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SHORT COMMUNICATION

Multidrug resistance and high genotypic diversity in *Campylobacter upsaliensis* from household dogs in Metro Manila, Philippines

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SUMMARY

Campylobacter upsaliensis is an emerging pathogen implicated in human gastroenteritis. Contact with pets, especially dogs, has been identified as a risk factor. Fourteen (14) *C. upsaliensis* isolates were obtained from household dogs in Metro Manila, Philippines. Antimicrobial susceptibility testing (AST) determined high resistance to ciprofloxacin (92.86%), erythromycin (100%), and tetracycline (100%). Multilocus sequence typing (MLST) identified 14 novel sequence types and 1 novel clonal complex, which is suggestive of a high genotypic diversity within *C. upsaliensis*. AST results indicate that antimicrobial stewardship and education of pet owners should be emphasised in veterinary practice.

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Campylobacter spp. is a zoonotic enteropathogen identified as one of the key causes of diarrhoeal diseases worldwide (Ahmed *et al*., 2018; Toledo *et al*., 2015). Clinical signs of campylobacteriosis include abdominal discomfort, nausea, vomiting, fever, and watery to bloody diarrhoea (Carbonero *et al*., 2012). Broiler chickens are an important source of contamination, and while consumption of contaminated meat is considered the primary risk factor for contracting campylobacteriosis, contact with domestic pets such as dogs and cats has also been associated with *Campylobacter* spp. infections (Montgomery *et al*., 2018; Lazou *et al*., 2017; Carbonero *et al*., 2012). Several reports have indicated the significance of *Campylobacter upsaliensis* in human gastroenteritis (Ahmed *et al*., 2018; Carbonero *et al*., 2012; Goossens *et al*., 1991). It is considered an emerging pathogen, especially in developing countries (*Amar et al*., 2014), with dogs and cats being the major carriers (Hald & Madsen, 1997). In dogs, reported *Campylobacter* spp. prevalence rates range from 2.7-87% (Ahmed *et al*., 2018; Carbonero *et al*., 2012; Tsai *et al*., 2007; Acke *et al*., 2006). Its mode of transmission is via the faecal-oral route through the ingestion of contaminated food and water or by direct contact with faecal matter from infected animals.

While campylobacteriosis is generally self-limiting, severe infection in immunocompromised patients and susceptible individuals necessitate antibiotic therapy (Amar *et al*., 2014). In severe or prolonged infections, macrolides are considered the drug of choice, but fluoroquinolones and tetracyclines are also recommended (Szczepanska *et al*., 2017). There have been reports, however, of *Campylobacter* spp. strains becoming resistant to some antibiotics included in the WHO list of critically and highly important antimicrobials for human medicine, such as nalidixic acid, ampicillin, streptomycin, and chloramphenicol (Ahmed *et al*., 2018; Tsai *et al*., 2007). While reports of drug resistance in *Campylobacter* spp. have been documented (Sharland *et al*., 2018; Amar *et al*., 2014), only a few studies have described the status of antibiotic resistance in *C. upsaliensis* isolated from dogs, and no information is currently available in the Philippines.

In monitoring *Campylobacter* spp. infections, genotyping methods such as multilocus sequence typing (MLST) have been used. It is based on the gene sequence analyses of at least seven housekeeping genes that are conserved within a species. *Campylobacter jejuni* and *C. coli* have been reported to have diverse genotypes based on different genotyping methods. Other *Campylobacter* species, such as *C. upsaliensis,* have also been mentioned to exhibit genotypic diversity

Key words:
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(Wassenaar & Newell, 2000). In the Philippines, there are no studies exploring the genotypic diversity in *Campylobacter* species.

Hence, the objectives of this study were to determine the antimicrobial resistance/susceptibility profiles of *C. upsaliensis* isolates from household dogs in Metro Manila, Philippines and to investigate the genotypic diversity of the *C. upsaliensis* isolates using MLST.

