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Occurrence of Antibiotic Resistant *C. jejuni* and *E. coli* in Wild Birds, Chickens, Environment and Humans from Orang Asli Villages in Sungai Siput, Perak, Malaysia

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Abstract: In developing countries, *Escherichia coli* and *Campylobacter jejuni* were found to be the prominent bacterial causes of gastroenteritis in human gastroenteritis, and they also showed an increased trend towards antibiotic resistance. Thus, the study aimed to determine the occurrence of *E. coli* and *C. jejuni* in wild birds, chickens, human and environment in villages in Perak and to determine the antibiotic resistance amongst *E. coli* and *C. jejuni* isolates. Three Orang Asli villages in Perak were chosen. Wild birds were trapped from nearby areas of the villages. We collected 52 human stool samples, 40 cloacal swabs from wild birds and 42 from chickens. For the environment, samples were collected from water (40 samples), flies (40 samples) and soil (40 samples). Two (5%) flies and 16 (38.1%) chickens were found positive for *C. jejuni*. Also, a total of 50 (96.2%) humans, 40 (100%) birds, 40 (95.2%) chickens were positive for *E. coli* and 111 (92.5%) of the 120 environmental samples tested positive for *E. coli*. The disk diffusion method was employed to determine the susceptibility of *C. jejuni* and *E. coli* isolates against ten antibiotics. All of the *E. coli* (100%) and *C. jejuni* (66%) isolates were found resistance towards at least one antibiotic. Hundred percent (100%) of the *E. coli* isolates obtained from wild birds were found to show multidrug resistance (MDR). *Campylobacter jejuni* isolates from flies and chickens showed high resistance towards nalidixic acid.

Keywords: Antibiotics, *Camplylobacter jejuni*, *Escherichia coli*, Chickens, Wild Birds and Human

Introduction

The most common causes of human gastroenteritis globally are *C. Jejuni*, *Salmonella* and pathogenic *E. coli*, especially in developed countries. It has been reported that they are categorised under the most frequent pathogenic bacteria isolated from both animals and humans (Haruna *et al*., 2013; Komba *et al*., 2015). A majority of these pathogenic bacteria cause acute but mostly self-limiting illnesses, with fever, diarrhoea and abdominal cramps; however, in certain cases, severe infections could also prevail (Shin *et al*., 2015). Treatment with an antimicrobial agent can reduce the illness duration, which could also prove lifesaving during serious infections (Komba *et al*., 2015). Chickens are one of the significant hosts *C. jejuni* and *E. coli* and contamination caused by these pathogens lead to high percentage of human colibacillosis and campylobacteriosis (Colles *et al*., 2016). These pathogenic bacteria find their way into chickens through air, water, litter, from adjacent poultry houses, other livestock that are in the farm, during transportation, wild birds and mechanical transmission via insects (Bull *et al*., 2006; Cox *et al*., 2012).

Wild birds may act as significant factor in terms of antibiotic resistance, mirroring the activities of humans and their effects on the environment due to diverse ecological niches of birds and their ability to easily carry environmental and human bacteria. Thus, wild birds may act as potential spreaders of antibiotic resistance since they can quickly migrate long distances; as a melting pot and reservoir of genes responsible for resistance as well as antibiotic-resistant bacteria; and as a possible source responsible for infecting human beings (Bonnedahl and Järhult, 2014).

Since the use of antimicrobial drugs has been widespread, developing antibiotic resistance in bacteria is of great concern and pose a potential threat to public health (EFSA, 2009; Jordan *et al*., 2009). In modern food animal husbandry, antibiotics are mostly employed for therapeutic, prophylactic and metaphylactic purposes (Aarestrup, 2004; Bronzwaer, 2008). Not only in the animal populations, even human populations are under the risk radar for developing and spreading antibiotic resistance (Holmes *et al*., 2016).

In recent years, a greatly growing concern is over foodborne pathogens not only because they cause foodborne diseases but also because there has been an increase in the frequency of antibiotic-resistant strains found in both animals and humans (Holmes *et al*., 2016; Snelling *et al*., 2005). Resistant bacteria continue to exist in the environment even after banning the use of antibiotic in feed because this only eases the selective pressure without losing resistance (Ahmed *et al*., 2010; Ghidan *et al*., 2008; Weese, 2011). In veterinary medicine, the growing resistance of bacteria to antibiotics has become a key issue, since animals could become the carriers for transferring resistant zoonotic agents, which would end up affecting humans (Weese, 2011).

Resistant *Campylobacter* and *E. coli* in food-producing animals could transfer genes to microorganisms in humans via multiple ways (Zhang and Plummer, 2008). For the same reason, the main vehicle responsible for transmitting resistant *Campylobacter* to humans through food chain is the food of animal origin (Luangtongkum *et al*., 2009). A better understanding can be gained through reporting of antibiotic resistance to determine the trends in their patterns to effectively control them and to study antibiotics' long-term efficiency.

