Prevalence and antimicrobial resistance in *Campylobacter* from different stages of the chicken meat supply chain in Morogoro, Tanzania

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SUMMARY

Poultry are recognized as a main reservoir of *Campylobacter* spp. However, longitudinal studies investigating the persistence of *Campylobacter* on broilers and retail chciekn meat in Tanzania are rare. The aim of the current work was to evaluate the prevalence and antimicrobial susceptibility of *Campylobacter* spp. isolated from broiler farms and retail chicken meat. Eight hundred samples were collected from broilers aged 1 week to slaughter and retail chicken carcasses, consisting of 600 fecal droppings and 200 carcass rinses. The overall *Campylobacter* prevalence was 43.3% (381/880). The isolation rate of *Campylobacter* from chicken faeces was 41.5%, from carcasses at the farm was 51.0% and from carcasses from retail stores was 37.5%. Biochemical testing by hippurate hydrolysis identified 72.4% of all isolates as *C. jejuni*, 20.5% as *C. coli*, and 7.1% as other *Campylobacter* spp. Multiplex polymerase chain reaction confirmed 75.1% of all isolates as *C. jejuni*, 17.8% as *C. coli*, 4.2% as both, and 2.9% as other *Campylobacter* spp. Antimicrobial susceptibility testing using disk diffusion assay and broth micro-dilution method revealed resistance to: ampicillin (41% and 44%, respectively), ciprofloxacin (56 and 59%), erythromycin (17 and 18%), gentamicin (6% and 12%), streptomycin (20 and 23%), and tetracycline (62 and 63%). Resistance to azithromycin (9%), chloramphenicol (7%) and nalidixic acid (72%) was determined using the disk diffusion assay only. Up to 5% and 4% of all isolate tested were pan-susceptible, while, 67% and 40% showed multidrug resistance using the disk diffusion assay and the broth microdilution method, respectively. These results reinforce the need of efficient strategy implementation to control and reduce *Campylobacter* in chickens at production and slaughter levels, and the necessity to reduce the use of antimicrobials in poultry sector.
Keywords: *Campylobacter*, poultry and poultry meat, Isolation rate, antimicrobial resistance

INTRODUCTION

Campylobacter spp. are Gram negative bacteria responsible for the greatest number of cases of bacterial gastroenteritis worldwide (WHO, 2013). It is estimated that 500 million *Campylobacter* infections occur every year globally (Ruiz-Palacios, 2007; WHO, 2013), about one million people in the USA (CDC, 2015; Cha *et al*., 2016), 40,000 cases for 100,000 children under 5 years old in developing country (Oberhelman and Taylor, 2000), and up to 20% of children under 5 years old in Tanzania (Jacob *et al*., 2011; Deogratias *et al*., 2014). Nonetheless, according to World Health Organization, human *Campylobacter* infections are underestimated in developing countries due to other reasons like absence of regular surveillance programs (Coker *et al*., 2002). *Campylobacter* is increasingly becoming a major problem in Sub-Sahara Africa where the number of infections is predicted to double by the year 2020 due to many factors including poor hygiene and sanitation, malnutrition, poor health status, poor immunity, and HIV and AIDS (Coker *et al*., 2002). The consumption of poultry and poultry products is the primary source

of sporadic human campylobacteriosis, while approximately 66% of *Campylobacter* outbreaks are attributed to dairy products, mostly raw milk or cheese (EFSA and ECDC, 2015; Kaakoush *et al*., 2015).

Many species of poultry, especially chickens and turkeys, frequently carry high levels of *Campylobacter* spp. (primarily *C. jejuni* and *C. coli*) in their intestine as part of the normal microbial flora without showing any signs of clinical disease (Sahin *et al*., 2002; Kashoma *et al*., 2014; Wei *et al*., 2014; Sahin *et al*., 2015). Prevalence of *Campylobacter*-positive poultry flocks are generally high but vary by regions, seasons, and the production types (conventional, free-range, and organic, etc.), with reported *Campylobacter*-positive flocks ranging from 2% to 100% (Berghaus *et al*., 2013; Kalupahana *et al*., 2013; Ma *et al*., 2014). Factors commonly associated with *Campylobacter* colonization in broiler flocks include lack of overall biosecurity on farms, presence of other animals in close proximity to poultry houses, use of old litter, farm personnel and equipment (Giombelli and Gloria, 2014; Torralbo *et al*., 2014; Sahin *et al*., 2015). Horizontal transmission is the main route of spread of *Campylobacter* with the poultry flock (Zhang and Sahin, 2013; Agunos *et al*., 2014). Once a poultry flock is infected with *Campylobacter*, colonization spreads rapidly with overall prevalence reaching the highest level (close to 100%) at the slaughter age (Barrios *et al*., 2006; Goddard *et al*., 2014).

The high numbers of *Campylobacter* in the intestinal tract results in contamination of poultry carcasses during the slaughter process due mainly to spillage of fecal material at defeathering and evisceration, as well as to cross-contamination from the abattoir environment (Elvers *et al*., 2011; Chokboonmongkol *et al*., 2013). The prevalence of *Campylobacter* on poultry carcasses at the end of the processing line (post-chill) ranges from 0% to 100% worldwide with variation attributed to countries, seasons and study set-up (Guerin *et al*., 2010; Hue *et al*., 2010; Ma *et al*., 2014). Carcass contamination by *Campylobacter* is attributable to the farm of origin, as a high prevalence on-farm is usually associated with high-level carcass contamination in processing plants (Rosenquist *et al*., 2006; Johannessen *et al*., 2007).

The common clinical campylobacteriosisis self-limiting gastroenteritis with vomiting, cramping, and diarrhea mostly lasting for 7–10 days. However, in a subset of patients *Campylobacter* may cause severe complications and increased risk for death and therefore requires treatment (Friedman *et al*., 2000; Guerrant *et al*., 2001). When clinical treatment is necessary, ciprofloxacin, a fluoroquinolone that inhibits DNA synthesis by targeting gyrA and macrolides such as azithromycin and erythromycin, which hinder bacterial protein biosynthesis by targeting 23S rRNA, have been recommended as the first line antimicrobials. Yet, resistance to both antimicrobials has emerged and increases in resistance frequencies have been reported (Cody *etal*., 2010; Rozynek *et al*., 2010; Zhou *et al*., 2015). The rise in antimicrobial resistant *Campylobacter* has been linked to the use of antimicrobials in veterinary medicine and in farming practices (White *et al*., 2002; Zhu *et al*., 2006).

