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Antibiotic resistance and plasmid profile of *Escherichia coli* isolated from ducks in Penang, Malaysia

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Abstract

Fifty five (n=55) isolates of *Escherichia coli* isolated from ducks in Penang, Malaysia were examined for their susceptibility to eleven different antibiotics and assayed for the presence of plasmid DNAs. All the 55 *Escherichia coli* isolates were resistant (100%) to vancomycin. Higher resistance (≥ 60) occurred for tetracycline 51 (92.7%), ampicillin 40 (72.7%), streptomycin 37 (67.3%), and sulfamethoxazole-trimethophrim 37 (67.3%). No and low resistance was observed for nitrofurantoin (0%) and gentamicin (1.8%), respectively. The isolates also showed some intermediate resistances to all antibiotics examined except for vancomycin. The 55 *Escherichia coli* isolates exhibited 23 different antibiotic resistant patterns with MAR index ranging from 0.09-0.82. Majority of the *Escherichia coli* isolates exhibited resistant pattern of VA-C-OFX-SXT-TE-AMP-NA-KF and VA-S-C-OFX-SXT-TE-AMP-NA-KF with MAR index of 0.73 and 0.82, respectively. The smallest plasmid DNA size was 1.2 kb and the largest plasmid DNA size was 81.5 kb. 51 (93%) of the duck *Escherichia coli* isolates harbored plasmids. The was no direct correlation between plasmid DNA sizes and antibiotic resistant among the duck *Escherichia coli* isolates. Thus, the antibiotic resistant of the *Escherichia coli* isolates could mostly be mediated by chromosomes instead of plasmids. This study also suggests that the use of antibiotics in duck farming in Penang, Malaysia needs to be controlled to prevent the spread of multiple antibiotic resistant *Escherichia coli* isolates.

Introduction

Escherichia coli are Gram negative rod shape bacteria and part of the *Enterobacteraceae* family (Neill *et al*., 1994; Feng *et al*., 2002). They are mostly found in the gastrointestinal tract of humans and warm blooded animals where they live as part of the normal microflora (Teophilo *et al*., 2002). Different groups of *Escherichia coli* are known to cause a variety of human infections including urinary infection, severe abdominal pain, hemorrhagic colitis, and haemolytic-uremic syndrome which can be fatal (Neill *et al*., 1994; Teophilo *et al*., 2002; Feng *et al.*, 2002). In the United States, it has been reported that about 265,000 illnesses and 100 deaths are caused by *Escherichia coli* infections each year (North Carolina Public Health, 2012). About 40% of these infections are caused by *Escherichia coli* O157:H7, a strain that is part of the shiga toxin-producing group of *Escherichia coli* bacteria (STEC) and the remaining 60% cases are caused by non-0157:H7 shiga toxin-producing *Escherichia coli* (North Carolina Public Health, 2012).

Escherichia coli have been reported to be resistant to a number of antibiotics such as tetracycline, naidixic acids, cefetoxin, chloramphenical, gentamicin, ampicillin, kanamycin, sulfamethoxazole-trimethophrim, etc (Alhaji *et al*., 2007; Lim *et al*., 2009; Sukhumungoon *et al*., 2011; Adzitey, 2011a). *Escherichia coli* and other foodborne pathogens also continue to show increase resistant to different antibiotics. This has been attributed to the widespread and misuse of antibiotics in the treatment of humans or animals, and as growth promoters in feed (Towner, 2000; Ahmadi *et al*., 2007). As such some countries have regulations that ban, limit or control the use of antibiotics in food production. It is also reported that antibiotic resistant can be chromosomal or plasmid mediated (Ahmadi *et al.*, 2007; Adzitey *et al*., 2012a). Plasmids are extra-chromosomal genetic element which can replicate independently of bacterial chromosome and play an important role in the spread of antibiotics resistant genes (Towner, 2000; Ahmadi *et al*., 2007). Dharmalingam *et al.* (2003) indicated that many pathogenic bacteria can survive antibiotic treatment due to the existence of antibiotic resistant

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encoding genes in plasmid.

Duck meats and eggs like other poultry species are important dietary sources with high quality protein, energy, and some vitamins and minerals (Adzitey, 2011b). The Department of Veterinary Services Malaysia (2009) reported that duck population increased around 154% between 1996 to 2004 in Malaysia. Currently, Malaysia is also the third world producer of duck meats (FAO, 2009). This presents opportunities for increase duck production and consumption in Malaysia. The consumption of duck meat, eggs or products contaminated with pathogenic *Escherichia coli* can lead to foodborne infection and if the pathogenic *Escherichia coli* involved in the infection is resistant to multiple antibiotics, treatment of infected persons can be difficult and fatal. The objective of this study was to determine the antibiotic resistance and plasmid sizes of *Escherichia coli* isolated from ducks in Penang, Malaysia.

