all birds had one or more isolates with at least one of the genetic markers assessed; 22/24 (92%) of poultry had specimens yielding multiple genetic markers (see Table 3).

Table 3

Occurrence and patterns of antibiotic resistance molecular markers among *Salmonella enterica* isolates of poultry tissue and environmental samples

| Genetic marker  | Environment samples<br>N = 31 | Poultry tissue samples<br>N = 126 | Isolates with only this marker |
|---|-------------------------------|-----------------------------------|--------------------------------|
| tetA  | 25 (81%)                      | 106 (84%)                         | 12(39%, E); 49 (39%, P)        |
| QnrS  | 11 (35%)                      | 53 (42%)                          | 0 (E); 1 (0.1%, P)             |
| mefA  | 3 (10%)                       | 10 (8%)                           | 0(E); 2 (0.2%, P)              |
| VIM-1   | 0                             | 1 (0.1%)                          | -                              |
| Both tetAandQnrS  | 10 (32%)                      | 48 (38%)                          |                                |
| Both tetAand mefA   | 2 (6%)                        | 4 (0.3%)                          |                                |
| Both tetAand VIM-1  | 0                             | 1(0.1%)                           |                                |
| Both tetA, QnrS, and mefA   | 1 (3%)                        | 4 (0.3%)                          |                                |
| *None of the isolates were positive for the AMR markers aacC1, NDM, OXA-62, IMP-1 |                               |                                   |                                |

## Discussion

This is the first study, to our knowledge that has collected a range of environmental samples from broiler farms in Nepal with the aim to assess AMR in *Salmonella enterica*. Our study findings reveal higher levels of *Salmonella* contamination and antimicrobial resistance than previously observed in poultry meat in Nepal. We also interviewed farm owners and found that few broiler farmers used or even knew of biosecurity measures. While Nepal regulates the use of antibiotics among food animals, enforcement is not generally maintained.

A high prevalence of NTS was detected in both poultry tissue (79%, 126/159) and environmental samples (50%, 31/62). The gastrointestinal tract of poultry is one of most common natural niche for several serovars of NTS. The high prevalence of NTS in poultry carcasses indicates possible high level of visceral contamination of the meat products. Another study conducted in retail meat shops of Kathmandu reported the detection of *Salmonella* in 14.5% of the chicken meat samples. A recent study conducted in 38 raw chicken meat samples collected from slaughter houses of Bharatpur, Chitwan reported that 26.2% of isolated organisms were *Salmonella* spp. Further, high prevalence of NTS in the environmental samples of the poultry farm suggests equally high level of fecal contamination of the surrounding suggesting lack of proper bio-security measures.

Similar studies conducted in other low- and middle-income Asian countries have reported a wide range of prevalence of NTS in poultry and environmental samples. Studies in India found a range of 0 to 10% NTS prevalence among poultry meat and tissue(18,19). Another study on NTS detected 0.6%, 1.7%, and 7.4% of NTS in poultry feces, drag swabs, and feed samples, respectively(19). In Vietnam, NTS prevalence among broiler farms was found to be 45.6%(20) in one study and 64.7% in another(21). A study of poultry meat carcasses in Yangon, Myanmar during 2014–2015 found a 98% prevalence of Salmonella among 141 retail markets(22).

Among Salmonella isolates from the previous study of retail meat in Chitwan, 85% (n = 23) were multi-drug resistant. The most common antibiotics for Salmonella resistance were ampicillin, nitrofurantoin, and doxycycline. Similar to our findings, the Vietnamese studies detected high percentages of NTS isolates resistant to tetracycline varying from 40%(20) to 78% (21) and low resistance to ciprofloxacin. Other studies of poultry and poultry farms from India found 100% of the NTS isolates to be tetracycline resistant(18,19); and one of these the routine use of oxytetracycline as a feed additive. In Myanmar, 54% and 9% of NTS isolates from retail chicken samples were resistant to tetracycline and ciprofloxacin, respectively(22).