Raising competitive commercial poultry requires maintaining a healthy flock and generating a safe product for consumption. However, production of healthy poultry in commercial farms, antimicrobial agents has been widely used either as therapeutics, prophylactics, metaphylactics or growth

promoter (Krishnasamy *et al*., 2015; Kassem *et al*., 2016). Many of the antimicrobials used for animal agriculture are also used for human medicine. The overuse of antibiotics in intensively produced farm animals is believed to play a major role in the emergence of antibiotic-resistant pathogens (Landers *et al*., 2012; WHO, 2015). Furthermore, studies have shown a close association between the prevalence of livestock-associated antibiotic-resistant bacteria in animals and in humans (Roz´ynek *et al*., 2010; Vieir *et al*., 2011; Chantziaras *et al*., 2014; Elliot, 2015).

Extensive research on *Campylobacter* in poultry farms and poultry carcasses has been undertaken over the last two decades, the majority of which were on commercial broiler production in developed countries with very limited report in developing countries (Sahin *et al*., 2015). In Tanzania, although poultry meat and particularly broiler chicken is a major proteins source for the population, limited data is available regarding the prevalence and antimicrobial resistance of *Campylobacter* from broiler farms and poultry meat. Prompted by the lack of data, we investigated the prevalence and antimicrobial resistance profiles of *Campylobacter* spp. recovered broiler farms and poultry carcasses widely available in Tanzania. Our results will constitute the basis for much-needed surveillance programs to monitor the trends of antimicrobial resistance in these food-borne pathogens.

MATERIALS AND METHODS

Study area

Between February and April 2014, four medium commercial broiler farms (A, B, C and D) belonged to different producers were monitored. The study farms were located in four wards of Morogoro Municipality, and with the same climatic condition (Figure 1). All farms followed similar biosecurity protocols. Farms used an "all in, all out" management systems. Each farm had approximately 900 broilers coming from the same hatchery that were reared in three houses/batches, each with 300 birds.

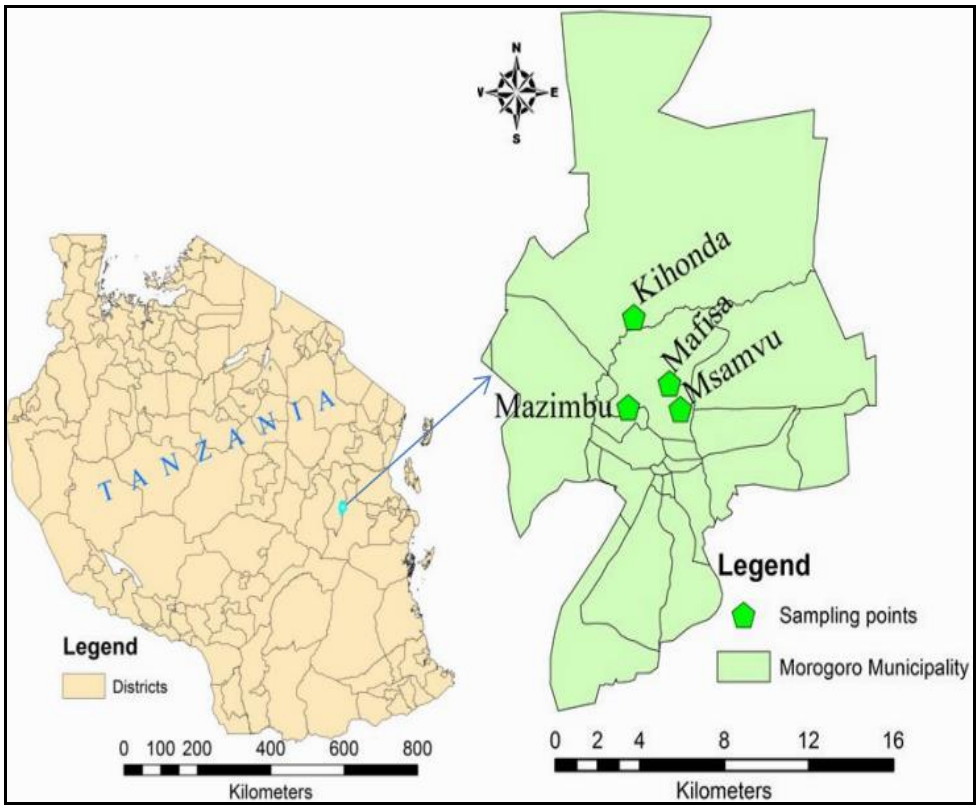

Figure 1: Map of the study area, Morogoro Municipality, Tanzania

Sample collection

Five samplings were made starting with one-week-old chicks to seven-week-old broilers. The sample size was calculated according to Thrusfield (1995) allowing the detection of *Campylobacter* spp. at a 95% confidence level and considering a within-flock prevalence of 70% (Mdegela *et al*., 2006). At 1 week of age, 50 fresh fecal droppings from each farm were randomly collected from brooder barns. Subsequently, 50 fecal samples were randomly collected in each farm at two week intervals up five-week-old birds. At seven weeks of age, 50 surface carcass swabs from each farm were aseptically collected during slaughter from randomly chosen carcasses immediately following evisceration. Fecal samples were placed in sterile polypropylene tubes. For surface swabs, each gauze pad was first premoistured with sterile maximum recovery diluents (MRD; Oxoid), swabbed at four parts of carcass, and then placed in a sterile plastic bag (Ziploc®; SC Johnson).