In Malaysia, there are several records of antibiotic resistance of *E. coli* and *C. jejuni* isolated from poultry as well as other food animal sources but information on multidrug resistance is less. Thus, the study aimed to determine the occurrence of *E. coli* and *C. jejuni* in the chickens, environment, wild birds and humans as well as the antibiotic resistance profiles of the isolates.

Materials and Methods

Study Design

This study was carried out as an observational cross-sectional study to investigate and record the exposures and observe the outcomes (such as presence or absence of the organisms understudy) as they occur (Khan *et al*., 2012).

Study Location

Orang Asli villages are situated in the mountains in Sungai Siput, Perak.

Sample Size

There were limitations in this study in particular the sampling of the Orang Asli villages. The Orang Asli villages are rather restricted to access; also the unwillingness of the villagers to participate affected the study. However, Prof. Dato Dr Abdul Rashid (Co-supervisor) had a good rapport with the head villages that we managed to include three Orang Asli villages in this study. Thus the collection of samples from human, wild birds, chickens and the environmental was carried out through convenient sampling. The sample size could not be determined because of the difficulty to estimate the target population.

Samples Collection

Three Orang Asli villages were selected to trap wild birds. A mist net was set up within the village or less than 5 km from the village. It was set up in the morning and placed for 6 hours. The trap was checked every 20 minutes for the trapped birds. These birds were photographed and marked and cloacal swabs were taken prior to their release. Forty birds were sampled.

In each village, 10-12 cloacal swabs or fresh stools samples were taken from chickens and humans respectively. A total of 52 cloacal swabs and 42 fresh stools were sampled.

Environmental samples consisted of water, flies, and soil were collected in the range of 10–20 per village. The total number of samples was 120 at 40 per each water, flies and soil samples.

Isolation of C. jejuni

Wild Bird, Chicken and Human Samples

For each cloacal swab or fresh stool, direct streaking was carried out onto the Campylobacter blood-free selective agar base (modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA)) (Oxoid) plate supplemented with CCDA that contained amphotericin and cefoperazone (Oxoid).

Water Samples

The procedure of Hudson *et al*. (1999) was employed 100 mL of each water sample was filter using a sterile 0.45 μm pore-size cellulose nitrate membrane filter (diameter: 47 mm) (Milipore, Sartorius Stedim, Biotech, Goettingen. Germany). Then, the membrane filter was placed in a sterile bottle containing 100 mL Bolton Selective Enrichment Broth (Oxoid), which was incubated at 42°C for 48 hours under microaerophilic condition. After incubation, loopfuls of each enriched culture was streaked onto mCCDA plate.

Pooled Flies Samples

A sterile cotton swab was used to crush each of the pooled flies and placed in a 10 ml Bolton Selective Enrichment Broth (Oxoid), and then loopfuls were plated onto mCCDA plates.

Soil Samples

A sterile spoon was used to pick soil samples, which were then placed in a bottle containing 19 ml of Bolton Selective Enrichment Broth (Oxoid); all bottles were incubated under microaerophilic condition at 42°C for 48 hours. Subsequently, loopfuls of each enriched culture was plated onto mCCDA plates.

All plates were placed in the anaerobic jars with microaerophilic atmosphere produced through a gas-generating sachet (CampyGen; Oxoid), and were incubated for 48 hours at 42°C. Plates that showed no growth were re-incubated for another 48 hours under the same condition.

Isolation of E. coli

Wild Bird, Chicken and Human

The bottles containing cloacal swabs or fresh stool and BPW (Oxoid) were incubated at 37°C for 24 hours aerobically.

Water Samples

About 100 ml BPW (Oxoid) was added to 50 ml of each water sample and incubated at 37°C for 24 hours aerobically.

Pooled Flies Samples

Crushing of the flies was done in the bottle containing 10 mL BPW (Oxoid) and then proceeded with incubation at 37°C for 24 hours aerobically.

Soil Samples

The soils in bottles were added with 10 mL of BPW (Oxoid) and incubated at 37°C for 24 hours aerobically.

Culture of Enriched Samples

The enriched cultures of fresh stool, cloacal swabs, water, flies and soil were streaked onto Brilliance *E. coli*/Coliform Selective Media (Oxoid), then incubated aerobically for 18-24 hours at 37°C. The suspected *E. coli* colonies that were dark blue to violet colour were sub-cultured on to Brilliance *E. coli*/Coliform Selective Media (Oxoid) and incubated aerobically for 18-24 hours at 37°C.

Identification of C. Jejuni

Gram staining showed gram negative slender, curved/s-shape and motility test using wet mount observed under a contrast microscope showed darting, cockscrew movement. The presumptive *C. jejuni*-like colonies were sub-cultured onto Columbia Blood Agar (Oxoid) containing 5% defibrinated horse blood (CBA), incubated microaerophilically for 48 hours at 42°C.

On the purified cultures, biochemical tests such as oxidase test, hippurate hydrolysis test, catalase test and indoxyl-acetate hydrolysis tests were conducted.