We detected the QnrS gene in 16% of samples overall. However, none of the specimens that were resistant to ciprofloxacin had the QnrS marker; the majority specimens with the marker tested sensitive to ciprofloxacin indicating possible alternative genetic mechanism for observed ciprofloxacin resistance. Whether genetic transmission may be a cause source for AMR surveillance data may depend on the background of antibiotic use in the area. It is possible that antibiotics used for longer periods of time have pressured bacterial genetics to incorporate genetic markers more widely. Markers may be a good harbinger of antibiotic pressure and possible emerging resistance. Antibiotic resistance among poultry in this area should continue to be closely monitored, and genetic markers should be evaluated to determine correlations with changes in resistance levels.

A limitation of this study is the lack of serotyping. We cannot rule out human contamination of samples that might have occurred during handling or processing. However, this study was aimed at assessing whether poultry meat meant of human consumption carries AMR salmonella, and as such findings from this study will provide insight into the same. Several logistical challenges also arose while carrying out the study, including malfunctioning air conditioning units in the local laboratory requiring the transport of specimens to Kathmandu for plating; monsoon rains causing mudslides and road blockages on the route between Kathmandu and Chitwan and causing delays in specimen collection, and floods causing severe damage at poultry farms and limiting their ability to participate. These challenges were overcome through consistent and persistent management of the field and laboratory teams, who are proficient at adapting plans to meet sudden needs. Limitations in the laboratory analysis included the lack of adherence to CLSI recommendations for Salmonella antimicrobial testing. Although limiting the usefulness of the results, lack of adherence to these standards is not unusual in low- and middle-income country settings, and has since been corrected in the current laboratory.

## Conclusions

In this study, we observed high prevalence of NTS in the poultry samples and in the farm environments. The high prevalence of antimicrobial resistance in NTS isolates was observed, particularly in specimens taken from transporting vehicles which could implicate in the spread of resistant NTS beyond farm. Our findings in poultry environments also raise concerns about the spread of AMR through the food supply, especially as similar resistance patterns were observed in meat intended for retail sale. Additionally, low levels of knowledge and practice regarding biosecurity amongst farmers found this study could further exacerbate the existing problem of high burden of antimicrobial resistant NTS in poultry industry. .

The continued use of antimicrobials among the quickly expanding farm industry is likely to exacerbate the potential AMR crisis in Nepal within the next several years. Research and prevention activities should be thoughtfully engaged in a short time frame to determine the extent of resistance to all main line classes of antimicrobials, use of antimicrobials in the wider farming community, and to engage communities and farms in antimicrobial stewardship.

## Abbreviations

AMR

Antimicrobial Resistance

CLSI

Clinical Laboratory Standards Institute

FAO

Food and Agriculture Organization

NTS

Non typhoidal Salmonella enterica

WHO

World Health Organization

XLD

Xylose Lysine Deoxycholate agar

## Declarations

### Ethical Approval and Consent to participate

This study did not involve official human questioner survey nor human biological sample acquisition. Wherever mentioned, consent was obtained prior to visiting a farm and or querying with farm owner on their farm capacity. Samples used for laboratory assessments were purchased from poultry meat and egg suppliers related to particular farm being assessed. As such ethical approval was not required as per Nepal Government rules.

### Consent for publication

All authors consented for the publication of this study.

### **Availability of supporting data**

The datasets during and/or analysed during the current study will be made available from the corresponding author on request

### **Competing interests**

All authors read and approved the final manuscript. Its contents are solely the responsibility of the authors. All authors declare that they have no competing interests.

### **Funding**

This publication was supported by RTI International.

### **Authors' contributions**

AN, JR, SMD and SM designed the study.

AG, BD, SD, SM carried out laboratory work

RC: Vet, supported field and survey

AN conducted the analysis

AN, SMD, and SM wrote the manuscript.

AN, JR, SMD, SM contributed to editing the manuscript.

### **Acknowledgements**

The authors of this paper would like to thank Dr. Sabin Shrestha, Nepal Poultry Federation, Dr. Swoyam Prakash Shrestha, AHD, NARC and the Agriculture and Forestry University, Chitwan.

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