Broiler carcasses were purchased from eight retail stores in Morogoro. These retail stores were selected to ensure complete coverage of all major outlets of poultry carcasses of the town. Sampling visits were made once a week collecting two carcasses per store from February 2014 to April 2014. The entire chicken carcasses were immediately placed in sterile plastic bags and transported in a cool box to the laboratory for further analyses within a maximum of 12 hr. On arrival at the College of Veterinary and Medical Sciences, Sokoine University of Agriculture, each sample was processed using standard procedures for isolation of

thermophilic *Campylobacter* spp. as described hereunder.

Isolation of thermophilic *Campylobacter* species

For isolation of *Campylobacter* from feces, approximately 2 g of feces were suspended with 9 mL of maximum recovery diluent (MRD) (Neogen, USA). One-mL suspension was added to 9 mL of Preston broth containing *Campylobacter* growth supplements (CM067, SR048, SR117, and SR232; Oxoid, England). The enrichment tubes were incubated at 42ºC for 48 hr under microaerophilic condition (5% O_2, 10% CO_2, and 85% N_2), which was generated using airtight jars containing the Campy Pouch system (Becton Dickinson and Co., Maryland, USA). For isolation of *Campylobacter* species from purchased carcass, upon arrival in the laboratory, each carcass was aseptically placed in a plastic bag that contained 200 to 500 ml of MRD, depending on the sample size. The bag was homogenized by squeezing for 3 min. A 5 ml aliquot of the resulting suspension was removed and added to 10 ml of Preston enrichment broth as described earlier.

After incubation, 100 µL of each culture was spread onto a modified charcoal cefoperazone deoxycholate agar (mCCDA) plate (CM 0739, Oxoid) containing the selective supplement (SR155E, Oxoid) and incubated for 48 hrs at 42ºC microaerobically. Where available, three presumptive *Campylobacter* colonies from each mCCDA plate were then subcultured onto Muller-Hinton (MH; Difco, MD) agar containing Selective Supplement (SR117, Oxoid) and incubated microaerobically at 42ºC for 48 hrs (Sanad *et al*., 2011). Pure cultures were stored at -80ºC in MH broth supplemented with 30% glycerol (vol/vol) until further identification and characterization.

Biochemical testing of *Campylobacter* spp

Suspected colonies on selective media were examined for morphology and biochemical tests, including catalase, oxidase and hippurate hydrolysis. In all testes, *C. jejuni* 81–176 (wild-type strain) and *C. coli* (ATCC 33559) were used as positive controls. Oxidase strips (MB0266A, OXOID LTD) were used to test the isolates. A dark deep blue/purple colour along the contact portion of the strip after few seconds of contact indicates a positive for oxidase reaction. For catalase test, a loop full of pure culture was transferred from the agar onto the surface of a clean, dry glass slide. A drop of 5% hydrogen peroxide was immediately placed onto the colony on the slide. Effervescence indicates a positive for catalase reaction. *Campylobacter* species such as *C. jejuni*, *C. coli*, *C. lari*and *C. hyointestinalis* are catalase positive while *C. upsailensis* is catalase negative.

For the hippurate hydrolysis test, a pure culture of the isolate was inoculated in 0.4 ml of 1% sodium hippurate substrate (1 g of sodium hippurate (Sigma) and 99 ml of distilled water) in a tube. The tube was then incubated for 2 h at 37°C and 0.2 ml of 2% ninhydrin solution (Sigma) were added and further incubated at 37°C for an additional 15 min. Color change from pale purple to deep purple or violet indicated hippurate hydrolysis, and was considered a positive test for *C. jejuni*, while those organisms that showed a negative reaction were considered either *C. coli* or *C. lari*.

Molecular characterization by Polymerase Chain Reaction

In order to confirm the biochemical identification, the isolated strains were submitted to multiplex Polymerase Chain Reaction (mPCR) as described previously

by Linton *et al*. (1997). Bacterial DNA lysates were prepared from fresh pure *Campylobacter* cultures using the boiling method as previously described (Kashoma *etal*., 2014). In cases where no PCR products were detected, template DNA was prepared using QIAampDNA MiniKit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Three genes selected for the identification of the *Campylobacter* spp., *C. jejuni*, and *C. coli* were the 16S rRNA gene, the mapA gene, and the ceuE gene, respectively. The sequences of the three sets of primers used for gene amplification are presented in Table 1. Amplification reactions were performed in a 25 µL mixture containing 12.5 µl of ready mix Taq polymerase Mastermix (Qiagen, MD, USA), 0.5 µl of each of forward and reverse primers (IDT, Iowa, USA), 6.0 µl of DNA extract and deionized water to make a final volume of 25 µl. Amplification reactions were carried out using a DNA thermal cycler (Eppendorf, Hamburg, Germany) with the following program: initial denaturing at 95 °C for 5 min followed by 35 cycles of denaturing at 95 °C for 2 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. All PCR products were resolved on a 1% agarose containing 0.5 µg/ml of ethidium bromide in TBE buffer (Promega Corporation, Madison, WI, USA). The size of the PCR products was determined using a 1 Kb DNA ladder (Invitrogen, California, USA). The amplification generated 857 bp, 589 bp, and 462 bp DNA fragments corresponding to the *Campylobacter* genus, *C. jejuni* and *C. coli*, respectively. Isolates those were positive for the genus-specific PCR but negative for the *C. coli* and *C. jejuni*-specific PCR were designated as other thermophilic campylobacters (OTC). *C. jejuni* 81–176 (wild-typestrain) and *C. coli* (ATCC33559) were used as positive controls, while standard-grade laboratory water was used as a no template (negative) control.

