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# Identification of Antimicrobial resistance profiles and Antimicrobial resistance genes of *Campylobacter* isolates from broiler farms in Sri Lanka

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**ABSTRACT:** Antimicrobial resistant (AMR) bacteria including *Campylobacter* has become an emerging global concern in human and animal health. There are very few researches on AMR *Campylobacter* conducted in Sri Lanka and none of them studied about AMR genes to the best of our knowledge. The present study focused on the detection of AMR *Campylobacter* from broiler in Sri Lanka, resistant against frequently used antimicrobials. Further, presence of AMR genes or mutations in responsible genes were compared to the resistant phenotypes. Cloacal swabs were collected from 118 broilers in nine farms covering three provinces in Sri Lanka. One *Campylobacter* colony per sample was isolated and the antimicrobial susceptibility test of the isolates was performed by inoculating the isolates onto agar plates with threshold concentrations of eight antimicrobial agents which belong to six antimicrobial classes. Three genetic markers for antimicrobial resistance, point mutations in 23S rRNA gene and *gyrA* gene, and the presence of resistant gene, *tet*(O) were also investigated. Altogether, 73 samples were *Campylobacter* positive of which 59 were *Campylobacter jejuni*, 13 were *Campylobacter coli* and one was unidentified *Campylobacter*. All isolates were resistant to three or more antimicrobials tested. The isolates were frequently resistant to ciprofloxacin, nalidixic acid, trimethoprim-sulfamethoxazole, amoxicillin and tetracycline, and susceptible to gentamicin and streptomycin, while the resistance to erythromycin was different between the species. Genetic screening revealed that most of the isolates possessed one or more of these genetic markers. This study urges the need of continuous surveillance for AMR *Campylobacter* in Sri Lanka.

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**KEYWORDS:** Antimicrobial resistant genes, Antimicrobial resistant profile, Broiler, *Campylobacter*

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## 1. INTRODUCTION

Poultry industry in Sri Lanka has remarkable boost over the past years because of increasing consumer demand for white meat and relatively lower market price than other meats (Alonso et al., 2005). Chicken is commonly consumed and exported meat type in Sri Lanka. At the same time, poultry meat is one of the most important infection sources of human campylobacteriosis, a major public health concern worldwide (Kottawatta et al., 2017; Perera et al., 2020). It has been estimated that 50% to 80% of the human campylobacteriosis cases may be attributed to the chicken reservoir (European Food Safety Authority, 2010).

Antimicrobial resistance of bacteria has become an emerging global concern in human and animal health over the recent decades. Antimicrobial resistant (AMR) *Campylobacter* spp. has also gradually become a major public health concern in both developed and developing countries (Khan et al., 2018; Kottawatta et al., 2017; Nisar et al., 2017). Antimicrobial resistance in

bacteria often occurs due to mutations in their genes or acquisition of resistant genes. The resistance to quinolones occurs due to point mutation (C257T) at quinolone resistance-determining region (QRDR) in DNA gyrase A gene resulting the replacement of threonine by isoleucine at amino acid 86 (Thr86Ile) (Iovine, 2013). Macrolide resistance of *Campylobacter* occurs due to a modification at 23S ribosomal RNA subunit gene. In *C. coli* and *C. jejuni*, point mutations at A2074C and A2075G in domain V 23S rRNA cause resistance to macrolides (Ekkapobyotin et al., 2008). The resistance mechanism of tetracycline is by the acquisition of *tet*(O) gene in *Campylobacter* spp. The *tet*(O) gene which is carried by plasmids causes resistance to TET by dislodging it from the binding site on the bacterial ribosomes (Gibreel et al., 2004).

There are very few researches on AMR *Campylobacter* conducted in Sri Lanka and none of them studied about AMR genes to the best of our knowledge. The present study focused on the detection of AMR *Campylobacter* resistant against frequently used antimicrobials for both humans and animals as a treatment remedy for campylobacteriosis, from broiler farms in Sri Lanka. Further, presence of AMR genes and mutations in responsible genes were compared to the resistant phenotypes. This study is the first to determine the correlation between antimicrobial resistances and their genetic markers against three commonly used antimicrobial classes in *Campylobacter* spp. isolated from poultry in Sri Lanka.