Identification of E. coli

Escherichia coli showed gram negative rods on gram staining.

The following biochemical tests were conducted on the purified cultures: Sulfide-Indole Motility (SIM), Tryptone Sugar Iron (TSI), Urease, Citrate, Methyl Red and Voges-Proskauer (MR-VP) tests.

Confirmation of *C. jejuni* and *E. coli* isolates using Polymerase Chain Reaction (PCR) assay.

DNA Extraction Method

The conventional boiling method was employed to extract the DNA. A bacterial suspension was boiled in sterile distilled water to extract bacterial DNA. To 1,000 μl sterile distilled water placed in a 1.5 ml Eppendorf tube (Eppendorf, Australia), a loop full of colonies was added, and incubated in dry water bath at 94°C for 10–15 min, and then cooled down to room temperature (37°C). Centrifugation of each bacterial suspension was done at 13,000 rpm for 3 min. The supernatant was then placed to a new 1.5 ml Eppendorf tube; after discarding the pellets, the supernatant was used as the template DNA (Yamazaki-Matsune *et al*., 2007).

Polymerase Chain Reaction (PCR) Assay for C. Jejuni

Positive control strain of *C. jejuni* (ATCC 29428) was used to optimise DNA templates and conduct PCR, which followed the protocol of Yamazaki-Matsune *et al* (2007). Modification was done to the final PCR amplifications to 50 μl reaction volume that included 1 μl

(10 mM) of each primer sets (Table 1), 25 µl of Master mix (QIAGEN), 13 µl of RNase-free water (QIAGEN) and 4 µl of DNA templates. In a DNA thermal cycler (Eppendorf), amplification of reaction mixtures was done for the subsequent cycling parameters: one cycle for 1.5 min at 95°C, 25 cycles each for 0.5 min at 95°C, 1.5 min at 58°C and 1 min at 72°C and finishing with an ultimate extension time for 7 min at 72°C.

Polymerase Chain Reaction (PCR) Assay for E. coli

In 50 µL reaction volume, amplification of DNA was done, which also included 1 µL (10 mM) of primer set as mentioned by Wang *et al*. (2002) employing the internal control targeted gene 16s rRNA of *E. coli* (Table 1), 4 µL of DNA templates, 25 µl of Master Mix (QIAGEN) and 19 µL of Rnase free water (QIAGEN). Adjustment of the mix volume was done to 50 µl with sterile water. In a DNA thermal cycler (Eppendorf), amplification of the reaction mixtures was done with the subsequent cycling parameters: starting denaturation step for 1.5 min at 95°C, followed by 30 amplification cycles with denaturation for 0.5 min at 95°C, annealing for 0.5 min at 58°C and extension for 0.5 min at 72°C and, at the end, an ultimate extension for 7 min at 72°C.

Agarose Gel Electrophoresis

PCR products that were already amplified were subject to resolving in a 2% agarose gel (Agarose, LE Analytical Grade) formulated in a 1× Tris-Borate-EDTA (TBE) buffer (2 mM EDTA, 40 mM Tris-Borate, PH 7.5) as well as Gel-red (3 µL/mL) for 90 min at 75 V. A gel documentation system Alpha Imager (BIO-RAD) was employed to observe the electrophoresed gel under transilluminator UV.

Antibiotic Susceptibility Test

All *E. coli* and *C. jejuni* isolates were subjected to an antibiotic susceptibility test by employing the disc diffusion test method. On CBA (Oxoid) supplemented with 5% defibrinated horse blood, all of the *C. jejuni* isolates kept at –40°C were revived, which was then incubated at 42°C for 48 hours under microaerophilic condition using CampyGen (Oxoid) gas pack. On the Brilliance *E. coli*/Coliform Selective Media (Oxoid), recovery was done for *E. coli* isolates.

The disc diffusion method used following Clinical and Laboratory Standards Institute (CLSI) (2010).

Ten antibiotics from eight different classes were chosen (Table 2) based on WHO recommendation for the Critically Important Antimicrobials for Human (2011) and the OIE recommendation for Veterinary Critically Important Antibiotic (2012) lists. The ten antibiotics were: ampicillin-sulfbactam (Sam), 10 µg; ciprofloxacin (Cip), 5 µg; erythromycin (E), 15 µg; tetracycline (Te), 30 µg; gentamicin (Cn), 10 µg; streptomycin (S), 10 µg; cefpodoxime (Cpd), 10 µg; enrofloxacin (Enr), 5 µg; nalidixic acid (Na), 30 µg; and trimetophrim-sulfamethoxazole (Sxt) 25 µg. The preparation of a suspension for each *C. jejuni* and *E. coli* isolates included introducing a loopful of the colonies to a 2 ml tube containing 0.9% NaCl. The inoculum turbidity was adjusted to a 0.5 McFarland standard (1.5×108 cfu/mL). Then, the bacterial suspension was spread in a gentle manner with a sterile cotton swab in three different directions over the surface of Mueller-Hinton (Oxoid) agar with addition of 5% defibrinated horse blood for *C. jejuni* isolates. The Mueller-Hinton (Oxoid) agar plate with no supplement was used for *E. coli*. Excess moisture was removed from the swab and the agar plates were allowed to dry for 3–5 min before the antibiotic discs were placed onto the agar surface. For each isolate, a minimum of two (2) Mueller-Hinton agar plates was needed, in which five (5) antibiotic discs were dispensed for each of the inoculated plate by using a disc dispenser.