Table 1. Primers for polymerase chain reaction (PCR) amplification of campylobacterial DNA for identification DNA

Organism	Primer	PCR product (bp)	Sequence
Campylobacter spp.	*16SrRNA*	857	5′ ATC TAA TGG CTT AAC CAT TAA AC 3′ 5' GGA CGG TAA CTA GTT TAG TAT T 3′
C. jejuni	*mapA*	589	5′ CTA TTT TAT TTT TGA GTG CTT GTG 3′ 5′ GCT TTA TTT GCC ATT TGT TTT ATT A 3′
C. coli	*ceuE*	462	5′ AAT TGA AAA TTG CTC CAA CTA TG 3′ 5′ TGA TTT TAT TAT TTG TAG CAG CG 3′

Antibiogram of the isolated species

Antibiogram of identified *Campylobacter* spp. was conducted using the Kirby-Bauer disk diffusion and the broth microdilution methods as described previously (Luber *et al*., 2003; Lehtopolku *et al*., 2012). Both tests were performed in accordance to the recommendations of the Clinical Laboratory Standards Institute (CLSI, 2012) and using the CLSI breakpoint interpretive criteria. In the cases when CLSI recommendations were not available, the ROSCO MIC for veterinary isolates was used to determine the breakpoints (ROSCO, 2007) (Table 2). The results were interpreted as susceptible, intermediately resistant, and Multi-drug Resistance (MDR) was defined as

resistance to three or more antimicrobial agents (Hakanen *et al*., 2003).

Table 2. The Guidelines Used to Determine the Antimicrobial Resistance Breakpoints Using the Disk-Diffusion and Broth Microdilution Methods

Antimicrobial agent	Result for Method:							
	Microdilution broth				Disk diffusion			
	Test range (µg/ml)	MIC breakpoints (µg/ml)			Disk conc. (µg)	Zone diameter breakpoint (mm)		
		S	I	R		S	I	R
Ampicillin	0.03 – 64.0	≤ 8	16	≥ 32	10	≥17	14-16	≤ 13
Ciprofloxacin	0.03 – 64.0	≤ 1	2	≥ 4	5	≥21	16-20	≤ 15
Erythromycin	0.03 – 64.0	≤ 8	16	≥ 32	15	≥23	14-22	≤ 13
Gentamycin	0.03 – 64.0	≤ 2	4	≥ 8	10	≥15	13-14	≤ 12
Streptomycin	0.03 – 64.0	≤ 2	4	≥ 8	10	≥15	12-14	≤ 11
Tetracycline	0.03 – 64.0	≤ 4	8	≥ 16	30	≥15	12-14	≤ 11
Azithromycin	NT	-	-	-	15	≥18	14-17	≤ 13
Chloramphenicol	NT	-	-	-	30	≥18	13-17	≤ 12
Nalidixic acid	NT	-	-	-	30	≥19	14-18	≤ 13

I, intermediate; MIC, minimum inhibitory concentrations; NT, Not tested; S, susceptible; R, resistance

In the Kirby-Bauer disk diffusion test, nine antimicrobial agents (Oxoid, UK) were tested at the following concentrations: 10 µg ampicillin (Amp), 5 µg ciprofloxacin (Cip), 15 µg erythromycin (Ery), 30 µg nalidixic acid (Nal), 10 µg streptomycin (Str), 30 µg tetracycline (Tet), 15 µg azithromycin (Azm), 10 µg gentamicin (Gen), and 30 µg chloramphenicol (Chl). Pure isolates were smeared on the surface of Mueller-Hinton agar supplemented with 5% defibrinated sheep blood with the help of sterile cotton swab. The plates were allowed to dry for few minutes. Antibiotic disc was placed on the agar surface within 15 min of inoculation of the plates. The plates were incubated overnight at 42°C under microaerobic condition. Sensitivity or resistance of an isolate for a particular antibiotic was determined by measuring the diameter of the zone of growth inhibition.

For the broth microdilution test and the determination of the minimum inhibitory concentration (MIC), 96-well plates containing two-fold serial dilutions of the antimicrobial agents were used as described previously (Ge*et al*., 2013). The antimicrobial agents tested included Amp, Cip, Ery, Gen, Str, and Tet. MIC values were defined as the lowest concentration of an antimicrobial agent that produced no visible growth. In both assays, *C. jejuni* 81–176 and *C. coli* (ATCC33559) were used as positive control strains.

Statistical Analysis

The prevalence and antimicrobial resistance of Campylobacter from poultry farms and stores were compared using the Chi-squared (X^2) test. A value of $P < 0.05$ was considered statistically significant. Agreement between the two antimicrobial resistance tests was determined using the Kappa statistic (Luber *et al*., 2003). A Kappa value of 100% indicates total agreement between the classifiers.

RESULTS

The Prevalence of *Campylobacter* in chicken farms and retail stores

The overall *Campylobacter* prevalence was 43.3% (381/ 880), with 41.5% (249/600) in chicken feaces, 51.0% (102/200) from Carcass rinses at farms and 37.5% (30/80) from retail store carcasses. All (381) of the presumptive *Campylobacter* isolates were positive for oxidase and catalase activities. The hippurate hydrolysis test identified 276 out of 381 isolates (72.4%) as *C. jujeni* and 78 isolates (20.5%) as *C. coli*. The remaining 27 (7.1%) *Campylobacter* spp. isolates belonged to other species. Ten isolates (2.6%) firstly identified as *C. coli* based on their inability to hydrolyze sodium hippurate, were later reconfirmed as *C. jejuni* by multiplex PCR. Similarly, 16 out of 27 isolates which were recognized as other *Campylobacter* species by hippurate test were then reclassified as *C. jejuni/C. coli* coexisting when subjected to multiplex PCR.

PCR revealed that the vastmajority of isolates was *C. jejuni* (75.1%; 286/381), whereas 17.8%(68/381) were *C. coli*. In addition, we also identified 4.2% (16/381) isolates that were positive for both *ceuE* and *mapA* PCR, and 2.9% (11/381) of isolates were *Campylobacter* spp., other than *C. jejuni* or *C. coli*. The frequency of *Campylobacter* isolation varied by farm/sources and by age within the farm (Table 3). There was no significant difference in *Campylobacter* prevalence between farms ($p > 0.05$), but farm D had a higher overall *Campylobacter* prevalence ($p < 0.05$) than other farms (A, B and C). Differences in *Campylobacter* spp. prevalence on farm A varied with age with highest prevalence (92%) observed on week 3. At week 1, farm C had higher prevalence (20%) than farms D (12%) and A (8%). No *campylobacter* species were isolated at week 1 from farm B. Prevalence increased rapidly in all farms reaching peak at week 5; 92% (farm A), 80% (farm D), 62% (farm C) and 54% (farm B). Prevalence of *Campylobacter* spp. recovered from carcass rinse after slaughter varied with farms, farm A had significant higher prevalence (60%) than other farms ($p < 0.05$). However, the overall prevalence of *Campylobacter* spp. recovered from chicken carcasses collected from stores was significantly lower (37.5%) than the average 51% isolated from all farms at slaughter.