## 2. MATERIAL AND METHODS

### 2.1 Sampling

Samples were collected from nine farms in three provinces, Western, North Western and Sabaragamuwa provinces, which are located in western and central part of Sri Lanka and famous for poultry production. Nine poultry farms were selected for sampling, based on the age of broilers (20 - 46 days old) and the type of management practice (semi-intensive farming, the most common rearing system for broiler in Sri Lanka (Iddamalgoda et al., 1998). Because *Campylobacter* spp. colonization is not detected in early life of poults due to biological protection received from the maternal immunity (Fonseca et al., 2016), sampling in this study was performed for poults from the age of 20 days, after establishing gut *Campylobacter* spp., to 46 days old, the age of shipping to cull.

In each farm, at least ten healthy broiler birds per poultry pen of different age groups were randomly selected and sampled. The cloacal content was collected using sterile swab, immersed in Amies agar transport medium with charcoal (Copan, Brescia, Italy) and transported on ice to the laboratory for culture and isolation at Department of Zoology, Faculty of Applied Sciences, The University of Sri Jayewardenepura, Sri Lanka.

This study was conducted according to the guidelines of the Institutional Animal Experiment Committee of Graduate School of Agricultural and Life Sciences, the University of Tokyo. The sampling from the birds was not invasive and conducted by a veterinarian in the routine health check at the farms. The birds went back to the regular rearing process after the sampling.

### 2.2 Culture and isolation of Campylobacter

Immediately after receiving the samples to the laboratory, each sample was inoculated onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid, Hampshire, UK) and incubated microaerobically using "Anaero Pack - MicroAero", (Mitsubishi Gas Chemical Company, Tokyo, Japan) for 48 hr at 42°C. After incubation, *Campylobacter* colonies were identified using colony morphology and Gram's staining. Identified colonies were sub-cultured on blood agar (Oxoid), determined as *Campylobacter* sp. by biochemical tests, catalase and oxidase tests (both positive), and stored in Nutrient broth (Oxoid) with 20% glycerol at -80°C. Isolates were brought to the Laboratory of Veterinary Public Health, The University of Tokyo, Japan on dry ice. *Campylobacter* species were identified by molecular analysis using polymerase chain reaction (PCR) method targeting *hip*O and *ceu*E genes, specific for *C. jejuni* and *C. coli*, respectively (Han et al., 2016).

### 2.3 Antimicrobial resistant profile

The antimicrobial susceptibility test (ABST) of the isolates was performed by inoculating the isolates onto agar plates with threshold concentrations of antimicrobial agents. Isolates were tested for antimicrobial susceptibility to eight antimicrobial agents which belong to six antimicrobial classes. Two aminoglycosides (streptomycin (STR) and gentamicin (GEN)) were selected because they are frequently used to various animal species. Two antimicrobials, nalidixic acid (NAL) and ciprofloxacin (CIP) were selected to represent the first- and second-generation quinolones. Other antimicrobials, namely erythromycin (ERY), tetracycline (TET), amoxicillin (AMX) and trimethoprim-sulfamethoxazole (SXT), were selected to represent macrolides, tetracyclines, penicillin and sulfonamide classes. The threshold concentrations of CIP (4 µg/ml), ERY (32 µg/ml) and TET (16 µg/ml) were recommended for *Campylobacter* by Clinical and Laboratory Standards Institute (CLSI) guidelines. Since there are no breakpoint criteria for other antimicrobials for *Campylobacter* by CLSI, the breakpoint values of GEN (16 µg/ml) for *Enterobacteriaceae* recommended by CLSI and those of AMX (32 µg/ml), NAL (32 µg/ml), STR (16 µg/ml) and SXT (40 µg/ml) used in the previous literatures (Deckert et al., 2013; Han et al., 2016; Holasova et al., 2007; Son et al., 2007) were applied. Mueller Hinton agar (Oxoid) plates containing 5% horse blood and respective concentrations of antimicrobial agents were prepared in triplicates. *Campylobacter* isolates were microaerobically cultured in nutrient broth at 42°C for 24 hr and multiple-inoculator was used to inoculate the strains onto each