For *C. jejuni* isolates, microaerophilic incubation was carried out for 48 hours at 42°C and for *E. coli*, aerobic incubation was for 24 hours at 37°C. After incubation, a digital caliper was used to measure the inhibition zone diameter. Reference strains of *E. coli* (ATCC 25922) and *C. jejuni* (ATCC 33560) were used as controls. The zone of inhibition diameter breakpoints were in accordance to CLSI, (2010) and classification of the isolates was done as resistant, intermediate and sensitive.

Resistance to at least one antibiotic in three or more classes is termed as multidrug drug resistance (MDR) (Magiorakos *et al*., 2012).

Table 1: The primers used in PCR assay to confirm *C. jejuni* and *E.coli* isolates

Species	Primer	Oligonucleotide sequence	Size
C. jejuni	C-1 C-3	5’-CCATAAGCACTAGCTAGCTGAT-3’	161bp
		5’-CCA TAA GCA CTA GCT AGC TGAT-3’	
E. coli	E16S-a E16S-b	CCCCCTGGACGAAGACTGAC	
		ACCGCTGGCAACAAAGGATA	401

Source: (Yamazaki-Matsune *et al*., 2007; Wang *et al*., 2002)

Table 2: Breakpoints to determine antimicrobial susceptibility of *E. coli* and *C. jejuni* isolates using the disc diffusion method

			Zone diameter breakpoint (mm)		
Classes of antibiotics	Antimicrobial agent	Disk concentration (µg)	S	I	R
Penicillin combinations	Ampicillin-sulfbactam	10	≥17	14-16	≤13
Tetracyclines	Tetracycline	30	≥19	15-18	≤14
Aminoglycosides	Gentamicin	10	≥15	13-14	≤12
	Streptomycin	10	≥15	12-14	≤11
Macrolides	Erythromycin	15	≥23	14-22	≤13
Fluoroquinolones	Ciprofloxacin	5	≥21	16-20	≤15
	Enrofloxacin	5	≥21	18-20	≤17
Quinolones	Nalidixic Acid	30	≥19	14-18	≤13
Sulfamethoxazole-Trimethoprim	Sulfamethoxazole-	25	≥16	11- 15	≤10
Cephalosporins/Cephamycins (Cephems)	Trimethoprim Cefpodoxime	10	≥21	18-20	≤17

Source: Clinical Laboratory Standards Institute (CLSI, 2010); S susceptible; I intermediate; R resistance

Table 3: *E. coli* isolated from different species of wild birds

Birds species (Scientific name) (No.)	Villages	*C. jejuni*	*E. coli*
Oriental Magpie Robin (*Copsychus saularis*) (24)	A(2), B(13), C(9)	0	24(100%)
White-rumped Shama (*Copsychus malabaricus*) (12)	A(4), B(8)	0	12(100%)
Little Spiderhunter (*Arachnothera longirostra*) (4)	A(3), C(1)	0	4(100%)
Total 40	40	0 (%)	40(100%)

Results

Isolation and Identification

Wild Bird

A total of 40 birds were trapped and were identified belonging to three species, namely White-rumped Shama 12 (31%), Oriental Magpie Robin 24 (60%) and Little Spiderhunter 4 (10%). None (0%) of the birds were positive for *C. jejuni* but all (100%) were positive for *C. jejuni* but all (100%) were positive for *E. coli* (Table 3).

Chickens

Escherichia coli and *C. jejuni* were found to occur in chickens at 95.2% (40/42) and 38.1% (16/42) respectively.

Humans

All fresh stool samples were negative (0%) for *C. jejuni* but 96.2% were positive for *E. coli*.

Environment

Of the 120 environmental samples collected, 111(92.5%) were positive for *E. coli* and only 2 (5%) were positive for *C. jejuni*, that is in flies. Thirty-five 35(87.5%), 37(92.5%) and 39(97.5%) of water, flies and soil respectively were positive for *E. coli*.

Table 4 showed the occurrence of *E. coli* and *C. jejuni* that were isolated in all sources. Figures 1 and 2 showed the PCR assay to confirm *C. jejuni* and *E. coli*.

Antibiotic Resistance

The antibiotic susceptibility test was conducted for a total of 18 *C. jejuni* isolates (16 isolates from chickens and 2 from flies) and 241 *E. coli* isolates (40, 40, 50, 39, 37 and 35 isolates from wild birds, chickens, human, soil, flies and water respectively). However, six *C. jejuni* isolates from chickens showed sensitivity towards all antibiotics and 100% of *E. coli* isolates were found be resistant to all antibiotics.