Antimicrobial Susceptibility of the *C. jejuni* and *C. coli* Isolates

Analysis of the Kirby-Bauer disk diffusion assay showed that 95 of the 100 isolates (95%) were resistant to one or more antimicrobial agents, whereas five (5%) isolates were pan- susceptible to all antimicrobials tested (Table 4). Ten isolates (10 %; nine *C. jejuni* and one *C. coli*) were resistant to a single antimicrobial agent and eighteen isolates (17*C. jejuni* and one *C. coli*) showed resistance to two antimicrobial agents. 67% of all isolates (48 *C. jejuni* and 19 *C. coli*) were classified as MDR. Of the MDR isolates, 48 (60.8%) were *C. jejuni* and 19 (90.5*%) C. coli*. Six isolates (four *C. coli* and two *C. jejuni*) were resistant to Gen, whereas 7.0% of isolates (four *C. jejuni* and three *C. coli*) were resistant to Chl. 56% (ten *C. jejuni* and 46*C. coli*) and 9% (two *C. jejuni* and seven *C. coli*) of the isolates were resistant to Cip and Azm, respectively. Up to 62% of all isolates (50 *C. jejuni* and 12 *C. coli*) were shown to be resistant to Tet, 17% to Ery, and 72% (56 *C. jejuni* and *16 C. coli*) to Nal. In addition, 20% (8*C. jejuni* and 12 *C. coli*) and 41% (30 *C. jejuni* and 11 *C. coli*) of isolates were resistant to Str and Amp, respectively. While resistance to Gen, Azm, Ery, and Strand was significantly higher ($P < 0.05$) in *C. coli* isolates in comparison to *C. jejuni*, but there were no significant differences ($P > 0.05$) in resistance associated with the remaining antimicrobials. Co-resistance to Cip and

Tet was the most overall predominant pattern (37%) followed by Tet and Amp (28%) combination.

Table 3. Prevalence of *Campylobacter* species isolated from broiler farms and retail stores

Source	Age of chicken (wks)	Total isolates Number (%)	*C. jejuni* Number (%)	*C. coli* Number (%)	*C. jejuni/C. coli* Number (%)	Other Campy spp. Number (%)
Farm A	1	4 (8.0)	3 (6.0)	0 (0.0)	0 (0.0)	1 (2.0)
	3	12 (24.0)	8 (16.0)	4 (8.0)	0 (0.0)	0 (0.0)
	5	46 (92.0)	39 (78.0)	5 (10.0)	1 (2.0)	1 (2.0)
	7	30 (60.0)	20 (40.0)	7 (14.0)	2 (4.0)	1 (2.0)
	Subtotal	92 (46.0)	70 (35.0)	16 (8.0)	3 (1.5)	3 (1.5)
Farm B	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	3	16 (32.0)	12 (24)	3 (6.0)	0 (0.0)	1 (2.0)
	5	27 (54.0)	20 (40)	5 (10.0)	1 (2.0)	1 (2.0)
	7	20 (40.0)	14 (28)	4 (8.0)	2 (4.0)	0 (0.0)
	Subtotal	63 (31.5)	46 (23)	12 (6.0)	3 (1.5)	2 (1.0)
Farm C	1	10 (20.0)	5 (10)	2 (4.0)	2 (4.0)	1 (2.0)
	3	20 (40.0)	16 (32)	2 (4.0)	2 (4.0)	0 (0.0)
	5	31 (62.0)	24 (48)	5 (10.0)	1 (2.0)	1 (2.0)
	7	24 (48.0)	19 (38)	4 (8.0)	0 (0.0)	1 (2.0)
	Subtotal	85 (42.5)	64 (32.0)	13 (6.5)	5 (2.5)	3 (1.5)
Farm D	1	6 (12.0)	4 (8.0)	1 (2.0)	0 (0.0)	1 (2.0)
	3	37 (74.0)	30 (60.0)	5 (10.0)	1 (2.0)	1 (2.0)
	5	40 (80.0)	31 (62.0)	7 (14.0)	2 (4.0)	0 (0.0)
	7	28 (56.0)	20 (40.0)	6 (12.0)	1 (2.0)	1 (2.0)
	Subtotal	111 (55.5)	85 (42.5)	19 (9.5)	4 (2.0)	3 (1.5)
Overall Farm Prevalence	1	20 (10.0)	12 (6.0)	3 (1.5)	2 (1.0)	3 (1.5)
	3	85 (42.5)	66 (33.0)	14 (7.0)	3 (1.5)	2 (1.0)
	5	144 (72.0)	114 (57.0)	22 (11.0)	5 (2.5)	3 (1.5)
	7	102 (51.0)	73 (36.5)	21 (10.5)	5 (2.5)	3 (1.5)
	Subtotal	351 (43.9)	265 (33.1)	60 (7.5)	15 (1.9)	11 (1.4)
Retail stores		30 (37.5)	21 (26.3)	8 (10.0)	1 (1.3)	0 (0.0)
Grand total		381 (43.3)	286 (32.5)	68 (7.7)	16 (1.8)	11 (1.3)

Note: A total of 50 fresh fecal droppings were collected from each farm in the four samplings (week 1, 3, 5 and 7- eviscerated/dressed carcasses) and 80 refrigerated/packed broiler carcasses. For each farm prevalence, numbers in the same column with different letters in the superscript were significantly different ($p < 0.05$), while numbers with the same letters did not differ significantly (chi-square test and Fisher's exact two-tailed test).

Table 4. Antimicrobial resistance phenotypes of *C. jejuni* and *C. coli* isolated from broiler firms and chicken meat. The antibiotic resistance was determined using the disk diffusion method.