Mueller Hinton agar plate. Mueller Hinton agar plate without antimicrobials was used as negative control. The resistance was judged by visual growth observation of *Campylobacter*.

### 2.4. Antimicrobial resistant gene profile

In the present study, three genetic markers for antimicrobial resistance, point mutations in 23S rRNA gene and *gyr*A gene, and the presence of resistant gene, *tet*(O), were investigated for three commonly used drug groups, namely macrolides, quinolones and tetracyclines. With respect to resistance to erythromycin, 23S rRNA gene point mutations at positions 2074 and 2075 in domain V (A2074C, A2075G) were examined by mismatch amplification mutation assay (MAMA-PCR) (Alonso et al., 2005; Zhang et al., 2016). Mutation of *gyrA* gene (C257T) with respect to ciprofloxacin resistance was also detected by MAMA-PCR describe by Zirnstein et al. (1999). Presence of *tet*(O) gene was examined with respect to tetracycline resistance using molecular methods describe by Nguyen et al. (2016).

## 3. RESULTS

### 3.1. Isolation and confirmation of Campylobacter sp.

Farm wise prevalence of *Campylobacter* species was shown in Table 1. Total of 118 cloacal samples were screened and 73 (61.9%) were positive for *Campylobacter* spp. From all of the farms and all of the poultry pens, at least three samples were positive for *Campylobacter* strains. Based on PCR confirmation for species, *C. jejuni* was positive in 59/118 (50.0%) samples and was *C. coli* in 13/118 (11.0%). One sample (1/118, 0.8%) contained unidentified *Campylobacter* sp. *C. jejuni* isolates were detected from all nine farms while *C. coli* was isolated from six poultry farms only.

**Table 1: Prevalence of *Campylobacter* species isolated from poultry farms in Sri Lanka**

| Farm | Age (days) | No. of *Campylobacter* positive/ No. of samples | No. of samples positive for *Campylobacter* species (%) | | |
|---|---|---|---|---|---|
| | | | *C. jejuni* | *C. coli* | Unidentified *Campylobacter* sp. |
| A | 20 | 5/5 | 2 (40.0%) | 3 (60.0%) | 0 |
| | 23 | 5/5 | 5 (100%) | 0 | 0 |
| B | 33 | 4/5 | 2 (40.0%) | 1 (20.0%) | 1 (20.0%) |
| | 43 | 4/7 | 3 (42.9%) | 1 (14.3%) | 0 |
| C | 28 | 5/5 | 4 (80.0%) | 1 (20.0%) | 0 |
| | 43 | 4/5 | 2 (40.0%) | 2 (40.0%) | 0 |
| D | 24 | 5/5 | 5 (100%) | 0 | 0 |
| | 38 | 7/7 | 7 (100%) | 0 | 0 |
| | 43 | 4/4 | 2 (50.0%) | 2 (50.0%) | 0 |
| E | 28 | 6/15 | 6 (40.0%) | 0 | 0 |
| F | 28 | 4/15 | 4 (26.7%) | 0 | 0 |
| G | 28 | 3/10 | 2 (20.0%) | 1 (10.0%) | 0 |
| H | 46 | 10/20 | 8 (40.0%) | 2 (10.0%) | 0 |
| I | 25 | 7/10 | 7 (70.0%) | 0 | 0 |
| **Total** | | **73/118 (61.9%)** | **59 (50.0%)** | **13 (11.0%)** | **1 (0.8%)** |