Isolates of *C. jejuni* from chickens showed resistance to all antibiotics with the exception of gentamicin, while *C. jejuni* isolates from flies were found to be sensitive to erythromycin, gentamicin and streptomycin. The resistance profile of isolates from chickens showed resistance to nine antibiotics, ranged from 50% to 3.5 %. Two *C. jejuni* isolates from flies were found 100% resistant to nalidixic acid and while one was resistant to tetracycline, ampicillin-sulfbactam, ciprofloxacin, sulfamethoxazole-trimethoprim, enrofloxacin and cefpodoxime (50% each) (Table 5).

The resistance profile of *E. coli* isolated from wild birds demonstrated that resistant to 8 antibiotics ranged from 77.5% to 2.3% while to erythromycin and tetracycline was 100% each. The isolates from chickens showed resistance to antibiotics ranged from 67.5% to 2.1% to erythromycin. Also, all isolates from human were resistant to erythromycin (100%) while to 9 antibiotics at 42% to 6%. In environmental samples, the isolates from flies also showed resistance to erythromycin (97.3%) while to other 9 antibiotics ranged from 59.5% to 2.7%. The isolates from water were susceptible to ciprofloxacin and gentamicin, were resistant to 7 antibiotics at 45.9% to 2.9% with 100% to erythromycin. All soil isolates were resistant against erythromycin (100%), while to other 9 antibiotics at 61.5% to 2.6%. Table 6 shows the antibiotic resistance profiles of *E. coli* isolates.

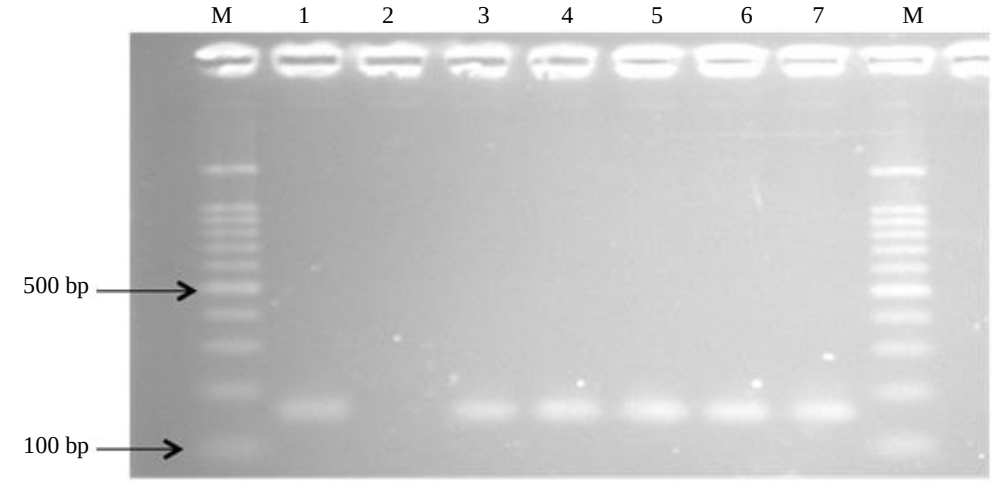

Fig. 1: PCR amplification of representative *C. jejuni* isolated from chickens and flies; Lane M: marker 100 bp ladder, Lane 1: *C. jejuni* ATCC 29428 as positive control, Lane 2: Negative control, Lane 3 to 7: *C. jejuni* isolates

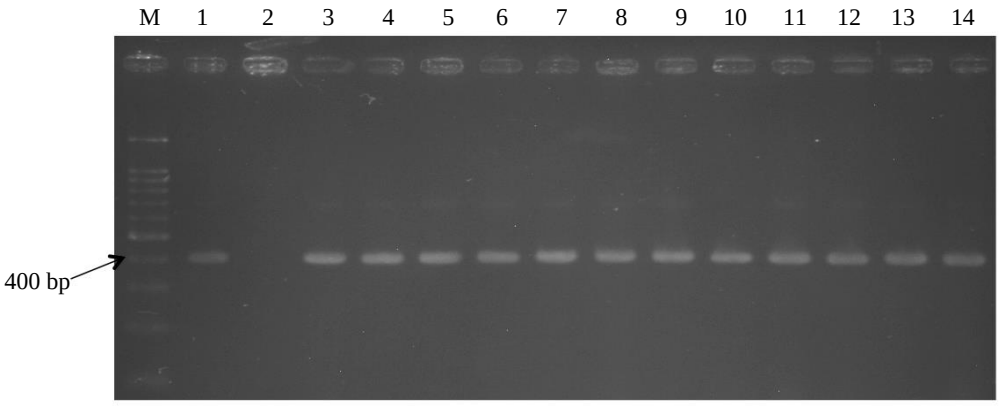

Fig. 2: PCR amplification of representative *E. coli* isolates; Lane M: Marker 100 bp ladder, Lane 1: *E. coli* ATCC 25922 as positive control, Lane 2: negative control, Lane 3 to 14: *E. coli* isolates