S/No	Resistance phenotype	*C. jejuni* No. (%)	*C. coli* No. (%)	Total No. (%)
1.	Pan-susceptible	4 (5.1)	0 (0.0)	4 (4.0)
2.	CIP	6 (7.6)	1 (4.8)	7 (7.0)
3.	AMP	2 (2.5)	1 (4.8)	3 (3.0)
4.	NAL / TET	6 (7.6)	0 (0.0)	6 (6.0)
5.	CIP/NAL	2 (2.5)	0 (0.0)	2 (2.0)
6.	CIP / AMP	4 (5.1)	0 (0.0)	4 (4.0)
7.	AMP / STR	2 (2.5)	0 (0.0)	2 (2.0)
8.	AMP/ CHL	1 (1.3)	1 (4.8)	2 (2.0)
9.	AMP / NAL / TET	8 (10.1)	1 (4.8)	9 (9.0)
10.	AMP / ERY / CHL	1 (1.3)	1 (4.8)	2 (2.0)
11.	AMP / NAL / STR	2 (2.5)	0 (0.0)	2 (2.0)
12.	CIP / ERY / AZM	1 (1.3)	1 (4.8)	2 (2.0)
13.	CIP / TET / NAL	16 (20.3)	0 (0.0)	16 (16.0)
14.	CIP / ERY / AMP	1(1.3)	1 (4.8)	2 (2.0)
15.	CIP / AMP / NAL	2 (2.5)	2 (9.5)	4 (4.0)
16.	ERY / TET / NAL	0 (0.0)	2 (9.5)	2 (2.0)
17.	AMP / ERY / TET / NAL	4 (5.1)	1 (4.8)	5 (5.0)
18.	CIP / TET / NAL / STR	3 (3.8)	3 (14.3)	6 (6.0)
19.	CIP / AZM / GEN / NAL	0 (0.0)	2 (9.5)	2 (2.0)
20.	CIP / ERY / TET / NAL	5 (6.3)	1 (4.8)	6 (6.0)
21.	CIP / TET / AMP / NAL	2 (2.5)	0 (0.0)	2 (2.0)
22.	AMP / AZM / TET / NAL	3 (3.8)	0 (0.0)	3 (3.0)
23.	AMP / CHL / TET / NAL / STR	2 (2.5)	1 (4.8)	3 (3.0)
24.	AMP / AZM / CHL / GEN / STR	0 (0.0)	1 (4.8	1 (1.0)
25.	CIP / AZM / GEN / NAL / TET	1 (1.63)	0 (0.0)	1 (1.0)
26.	CIP / AMP/ ERY / TET / NAL	2 (2.5)	0 (0.0)	2 (2.0)
27.	ERY / AZM/ GEN / TET / NAL / STR	0 (0.0)	1 (4.8)	1 (1.0)

With regards to broth microdilution test, a total of twenty-three different antimicrobial-resistant patterns were found among the 100 *Campylobacter* isolates tested (Table 5). Four isolates (*C. jejuni*) were pan-susceptible to all antimicrobials, while 96 isolates were resistant to at least one antimicrobial agents tested. The *Campylobacter* isolates displayed resistance most frequently to Tet (63%) and less frequently to Gen (12%). In comparison to *C. jejuni*, significantly more ($P < 0.05$) *C. coli* isolates displayed resistance to Gentamycin regardless of the source of the isolates, however, there were no significant differences ($P > 0.05$) in resistance associated with the remaining antimicrobials. Twenty-five of 79 *C. jejuni* (31.7%) isolates were resistant to three or more antimicrobials, while, 71.4% (15/21) of *C. coli* isolates were resistant to three or more antimicrobials. Approximately 60.8% and 5.1% of *C. jejuni* isolates were resistant to Cip and Gen, respectively. Additionally, 52.4% and 38.1% of *C. coli* strains were resistant to Cip and Gen, respectively. The co-resistance to Cip and Tet was the most overall predominant pattern (33%) followed by Amp and Tet (23%) combination.

Table 5. Antimicrobial resistance phenotypes of *C. jejuni* and *C. coli* isolated from broiler firms and chicken meat. The antibiotic resistance was determined using the microdilution method.

S/No	Resistance phenotype	*C. jejuni* No. (%)	*C. coli* No. (%)	Total No. (%)
1.	Pan-susceptible	4 (5.1)	0 (0.0)	4 (4.0)
2.	CIP	8 (10.1)	0 (0.0)	8 (8.0)
3.	AMP	5 (6.3)	0 (0.0)	5 (5.0)
4.	TET	5 (6.3)	1 (4.8)	6 (6.0)
5.	AMP / TET	8 (10.1)	1 (4.8)	9 (9.0)
6.	AMP / STR	2 (2.5)	0 (0.0)	2 (2.0)
7.	CIP / STR	3 (3.8)	1 (4.8)	4 (4.0)
8.	CIP / TET	11 (13.9)	2 (9.5)	13 (13.0)
9.	CIP / AMP	5 (6.3)	2 (9.5)	7 (7.0)
10.	ERY / TET	1 (1.3)	1 (4.8)	2 (2.0)
11.	AMP / STR / TET	1 (1.3)	1 (4.8)	2 (2.0)
12.	AMP / ERY / TET	1 (1.3)	2 (9.5)	3 (3.0)
13.	AMP / GEN / TET	3 (3.8)	1 (4.8)	4 (4.0)
14.	CIP / AMP / STR	2 (2.5)	0 (0.0)	2 (2.0)
15.	CIP / AMP / TET	4 (5.1)	1 (4.8)	5 (5.0)
16.	CIP / GEN / TET	1 (1.3)	1 (4.8)	2 (5.0)
17.	CIP / STR / TET	3 (3.8)	3 (14.3)	6 (6.0)
18.	CIP / ERY / TET	5 (6.3)	1 (4.3)	5 (5.0)
19.	ERY / STR / TET	2 (2.5)	0 (0.0)	2 (2.0)
20.	ERY / GEN / TET	1 (1.3)	1 (4.3)	2 (2.0)
21.	CIP / GEN / STR / TET	3 (3.8)	0 (0.0)	3 (3.0)
22.	CIP / GEN / AMP / TET	2 (2.5)	1 (4.3)	3 (3.0)
23.	CIP / GEN / ERY / STR / AMP	0 (0.0)	1 (4.3)	1 (1.0)

A comparison between the disk-diffusion and microdilution methods showed no significant differences ($p > 0.05$) in the number of isolates that were resistant to all antimicrobials. Additionally analysis using the Kappa statistics showed that the results obtained using the two tests was mostly in high agreement. The lowest agreement was noted for GEN (Kappa value = 0.6377), while the highest agreement was noted for Cip and Amp (Kappa value = 0.9386 and 0.9387, respectively) (Table 6).