### 3.2 Antimicrobial resistance

All 73 *Campylobacter* isolates showed resistance against any of the antimicrobials tested. The percentages of the *C. jejuni* and *C. coli* isolates resistant to each antimicrobial are shown in Table 2. The highest resistance (100% of *C. jejuni*, *C. coli* and unidentified *Campylobacter* isolates) was observed to CIP and NAL which belongs to the class Quinolones. All *C. jejuni* isolates were susceptible to ERY and GEN and only one isolate was resistant to STR. *C. coli* isolates were more frequently resistant to TET than *C. jejuni* isolates (92.3% vs. 72.1%), and isolates showed resistance to ERY were only *C. coli* strains.

**Table 2: Percentages of the *Campylobacter jejuni* and *C. coli* isolates resistant to antimicrobials**

| Antibiotic | % *C. jejuni* resistance (n=59) | % *C. coli* resistance (n=13) | % Unidentified *Campylobacter* resistance (n=1) | Total resistance % (n=73) |
|---|---|---|---|---|
| Ciprofloxacin | 100 | 100 | 100 | 100 |
| Nalidixic acid | 100 | 100 | 100 | 100 |
| Trimethoprim-Sulfamethoxazole | 98.3 | 76.9 | 100 | 95.0 |
| Amoxycillin | 78.0 | 85.6 | 100 | 79.5 |
| Tetracycline | 72.1 | 92.3 | 100 | 76.7 |
| Erythromycin | 0 | 61.5 | 0 | 11.0 |
| Streptomycin | 1.7 | 15.4 | 0 | 4.1 |
| Gentamicin | 0 | 7.7 | 0 | 1.4 |

### 3.3. Antimicrobial resistant profiles

Based on the resistance of the *Campylobacter* isolates, antimicrobial resistance profiles were created (Table 3). Among 59 *C. jejuni* isolates, 43 of them (72.9%) showed resistant to five antimicrobials of four classes, and three isolates (5.1%) were resistant to four antimicrobials of three antimicrobial classes. Other 13 isolates (22.0%) were resistant to three antimicrobials of two classes (CIP, NAL and SXT or AMX). Among *C. coli* isolates, two out of 13 (15.4%) isolates were resistant to seven antimicrobials followed by six (46.2%) and two (15.4%) isolates which were resistant to six and five antimicrobials. Two isolates were resistant to four antimicrobials of three classes, and one isolate was resistant only to two antimicrobials classes. Hence, *C. coli* isolates were resistant to wide range of antimicrobials compared to *C. jejuni*. Of *C. jejuni* and *C. coli* isolates, highest percentages of resistance profiles were CNTrAT (42/59, 71.2%) and CNTrATE (6/13, 46.2%), respectively (Table 3). Altogether 80.8% of isolates (59/73) were resistant to three or more antimicrobial classes, indicating the high prevalence of multidrug resistance.

**Table 3: Percentage of *Campylobacter* isolates with respect to their antimicrobial resistance profiles**

| Antimicrobial resistant profile | Percentage of *Campylobacter* isolates % (No. of isolates) |
|---|---|
| ***C. jejuni* (n=59)** | |
| CNA | 1.7 (1) |
| CNTr | 20.3 (12) |
| CNTrT | 1.7 (1) |
| CNTrA | 3.4 (2) |
| CNTrAS | 1.7 (1) |
| CNTrAT | 71.2 (42) |
| ***C. coli* (n=13)** | |
| CNT | 7.7 (1) |
| CNTrT | 15.4 (2) |

| | |
|---|---|
| CNTrAT | 7.7 (1) |
| CNTrAG | 7.7 (1) |
| CNTrATE | 46.2 (6) |
| CNTrATES | 15.4 (2) |
| **Unidentified *Campylobacter* sp. (n=1)** | |
| CNTrAT | 100 (1) |

**C:** ciprofloxacin, **N:** nalidixic acid, **Tr:** trimethoprim-sulfamethoxazole,
**A:** amoxicillin, **T:** tetracycline, **E:** erythromycin, **S:** streptomycin,
**G:** gentamicin

The most abundant profile in *C. jejuni* was observed in all age groups, and that more than two different antimicrobial resistance profiles can often be found from the poults in the same farm. There was no difference among the farms in terms of antimicrobial resistance profile.