Table 4: *C. jejuni* and *E. coli* positive sources in the villages

Location	Human No./+ (%)	Chicken No./+ (%)	Wild bird No./+ (%)	Fly No./+ (%)	Water No./+ (%)	Soil No./+ (%)
			C. jejuni			
A	22/0 (0)	10/3 (30)	9 (0)	15/0 (0)	10/0 (0)	10/0 (0)
B	20/0 (0)	12/0 (0)	21 (0)	10/0 (0)	10/0 (0)	10/0 (0)
C	10/0 (0)	20/13(65)	10 (0)	15/2 (13.3)	20/0 (0)	20/0 (0)
Total	52/0 (0)	42/16(38.1)	40/0 (0)	40/2 (5)	40/0 (0)	40/0 (0)
			E. coli			
A	22/22 (100)	10/10 (100)	9 (100)	15/15 (100)	10/7 (70)	10/10 (100)
B	20/18 (90)	12/10 (83.3)	21 (100)	10/7 (70)	10/9 (90)	10/10 (100)
C	10/10 (100)	20/20 (100)	10 (100)	15/15 (100)	20/19 (95)	20/19 (95)
Total	52/50 (96.2)	42/40 (95.2)	40 (100)	40/37 (92.5)	40/35 (87.5)	40/39 (97.5)

Table 5: Antibiotic resistance of *C. jejuni* isolated from chicken and flies

Antibiotics	Chickes n = 16	Percentage of antibiotic resistance	Flies n = 2	Percentage of antibiotic resistance
Ampicillin- sulfbactam (Sam)	2	14.3%	1	50%
Tetracycline (Te)	3	21.4%	1	50%
Gentamicin (Cn)	0	0%	0	0%
Erythromycin (E)	1	7.1%	0	0%

Table 5: Continue

Ciprofloxacin (Cip)	2	14.3%	1	50%
Nalidixic Acid (Na)	7	50%	2	100%
Enrofloxacin (Enr)	4	28.8%	1	50%
Sulfamethoxazole Trimethoprim (Sxt)	1	7.1%	1	50%
Cefpodoxime (Cpd)	4	28.8%	1	50%
Streptomycin (S)	2	14.3	0	0%

Table 6: Antibiotic resistance of *E. coli* isolates

Antibiotics	Human n = 50	Percentage of antibiotic resistance	Chickens n = 40	Percentage of antibiotic resistance	Wild birds n = 40	Percentage of antibiotic resistance	Flies n =37	Percentage of antibiotic resistance	Water n = 35	Percentage of antibiotic resistance	Soil n =39	Percentage of antibiotic resistance
Ampicillin-sulfbactam (Sam)	6	12%	4	10%	18	45%	7	18.9%	4	11.4%	10	25.6%
Tetracycline (Te)	21	42%	27	67.5%	40	100%	22	59.5%	16	45.7%	24	61.5%
Gentamicin (Cn)	3	6%	1	2.1%	1	2.3%	1	2.7%	0	0%	1	2.6%
Erythromycin (E)	50	100%	40	100%	40	100%	36	97.3%	35	100%	39	100%
Ciprofloxacin (Cip)	3	6%	2	5%	7	17.5%	3	8.1%	0	0%	2	5.1%
Nalidixic Acid (Na)	13	26%	8	20%	14	35%	8	21.6%	3	8.6%	11	28.2%
Enrofloxacin (Enr)	6	12%	6	15%	27	67.5%	13	35.3%	6	17.1%	6	15.4%
Sulfamethoxazole Trimethoprim (Sxt)	11	22%	12	30%	30	75%	11	29.7%	5	14.3%	10	25.6%
Cefpodoxime (Cpd)	3	6%	9	22.5%	4	10%	9	18.9%	1	2.9%	7	17.9%
Streptomycin (S)	10	20%	12	30%	31	77.5%	13	35.1%	5	14.3%	22	56.4%

Discussion

Occurrence of C. jejuni

In this study, *C. jejuni* were isolated from chickens and flies and not from other sources. The occurrence of *C. jejuni* in the chicken was 38.1%, while it was 5% in the flies. The results were similar to those found in other studies such as by Nwankwo *et al*. (2017) and Osbjer *et al*. (2016). In chickens, the rates were higher as reported by Huat *et al*. (2010) and Keramas *et al*. (2004). This may be due to the poor hygiene and several sources of *C. jejuni* in the surroundings which included insects and wild animals. Additionally, many investigations suggested that the presence of *C. jejuni* in chickens could be because of the contaminated drinking water (Cokal *et al*., 2011), flies (Hald *et al*., 2004) or from wild birds (Choi *et al*., 2011).

The flies are regarded as one of the several sources of *C. jejuni* present in the chickens in the villages and may brought to human residences and eating-places. This because was several flies were noticed in and around the chicken houses and human residences as well as their kitchens. As per Royden *et al*. (2016), the threat of transmission through route of flies is likely to be high, especially at the time of summer when the populations of the flies are the highest.