Faecal samples were collected from 195 household dogs from the cities of Caloocan, Las Piñas, Makati, Malabon, Mandaluyong, Manila, Marikina, Muntinlupa, Navotas, Parañaque, Pasay, Pasig, Quezon, San Juan, Taguig, Valenzuela, and the municipality of Pateros in the National Capital Region of the Philippines. Households that participated in the study were randomly sampled and only those that had healthy dogs were included. About 10-12 dogs were sampled per city. The isolation protocol for *Campylobacter* spp. using modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) described in ISO 10272:2006-1 was followed (ISO, 2006). Prior to isolation, selective enrichment in Bolton Selective Enrichment Broth (HiMedia™, Mumbai, India) supplemented with 5% (v/v) mechanically-defibrinated lysed horse blood and Bolton Selective Supplement (HiMedia™, Mumbai, India) containing cefoperazone (20 mg/L), vancomycin (20 mg/L), trimethoprim (20 mg/L), and amphotericin B (10 mg/L) was done. Samples were initially incubated at 37°C for 4 h under microaerobic conditions and then at 42ºC for 48 h, also under microaerobic conditions. After selective enrichment, 100 µL of the enrichment was plated onto mCCDA (HiMedia™, Mumbai, India) plates supplemented with mCCDA Selective Supplement (HiMedia™, Mumbai, India) containing cefoperazone (32 mg/L) and amphotericin B (10 mg/L). The plates were incubated at 42°C for 48 h under microaerobic conditions. Colonies typical of *Campylobacter* spp. were purified and maintained on Mueller-Hinton agar (MHA) plates supplemented with 5% (v/v) mechanically-defibrinated lysed horse blood. Primer pair UpsF and UpsR (Goossens et al., 1991) was used to identify *C. upsaliensis*. Antimicrobial susceptibility testing using the broth microdilution method was performed, following the standards recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *C. jejuni* and *C. coli* (EUCAST, 2017), since there is no standard available for *C. upsaliensis*. The antibiotics used were ciprofloxacin (CIP; 0.125-16 mg/L) (Ciprofloxacin hydrochloride monohydrate; HiMedia, India), erythromycin (ERY; 1-128 mg/L) (Erythromycin; HiMedia, India), and tetracycline (TET; 0.5-64 mg/L) (Tetracycline hydrochloride; HiMedia, India).

The genomic DNA of the isolates was extracted using G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea). PCR amplification of the seven housekeeping genes (Miller *et al.*, 2005) was carried out in 25 µL reactions. *Campylobacter upsaliensis* ATCC 43953 was used as positive control. The PCR products were sent to Macrogen, Inc. (10F World Meridian Center, 60-24 Gasan-dong, Geumcheon-gu, Seoul 153-781, Republic of Korea) for purification and DNA sequencing. The consensus sequence of each gene was used to query the *Campylobacter* PubMLST database (https://pubmlst.org/campylobacter/) to assign an allelic profile for each isolate.

A total of 14 *C. upsaliensis* isolates were obtained and found to be resistant to the antibiotics used. The majority of the isolates (92.86%) were multidrug-resistant. These isolates exhibited resistance to fluoroquinolones (CIP), macrolides (ERY), and tetracyclines (TET). One isolate (CUMI01) was susceptible to ciprofloxacin but resistant to erythromycin and tetracycline. High levels

Table 1 - *MLST of the 14 C. upsaliensis isolates. Included are their respective allele numbers, arbitrary sequence types and clonal complex, and resistance profiles.*

Isolate	*adk*	*aspA*	*atpA*	*glnA*	*glyA*	*pgi*	*tkt*	*ST*	*Clonal Complex*	*Resistance Profile*
CUC01	1	15	36	41	1	12	8	*NewST1*	undefined	CIP+ERY+TET
CUQ08	38	39	1	37	1	12	8	*NewST2*	undefined	CIP+ERY+TET
CUQ12	13	14	1	28	51	38	9	*NewST3*	undefined	CIP+ERY+TET
CUP06	38	36	1	13	51	27	9	*NewST4*	undefined	CIP+ERY+TET
CUM01	13	15	1	41	51	12	8	*NewST5*	*NewCC5*	ERY+TET
CUL06	8	35	2	37	51	12	8	*NewST6*	undefined	CIP+ERY+TET
CUL09	1	64	36	28	51	12	8	*NewST7*	undefined	CIP+ERY+TET
CUM08	1	13	36	28	51	48	9	*NewST8*	undefined	CIP+ERY+TET
CUM10	1	13	24	13	55	12	8	*NewST9*	undefined	CIP+ERY+TET
CUN05	1	39	24	37	33	27	8	*NewST10*	undefined	CIP+ERY+TET
CUP03	8	1	4	45	55	12	8	*NewST11*	undefined	CIP+ERY+TET
CUP04	6	1	4	45	51	12	9	*NewST12*	undefined	CIP+ERY+TET
CUS10	6	14	1	41	51	12	8	*NewST13*	*NewCC5*	CIP+ERY+TET
CUS11	6	14	1	41	1	12	9	*NewST14*	*NewCC5*	CIP+ERY+TET