### 3.4. Detection of genetic markers for antimicrobial resistance

Among 59 *C. jejuni* isolates, 52 (88.1%) had a mutation in *gyrA* gene and 47 (79.7%) possessed *tet*(O) gene (Table 4). Forty-one (69.5%) showed both mutation of *gyrA* and presence of *tet*(O) genes, and only *gyrA* mutation or *tet*(O) gene was detected in 11 (18.6%) and six (10.2%) isolates, respectively. Because none of the *C. jejuni* isolates were resistant to ERY, the MAMA-PCR for 23S rRNA gene was performed on randomly selected nine *C. jejuni* isolates and none of them possessed the mutation (Table 4). Comparatively, in *C. coli*, 12 isolates (92.3%) possessed *tet*(O) gene followed by eight (61.5%) and two (15.4%) isolates having 23S rRNA (A2075G mutation only) and *gyrA* (C257T) mutations (Table 4). One isolate each of *C. jejuni* and *C. coli* did not have either of the gene markers.

**Table 4: Relationship between drug resistance and resistance-related genes of *Campylobacter* isolates**

| **Resistant gene/mutation** | **Resistant to** | | | | | |
|---|---|---|---|---|---|---|
| | **ERY** | | **TET** | | **CIP** | |
| | **R** | **S** | **R** | **S** | **R** | **S** |
| *C. jejuni* (n=59) | | | | | | |
| A2074C/G, A2075G | 0/0 | 0/9 | 0/8 | 0/1 | 0/9 | 0/0 |
| *Tet*(O) | 0/0 | 47/59 | 42/43 | 5/16 | 47/59 | 0/0 |
| *gyrA* | 0/0 | 52/59 | 38/43 | 14/16 | 52/59 | 0/0 |
| None | 0/0 | 1/59 | 1/43 | 0/16 | 1/59 | 0/0 |
| *C. coli* (n=13) | | | | | | |
| A2074C/G, A2075G | 8/8 | 0/5 | 8/12 | 0/1 | 8/13 | 0/0 |
| *Tet*(O) | 8/8 | 4/5 | 12/12 | 0/1 | 12/13 | 0/0 |
| *gyrA* | 1/8 | 1/5 | 2/12 | 0/1 | 2/13 | 0/0 |
| None | 0/8 | 1/5 | 0/12 | 1/1 | 1/13 | 0/0 |
| Unidentified *Campylobacter* sp. (n=1) | | | | | | |
| A2074C/G, A2075G | 0/0 | 0/1 | 0/1 | 0/0 | 0/1 | 0/0 |
| *Tet*(O) | 0/0 | 1/1 | 1/1 | 0/0 | 1/1 | 0/0 |
| *gyrA* | 0/0 | 0/1 | 0/1 | 0/0 | 0/1 | 0/0 |
| None | 0/0 | 0/1 | 0/1 | 0/0 | 0/1 | 0/0 |

ERY: erythromycin, TET: tetracycline, CIP: ciprofloxacin, R: resistant, S: susceptible

## 4. DISCUSSION

In the present study, we detected *Campylobacter* from all pens of all farms. *C. jejuni* and *C. coli* are the main *Campylobacter* species present in poultry. It has been reported that *C. jejuni* was more abundant compared to *C. coli* (Alonso et al., 2005; Nguyen et al., 2016), which were similar results with that in the present study. In Sri Lanka, a study done on non-human primate (*Macaca sinica*) also showed higher proportion of *C. jejuni* isolates compared to *C. coli* (Tegner et al., 2019). However, a study reported in 2017 by Sri Lankan research group mentioned that higher proportion of *C. coli* was isolated from poultry meat and cecal samples in Sri Lanka (Kottawatta et al., 2017). Continuous monitoring of *Campylobacter* is necessary in Sri Lanka.