The nonexistence of *C. jejuni* in human samples might be because the samples were attained from healthy humans. According to Huttenhower *et al*. (2012), infectious microbes including Salmonella enterica, Mycobacterium avium, Vibrio cholerae and *C. jejuni* were generally not found in the stool of healthy humans, while Helicobacter pylori were detected in only a couple of stool samples while *E. coli* was found high in the stool.

Furthermore, all wild birds were found to be negative for *C. jejuni* possibly because the bird species feeding habits including vegetables and insects. Nevertheless, a study carried out in Selangor, Malaysia on the Oriental Magpie Robin as well as other insect-eating birds were found negative for *C. jejuni* (Mohamed-Yousif *et al*., 2018); however, other bird species in their study were found positive for *C. jejuni* like Eurasian Tree Sparrow, Spotted Dove and Rock Pigeon. Moreover, Sensale *et al*. (2006) did not find *C. jejuni* in the insect-eating birds. Other research works performed on various bird species and their feeding habits, such as by Rahimi *et al*. (2011), isolated 16.7% *C. jejuni* from the pigeon. A research carried out in the UK by Colles *et al*. (2016) confirmed that of the 331 geese sampled, 50.2% were found to be positive for *C. jejuni*. In a study in Malaysia, Saleha *et al*. (2001) isolated 18.1% Campylobacter spp. from flying birds around the poultry farms. Besides that, Ganapathy *et al*. (2007) isolated, 57.3% Campylobacter spp. from crows. Elnohi *et al*. (2013) found 27.2% Campylobacter spp. in wild birds in the open surroundings. Therefore, possibly the types of bird species and their eating habits suggest the presence of *C. jejuni*. The nonappearance of *C. jejuni* in the samples from the environment (water and soil) could possibly be because of the unsuitable atmospheric condition and other surrounding pressures that would have caused them in viable but non culturable (VBNC) state (Magajna and Schraft, 2015). Several investigations have found low number of *C. jejuni* in the water

(Banting *et al*., 2016; Nilsson *et al*., 2017) as well as soil (Guévremont *et al*., 2017) is possibly because they are more possibly in the form of VBNC in water and soil. According to Magajna and Schraft (2015), after the humans or chickens pick the *C. jejuni* cells from the soil or water, they still continued to be viable and potentially infectious for a long time than they were before.

Escherichia coli is the most common commensal enteric bacteria in humans and animals, and the pathogenic ones are significant zoonotic pathogens and considered of public health concern (Costa *et al*., 2008). In the current study, prevalence of *E. coli* found in wild birds was high, up to 100%. Other investigations showed low occurrence of *E. coli* in birds. A research on wild birds conducted in France (Bonnedahl *et al*., 2009) stated a prevalence of 47.1% of *E. coli* from wild bird species Yellow-legged Gulls.

Different eating habits affect the occurrence of *E. coli* found in wild birds, as reported by some studies (Sharma *et al*., 2018; Vittecoq *et al*., 2017). From this current study, it was found that the birds displayed higher occurrence of *E. coli*. It maybe because these birds consumed items ranging from vegetation (Whitman *et al*., 2003) to human food and wastes (Waturu *et al*., 2017) that were possibly contaminated with *E. coli*. Additionally, these birds may have been infected by *E. coli* from the river water and water from other sources, as shown by some studies by Carter *et al*. (2009; Gomi *et al*., 2017) and McLellan (2004).

In the current research, the insect-eating birds (Oriental Magpie Robin, Little Spider Hunter and White-rumped Shama) are regarded as one of the sources of *E. coli* in the villages. Flies and beetles have been shown to be carrying *E. coli* (Choi *et al*., 2011; Jones *et al*., 2015; Puri-Giri *et al*., 2017).

The research confirmed that *E. coli* was highly prevalent (95.23%) in chickens as also shown by other research works. It was reported that the chickens were highly infected in Japan (100%), Netherlands (94%), Minnesota, U.S.A (89%) and a couple of states in Australia (90%) according to HCV New Drug Research (Leverstein-van *et al*., 2011) and Zhao *et al*. (2005), Vietnam (100%) and the North East region in India (98%) (Nzouankeu *et al*., 2010; Thangh Huong *et al*., 2009). It was reported that the rate of contamination was low in Nigeria, which was 11.1% (Adesiji *et al*., 2011) and Calabar Metropolis had a rate of 16% (Ukut *et al*., 2010), whereas in Cameron, it was found to be 11.3% (Nzouankeu *et al*., 2010). The rates of contamination were greater for the chickens as reported by researchers like Nzouankeu *et al*. (2010) and Zhao *et al*. (2005).

In this study, the high rates of *E. coli* in the chickens may be due to the poor hygiene in the chicken houses and because they release the chickens from morning to evening to eat from the open environment; thus, they might pick up the bacteria from the soil, water, and food as there are many sources of *E. coli* in the village environment. They may also shed the bacteria through feces, spreading to the environment and among animal species in the village.