of resistance across antibiotic classes were observed among samples: 92.86% were resistant to ciprofloxacin and 100% were resistant to erythromycin and tetracycline (*Table 1*). The 14 *C. upsaliensis* isolates were assigned their respective ST and CC, all of which were novel (*Table 1*), after being queried against the PubMLST non-*jejuni/coli* database. Fourteen novel STs and one novel CC were arbitrarily assigned. Currently, there are only 204 *C. upsaliensis* profiles deposited in the database (https://pubmlst.org/campylobacter/); the small number of curated data limited ST assignment. Furthermore, the absence of a database curator meant that data submissions cannot be made, and arbitrary designation of ST was necessary, as advised by the former database curator (personal communication, 09 May 2019).

In this work, *C. upsaliensis* from household dogs was found to be resistant to drugs that are commonly prescribed to treat campylobacteriosis, such as ciprofloxacin (92.86%), erythromycin (100%), and tetracycline (100%). Vandenburg *et al.* (2006) reported that the *C. upsaliensis* strains in their work were resistant only to erythromycin (12.9%). In another report, only one *C. upsaliensis* isolate was found to be resistant to ciprofloxacin, while resistance to streptomycin was determined to be 79% (Olkkola *et al*., 2015). Overall, there are limited studies on the antibiotic resistance of *C. upsaliensis* recovered from dogs, despite it being a potential source of infections in humans. Reported resistance rates of *Campylobacter* spp. isolates from dogs vary across geographic locations, isolation methods, and antimicrobial susceptibility testing methods. *Campylobacter* spp. isolated from dogs in Brazil showed high levels of resistance to fluoroquinolones, macrolides, and tetracycline. *Campylobacter jejuni* from pets were at least 40%, 45%, and 60% resistant to fluoroquinolones, macrolides, and tetracycline, respectively. *Campylobacter coli* from pets, on the other hand, registered resistance rates of >50%, 67%, and 50% to fluoroquinolones, macrolides, and tetracycline, respectively (Rodrigues et al., 2015). A study in India reported that *Campylobacter* spp. isolated from healthy and sick client-owned dogs were resistant to various drugs, which included tetracycline and ciprofloxacin (Ahmed *et al*., 2014). In Spain, resistance rates of 34.8% and 31.5% to ciprofloxacin and tetracycline, respectively, were reported among *Campylobacter* spp. isolates, all of which were isolated from client-owned dogs (Carbonero *et al*., 2012). *Campylobacter* spp. isolated from fresh-looking dog faeces in public parks were also found to be resistant to tetracycline (85.7%) and ciprofloxacin (100%) (Toledo *et al*., 2015). In another report, *C. jejuni* isolates from asymptomatic and symptomatic dogs were determined to be resistant to ciprofloxacin (19.6%), tetracycline (13.7%), and erythromycin (11.8%) (Acke *et al*., 2009). In the work of Lazou *et al.* (2017), 66.7% of *Campylobacter* spp. isolates from diarrhoeic and non-diarrhoeic dogs were resistant to tetracycline and quinolones. Fluoroquinolone resistance (13.9%) was also observed among *Campylobacter* spp. isolates from healthy dogs in Chile, albeit low, compared to other reports (Fernández & Oval, 2013). Another study in Taiwan determined that *Campylobacter* spp. isolated from household and shelter dogs had resistance rates of 81.8%, 78.8%, and 18.2% to erythromycin, tetracycline, and ciprofloxacin, respectively (Tsai *et al*., 2007). In a *C. jejuni* outbreak report in the US that was linked to puppy exposure, all the isolates were resistant to antibiotics commonly prescribed in the treatment of campylobacteriosis (Montgomery *et al*., 2018). The World Health Organization (2017) published a list of priority pathogens against which new antibiotics are needed. Fluoroquinolone-resistant *Campylobacter* spp. are included in the list and have been placed under Priority 2 (High). In general, the high resistance rates mentioned in various reports corroborate the results of this study. It is also important to note that many of the drugs that were tested against *Campylobacter* spp. isolates from animals are those that are prescribed for treating a range of human infections, including campylobacteriosis.