*Campylobacter* isolates from Sri Lankan poultry farms showed 100% resistance to quinolones (NAL and CIP) and frequent resistance to SXT, AMX and TET, among both *C. jejuni* and *C. coli*. A study done on poultry meat, skin and cecal samples obtained from farms and retail markets in 2006-2007 in Sri Lanka has reported that over 80% of the isolates were resistant against quinolones (Kottawatta et al., 2017). *Campylobacter* might be acquiring multidrug resistance in Sri Lanka.

The overuse, misuse and poor knowledge on antimicrobial usage leads to occurrence of antimicrobial resistance in bacteria. Many researchers have linked the use of antimicrobial agents in animal husbandry to the emergence and spread of resistance among *Campylobacter* spp. (Fonseca et al., 2016; Luangtongkum et al.,2009). It is suggested that some farmers in Sri Lanka misuse antimicrobials as a prophylactic antimicrobial to control infections which may occur due to their poor hygienic and biosecurity measures (De Silva, 2013; Priyankarage, 2016; Priyankarage, 2019). In Sri Lanka, antimicrobials have been able to be purchased over the counter from pharmacists despite prescription from a medical or veterinary doctor is required. Antimicrobials, even though it is prohibited, might be illicitly used as a feed additive and/or included in growth supplements in poultry industry in Sri Lanka. Such malpractices may have induced the multidrug resistance and multiple gene mutations/resistant genes in *Campylobacter*.

The antimicrobial resistant profiles were present irrespective of age group in the present study, indicating that there were possibilities to acquire AMR *Campylobacter* spp. throughout all growing periods. Once AMR bacteria spread in farms, they will be at a high risk for contaminating the food production chain and causing human infection (Fonseca et al., 2016).

In the present study, we applied MAMA-PCR method, which characterizes the mutations rapidly and accurately without sequencing PCR products (Alonso et al., 2005; Nguyen et al., 2016). It is cost effective, less time consuming, not labour intense and able to be performed in most of the diagnostic laboratories in developing countries like Sri Lanka, although there are some limitations in this method. Interestingly, all *Campylobacter* isolates carrying *gyrA* gene mutation, 52 *C. jejuni* and two *C. coli*, were resistant to CIP, while 19 out of 73 CIP resistant isolates were without *gyrA* gene mutation (Table 4). Although it is possible that there is a mutation where MAMA-PCR in the present study cannot detect, this might be due to acquiring other resistant determinants including multidrug efflux pumps (Levy, 2002), which are known to be involved in resistance mechanisms on antimicrobial classes of tetracyclines, quinolones and macrolides (Elhadidy et al., 2019; Iovine, 2013;).

In 43 *C. jejuni* isolates resistance to TET, 42 isolates possessed *tet*(O) gene. On the other hand, five out of 47 (10.6%) *C. jejuni* isolates having *tet*(O) gene were susceptible to TET (Table 4). Similar results have been reported by Wozniak-Biel et al. (2017) in Poland that 27% of *Campylobacter* isolates having *tet*(O) gene from poultry were susceptible to TET. Obeng et al. (2012) also reported 3.8% of *C. jejuni* from chicken showing *tet*(O) gene in PCR susceptible to TET. These findings reveal that *tet*(O) gene may either being silent (not expressed) or inactivated in some cases of poultry *Campylobacter* spp. (Wozniak-Biel et al. 2017).

## 5. CONCLUSION

This study revealed the presence of *Campylobacter* with high frequency in poultry cloacal samples in Sri Lanka. Most of the *Campylobacter* isolates were resistant to three or more antimicrobial classes and possessed one or more genetic markers causing antimicrobial resistance. This study urges the need of a continuous surveillance program to be adopted to monitor AMR *Campylobacter* in poultry and to emphasize on avoiding the use of antimicrobials unnecessarily in Sri Lanka.

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