Table 6. Comparison of antimicrobial resistance of *Campylobacter* spp. identified by disk diffusion and broth microdilution methods.

Antimicrobial agent	Disk diffusion				Broth microdilution				Agreement between methods Kappa value
	No. of isolates			% of resistant isolates	No. of isolates			% of resistant isolates	
	S	I	R		S	I	R		
Aminoglycosides									
Gentamicin	82	11	7	7.0	78	10	12	12.0	0.6377
Streptomycin	73	9	18	18.0	63	14	23	23.0	0.8472
β- lactam									
Ampicillin	50	9	41	41.0	47	9	44	44.0	0.9387
Macrolides									
Azithromycin	76	15	9	9.0	-	-	-	-	-
Erythromycin	74	9	17	17.0	74	8	18	18.0	0.9005
Quinolones									
Ciprofloxacin	35	9	56	56.0	78	25	59	59.0	0.9386
Nalidixic acid	21	7	72	72.0	-	-	-	-	-
Phenicol									
Chloramphenicol	87	6	7	7.0	-	-	-	-	-
Tetracycline									
Tetracycline	24	14	62	62.0	22	15	63	63.0	0.8507

DISCUSSION

Campylobacter is the leading cause of bacterial gastroenteritis in the world, and is estimated to affect about 20% of children of less than 5 years old with diarrhoea in Tanzania (Jacob *et al*., 2011; Deogratias *et al*., 2014). In the present study, *Campylobacter* was recovered from the broiler faeces starting from one-week-old birds (10%) and continuing to slaughter age (72%). The observed timing of colonization of *Campylobacter* in this study is earlier than of two for four weeks reported elsewhere (Jacobs-Reitsma *et al*., 1995; van Gerwe *et al*., 2009). Since there is unlikelihood of vertical transmission for contamination of chicken flocks with *Campylobacter* (Sahin *et al*., 2003; Patriarchi *et al*., 2011), environmental contamination can act as a reservoir and source for *Campylobacter*, which may be especially important under managements that exploit the same houses for multiple rearing cycles with low biosecurity. All 3 flocks in our study remained positive throughout, with the highest prevalence after week 5. Prevalence of *Campylobacter*-positive chicken flocks are generally high but vary by regions, seasons, and the production types (conventional, free-range, and organic), with reported *Campylobacter*-positive flocks ranging from 2% to 100% (Ansari-Lari *et al*., 2011; Berghaus *et al*., 2013; Kalupahana *et al*., 2013; Thakur *et al*., 2013; Ma *et al*., 2014).

The most common route of transmission of *Campylobacter* infection is via consumption of contaminated chicken products (Doorduyn *et al*., 2010; Taylor *et al*., 2013).Our data show clearly that a significant percentage (51%) of the chicken carcasses at slaughter and 37.5% of the chicken carcasses available on the shelves of retail stores in Morogoro carry these bacteria. This is consistent with other studies on the contamination of chicken carcasses with *Campylobacter* species: for example 52.25% of chickens in Saudi Arabia (Yehia *et al*., 2014), 48% in Qatar (Abu-Madi *et al*., 2016), and between 36.5%-76% in Iran (Rahimi *et al*., 2010;

Ansari-Lira *et al*., 2011) of chicken meat at stores were contaminated. However, our results are lower than 90% prevalence of *Campylobacter* spp. recovered from chicken carcasses in Yaounde, Cameroon (Nzouankeu *et al*., 2010) but higher than 10.8% in prepackaged chicken samples from grocery stores in USA (Mollenkopf *et al*., 2014) and 17% from chicken meat in Brazil (Salva *et al*., 2016).

Multiplex PCR analysis identified 32.5% of all samples as positive for *C. jejuni* and 7.5% as positive for *C. coli*. *Campylobacter jejuni* has been reported to be the most frequent species recovered from chicken farms (Colles *et al*., 2015; Prachantasena *et al*., 2016; Vidal *et al*., 2016) and chicken carcasses (Johnsen *et al*., 2006; Rahimi *et al*., 2010). Both *C. jejuni* and *C. coli* are well adapted to the avian host and reside mainly in the intestinal tract of birds (Hermans *et al*., 2012).

Biochemical tests are the basis of *Campylobacter* identification; however, these tests have low discriminatory power compared to molecular techniques (Engvall *et al*., 2002). Applying biochemical tests, 354out of 381 (92.9%) isolates were identified correctly. Ten (2.6%) and sixteen (4.2%)of isolates that were biochemically determined as *C. coli* and other *Campylobacter* spp. were then proved to be*C. jejuni* and *C. jejuni*/*C. coli* co-existing, respectively, by mPCR. The finding of such reclassification is consistent with previous reports. Adzitey and Corry, (2011) reported that 5.5% of *Campylobacter* which yielded negative to hippurate test were reclassified as *C. jejuni* by mPCR. Similarly, Rönner *et al*. (2004) reported that 5% of human *Campylobacter* isolates and 10% of chicken isolates that were hippurase negative (presumptive *C. coli* isolates) were further reconfirmed as *C. jejuni*by mPCR. The PCR method offers more accurate results for species identification since the hippurate test could yield misleading reactions. Therefore, hippurate hydrolysis test can only be used to differentiate between *C. jejuni* and *C. coli* especially in areas where molecular equipment are unavailable.