In humans, the presence of *E. coli* was 96.2%. These *E. coli* are generally commensal, however some investigations demonstrated low amount of the infection (Kolenda *et al*., 2015; Nielsen *et al*., 2018) possibly due to presence of pathogenic *E. coli*, which may be acquired from contaminated food, water or environment.

The high presence of *E. coli* in the environment (92.5%) is possibly because of several *E. coli* sources in the surroundings like wild animals, flies and chickens; also the atmosphere is suitable for the bacteria to survive and propagate in high humidity (Guo *et al*., 2002) and low temperature (Choi *et al*., 2011).

Antibiotic Resistant C. jejuni and E. coli

The Campylobacter jejuni isolates from chickens were resistant to to 9 antibiotics (6.3-44%) and were susceptible to gentamicin. On the other hand, in flies, one of the two isolates was resistant to 6 antibiotics and susceptible to gentamicin, erythromycin and streptomycin. It may be due to the exposure of the chickens to resistant *C. jejuni*, present in the open environment, as well as to the wastes produced by humans or other animals in the village.

Adzitey *et al*. (2012) reported, *C. jejuni* from ducks in Penang demonstrated resistance to sulfamethoxazole-trimethoprim (96%), tetracycline (96%), nalidixic acid (84%) as well as ampicillin (81%). It is possible that the chickens in the villages were less exposed to antibiotics.

Commensal bacteria in the gut are reported to harbor antibiotic resistant genes while may be transferred to pathogenic bacteria (Martinez, 2009). It is believed that the high percentages of resistance are a useful indicator for the pressure selection such as brought about by the use of antibiotics. The inappropriate use of antibiotics should be stopped as antibiotics may entirely lose their effectiveness against pathogens. It is reported that *E. coli* can quickly acquire resistance to antibiotic faster than any other bacteria (Martinez, 2009). In this study, the resistance against antibiotics demonstrated by *E. coli* was quite high. This has been shown globally (Andersson and Hughes, 2010; Chai *et al*., 2007; Laxminarayan *et al*., 2013; Saleha, 2002; Sallam, 2007; Taremi *et al*., 2006). The *E. coli* found in wild birds demonstrated resistance against all the antibiotics tested. Almost all the isolates were resistant to erythromycin. It was similar to a study by Nhung *et al*. (2015), in which there were high resistance to ampicilin, sulfamethoxazole-trimethoprim and tetracycline. This high occurrence of resistant *E. coli* in wild birds is possibly because these birds were exposed to contaminated environment.

All isolates from chickens were resistant to erythromycin (100%). This high occurrence of erythromycin resistant *E. coli* could be due to these chickens were exposed to the wild birds, contaminated water and soil.

In this current study, the isolates from human were found resistant to erythromycin at 100%, similar to isolates from chickens and wild birds.

Almost all isolates from flies, soil and water isolates were also found to be resistant to erythromycin (97.3%, 100% and 100% respectively). It was similar to a report by Milanović *et al.* (2016), which showed high resistance to tetracycline and erythromycin. Also, quite a high percentage of *E. coli* isolates from all sources except wild birds in which all showed resistance to tetracycline.

Likewise, low percentage of resistance against aminoglycosides (gentamicin) could possibly be due to little use of the antibiotic in poultry or human treatment or because of its intramuscular route of dispensation, which may not be possible for wide-ranging applications (Rodrigo *et al*., 2007).

Conclusion

In the Orang Asli villages, *C. jejuni* were fount present in chickens and flies while they were not isolated from human, wild bird, water and soil. However, *E. coli* were isolated from human, wild bird, flies, water and soil. Most *C. jejuni* isolates were resistant to antibiotics except to gentamicin. The *E. coli* also showed resistance to all antibiotics and in particular almost all isolates were resistant to erythromycin and quite high to tetracycline. Thus, wild birds may play a role in the spread of resistant bacteria in the Orang Asli villages in Sungai Siput, Perak, Malaysia. The presence of multidrug resistance *C. jejuni* and *E. coli* in chickens and flies in the villages environment may pose hazard to human health upon exposure to these organisms. More epidemiology studies are needed to fully understand the role of environment in the epidemiology of antibiotic resistant *C. jejuni* and *E. coli* in villages in Malaysia.

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Author's Contributions

Ibrahim Mohamed Mohamed-Yousif, Abu Jalila and Saleha Abdul-Aziz: designed and carried out the experiment..

Zunita Zakaria, Abdul Rashid Khan and Elmutaz Atta Awad: Critically analysed the manuscript and provided guidance. Additionally, the authors have read and approved the final version of this manuscript.

Ethics

The study was approved for animals by Institution Animal Care and Use Committee (IACUC) (AUP No.: R089/2016), and for human by Ethics Committee for Research Involving Human Subjects (Ref No.: FPV(EXP16)P168).

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