In veterinary practice, usage in companion animals of critically important antimicrobials used in human medicine has been found to be prevalent, with prescription behaviour frequently deviating from existing guidelines (Joosten *et al*., 2020). In the Philippines, the Inter-Agency Committee on Antimicrobial Resistance (2015) described the regulations on the use of antimicrobials in animals as generally "lacking and not properly enforced" and cites the inappropriate use of antimicrobials as the cause of the increasing problem with antimicrobial resistance. In Greece, fluoroquinolone-resistant *C. jejuni* strains have been attributed to the use of enrofloxacin in veterinary practice. The observed resistance to tetracycline, on the other hand, may be due to cross-resistance with doxycycline or to primary infection of pets with tetracycline-resistant isolates from other animals or food sources (Lazou *et al*., 2017). The *Campylobacter* spp. outbreak reported in the US revealed the widespread use of different antibiotic classes in the commercial dog industry. Such practice promotes the transmission of multi-drug resistant pathogens over a large network consisting of breeders, distributors, transporters, stores, and consumers. This finding highlights the need for industry-wide implementation of the prudent use of antibiotics (Montgomery *et al*., 2018).

This work also presents an overview of the genotypic diversity of *C. upsaliensis* from household dogs *i*n the Philippines. MLST analysis revealed 14 novel STs and 1 novel CC, which indicate high genotypic

diversity, as in the case of *C. jejuni* and *C. coli* (Duarte *et al*., 2014; Leblanc-Maridor *et al*., 2011; Colles *et al*., 2003). The detection of novel STs and CC in this study highlights the very limited size of the non-*jejuni/coli* database, as well as the high genotypic diversity observed in *C. upsaliensis* cited in a few studies. A similar study by Parsons *et al.* (2012) reported new STs of *C. upsaliensis* from dogs, as well as the high genotypic diversity observed. It also cited the small size of the *C. upsaliensis* database for the apparently high genotypic diversity reported. It noted that the diversity within *C. upsaliensis* from dogs appears to be greater than those of dog-derived *C. jejuni* isolates. Miller et al. (2005) further commented that at each locus in *C. upsaliensis*, there are approximately 4 times more alleles than in *C. coli*. Similarly, Olkkola *et al*. (2015) reported a high number of allelic differences in *C. upsaliensis* isolates from Finnish dogs with the same ST through whole genome MLST. Overall, these reports underpinned the substantial diversity in *C. upsaliensis* populations from dogs. Due to ST assignment constraints, epidemiological information such as source or overlap between human and non-human isolates or correlation with antibiotic resistance cannot be fully determined and described. Wassenaar & Newell (2000) cited that campylobacters in general possess the potential for genetic instability, possibly through the uptake of exogenous DNA by natural transformation, programmed DNA recombination or random genomic reassortment or rearrangement, which may result in the generation of "mosaic" alleles (Sheppard *et al*., 2011). Data obtained by Leblanc-Maridor *et al.* (2011) indicated the high genomic variability of *Campylobacter*, which may be an adaptation to environmental pressures. Their work suggested that genotypic diversity could improve survival and the ability to colonize hosts. Kashoma *et al.* (2015) suggested that the high genotypic diversity observed in their work with *C. jejuni* and *C. coli* may have been due to the isolates evolving and adapting to their host and factors affecting the host's immediate environment. High genetic instability may also be observed in certain animal isolates that co-exist with other strains, and this is brought about by the exchange of genetic material and other selective pressures from the environment (Leblanc-Maridor *et al*., 2011). The work by Sheppard *et al.* (2011) detected mosaic alleles in *C. jejuni* and *C. coli* and identified a bidirectional horizontal gene transfer. It is likely that the mosaic alleles were created among other closely related transformable bacteria. It is worth exploring whether the same events can also be definitely said for other campylobacters, specifically *C. upsaliensis*.

Given the results of the study, the importance of antimicrobial stewardship should be emphasised in veterinary practice. Other critical aspects include education of pet owners regarding regular veterinary care, hygiene practices when handling pets, clean living environment, and proper waste disposal.

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