Macrolides, quinolones and tetracycline are among the common antimicrobials recommended for testing, because they can be of therapeutic relevance in severe cases of infection. In this study, high levels of resistance of *Campylobacter* to Cip (56-59%) and Tet (62-63%) but low resistance to Ery and Gen were revealed. The High resistance to Cip and Tet observed in this study can be associated with the authorization use of enrofloxacin (which is closely related to ciprofloxacin) and chlortetracycline for either therapeutic, metaphylactic or prophylactic use in chicken production systems in Tanzania (Mubito *et al*., 2014). Nevertheless, moderate to high prevalence of Cip and Tet resistance in *Campylobacter* isolates from chickens has also been reported elsewhere (Kim *et al*., 2010; Carmelo *et al*., 2013; Nguyen *et al*., 2016b). In this study, resistance to nalidixic acid was found in both *C.jejuni* (70.1%) and *C. coli* (76.2%) isolates. A wide-spread of *Campylobacter* isolates resistance to nalidixic acid has been reported from a variety of sources including chicken, food animals and products in different countries (Bostan *et al*., 2009; Dabiri *et al*., 2014; Kashoma *et al*., 2015; Kashoma *et al*., 2016).

Campylobacter spp. are inherently resistant to β-lactams (including ampicillin) due to their ability to produce β-lactamases, low affinity binding of β -lactams to the target (penicillin-binding proteins [PBPs]), or failure of the drugs to penetrate the outer membrane porins (Engberg *et al*., 2006; Li *et al*., 2007). Consequently, the reasonable resistance to ampicillin (41 – 44%)

observed in this study might be due to the frequent use of β -lactams such as Amoxicillin trihydrate (Novamox®) in chicken production system in Tanzania (Mubito *et al*., 2014). Similarly, the license and extensive use of Streptomycin sulphate (Aliseryl®) in chicken production (Mubito *et al*., 2014) might be associated with the moderate *Campylobacter* resistance to streptomycin observed in this study. However, low to moderate *Campylobacter* resistance to ampicillin and streptomycin has been reported elsewhere (Han *et al*., 2007; Carmelo *et al*., 2013).

In this study, low antimicrobial resistance was observed for different antimicrobials. Specifically, a relatively low number of isolates were resistant to Gen (6 –12%), Azm (9%) and Chl (7%), respectively. Generally, *Campylobacter* resistance to Chl and Gen has been reported to be low (Fallon *et al*., 2003; Kassa *et al*., 2007; Nguyen *et al*., 2016a). Furthermore, previous studies in Tanzania showed that 4 - 13% and 11 13% of the *Campylobacter* isolated from food animals and animal products were resistant to Chl and Gen, respectively (Kashoma *et al*., 2015; Kashoma *et al*., 2016). A moderate number of *Campylobacter* isolates in this study was resistant to macrolides (9% Azm and 17 – 18% Ery). Macrolides such as Erythromycin thiocyanate (Aliseryl®) is licensed and is extensively used in Tanzania as therapeutic agents for treatment of respiratory conditions in chicken (Mubito *et al*., 2014). The use of Ery in chicken for the purpose of either treatment or growth promotion contributes to the selection of resistant *Campylobacter* strains to other macrolides including Azm (Juntunen *et al*., 2010). Furthermore, the high resistance to macrolides (Ery and Azm) in *Campylobacter* isolated from humans in Tanzania (Komba *et al*., 2015) have been reported and highlights the need for understanding the impact of the use of antimicrobials in animal agriculture on the rise of resistant pathogens in food animals and humans. This further emphasizes the need for *Campylobacter* surveillance and control studies in Tanzania.

In *vitro* antimicrobial susceptibility testing involves measuring the antimicrobial's activity against the test microorganism by determining the MIC or inhibition zone diameter (Ge*et al*., 2013). Although the disk-diffusion method is more convenient, flexible, cheap, and widely used for testing pathogens, several researchers have reported different results when the method was compared with the broth microdilution method (Van der Beek *et al*., 2010; Lehtopolku *et al*., 2012; Kashoma *et al*., 2016). In this study, 2 - 5% of the *Campylobacter* isolates that were classified to either susceptible or intermediate resistance to different antimicrobial agents (2% Cip and Ery, 3% Tet and Amp, 5% Gen and Str) by the disk diffusion method were found to be resistant to the respective antimicrobial agents using the broth microdilution methods. Since accurate determination of *Campylobacter* susceptibility is of vital importance to ensure an adequate therapy and effectively monitor the antimicrobial resistance trends worldwide (Lehtopolku *et al*., 2012), it is important to use multiple approaches to limit methodological biases and to interpret the data adequately.

In recent years multidrug resistant *Campylobacter* strains have been increasingly reported worldwide, which is now recognized as a major emerging public health concern. In the current study, 40 - 67% *C. jejuni* and 71 – 90% *C. coli* isolates showed resistance to three or more classes of antimicrobials. Our data are in agreement with previous reports that showed the presence of higher proportion of *Campylobacter* isolates being resistant to three or more antimicrobial agents

(Luangtongkum *et al*., 2006; Usha *et al*., 2010; Fraqueza *et al*., 2014; Kashoma *et al*., 2015; Kashoma *et al*., 2016; Nguyen *et al*., 2016a). Furthermore, analysis of human-associated *Campylobacter* in Tanzania showed that 77.9% of the isolates were resistant to more than six of the tested antimicrobials (Komba *et al*., 2015).While the contribution of food animal-associated isolates to the MDR in human isolates is currently unknown, this is a point of serious concern that suggests that Tanzania, like other countries, has to devise stringent control and regulatory measures to reduce MDR isolates in the food chain.

In conclusion, antimicrobial resistance is highly prevalent in the chicken *Campylobacter* isolates from Morogoro, Tanzania, and many of them are resistant to multiple antimicrobial agents tested. Although *Campylobacter* as a cause for food-borne diseases is still underestimated in Tanzania, the high prevalence of multidrug resistant *Campylobacter* in broilers is alarming, given the fact that contaminated chicken meat is the major source of human *Campylobacter* infections. Food-borne transmission of antibiotic-resistant *Campylobacter* to humans compromises the clinical treatment of human campylobacteriosis. Thus, prudent measures for antimicrobial usage and active surveillance should be established to reduce the prevalence and spread of antimicrobial resistant *Campylobacter*.

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