Material and Methods

Bacterial strains

A total of 55 *Escherichia coli* isolated from ducks in a wet market and two farms in Penang, Malaysia were used for this study (Adzitey *et al*., 2012b). They were isolated from duck faeces (n=18), intestines (n=14), soil (n=13), and wash water (n=10). *Escherichia coli* isolates were stored at -18°C in Tryptic Soy Broth (TSB) containing 15% glycerol and beads. They were recovered by enrichment in EC broth incubated at 37°C for 24 h, followed by streaking onto Levine Eosin Methylene Blue (L-EMB) agar and incubated at 37°C overnight. Single colonies showing metallic sheen were purified on Tryptic Soy Agar (TSA) incubated at 37°C for 24 h and confirmed using recommended biochemical tests. The biochemical tests carried out were Indole, Methyl Red, Voges-Proskauer, Simmons citrate and MacConkey Sorbitol tests according to Feng *et al.* (2002). All media used was purchased from Merck (Darmstadt, Germany).

Antibiotic susceptibility test

The disk diffusion method of Bauer *et al*. (1966) was used to determine the antibiotic resistance of 55 *Escherichia coli* against the following 11 antimicrobial agents: vancomycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), chloramphenical (30 µg), ofloxacin (5 µg), sulfamethoxazole-trimethoprim (1.25-23.75 µg), tetracycline (30 µg), ampicillin (10 µg), nalidixic acid (30 µg), nitrofurantoin (30 µg), and cephalothin (30 µg) purchased from Oxoid (Basingstoke, UK). Pure cultures of *Escherichia coli* were grown overnight in TSB at 37°C and the concentration adjusted using sterile TSB until a 0.5 McFarland turbidity was attained. One hundred µl of the culture suspension was spread plated onto Mueller Hinton Agar (Basingstoke, Oxoid, UK) and three or four antimicrobial disks were placed on the surface of the agar plate. The plates were incubated at 37°C for 16 to 18 h and the results were interpreted as sensitive, intermediate, or resistance according to Clinical and Laboratory Standards Institute Guidelines (CLSI, 2006).

The multiple antibiotic resistance (MAR) index of each strain was calculated and interpreted according to the method described by Krumperman (1983) using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolates was resistant and 'b' the total number of antibiotics tested.

Determination of plasmid size(s)

Single colony of pure *Escherichia coli* was inoculated into 5 ml Luria-Bertani (Merck, Darmstadt, Germany) and incubated in orbital shaker with vigorous shaking (250 rpm) at 37°C for 16 to 18 h. The cell density was adjusted between 1.6 to 1.9 using spectrophotometer at 600 nm. The overnight culture (1.5 ml) was centrifuged for 5 min at 1000 x g to obtain pellets. Pellets were dried and subjected to plasmid DNA extraction and purification using Promega Wizard® Plus Minipreps DNA Purification System by following the manufacturer's instructions available at *http://www.promega.com/tbs/tb225/tb225*. Purified plasmids extracted were temporarily stored at -20°C for further analysis.

Escherichia coli plasmid DNAs were loaded on 0.7% agarose gel, separated using horizontal gel electrophoresis system (ELITE 300, USA) and stained with ethidium bromide. Plasmid DNA bands were visualized using UV transilluminator (UV TEC Gel Imaging System, UK). Lambda DNA/HindIII marker was used as the molecular weight marker and plasmid size was determined using UVI TEC UVIBand.

Results and Discussions

The percentage antibiotic resistant of the 55 *Escherichia coli* isolates isolated from duck intestines, faeces, wash water and soil is shown in Table 1. From Table 1, all *Escherichia coli* isolates were resistant to vancomycin. A high proportion of the isolates were also resistant to tetracycline 51 (92.7%), ampicillin 40 (72.7%), streptomycin 37 (67.3%), sulfamethoxazole-trimethoprim 37 (67.3%), chloramphenicol 31 (56.4%), cephalothin 30

Table 1. Percentage antibiotic resistant isolates of duck *Escherichia coli* isolates

Antibiotics	*Escherichia coli*		
	[a]No. (%) R[a]	No. (%) I[b]	No. (%) S[c]
Vancomycin	55(100)	0(0)	0(0)
Gentamicin	1(1.8)	25(45.5)	29(52.7)
Streptomycin	37(67.3)	14(25.5)	4(7.3)
Chloramphenicol	31(56.4)	4(7.3)	20(36.4)
Ofloxacin	23(41.8)	3(5.5)	29(52.7)
Sulfamethoxazole-trimethoprim	37(67.3)	10(18.2)	8(14.6)
Tetracycline	51(92.7)	4(7.3)	0(0)
Ampicillin	40(72.7)	9(16.4)	6(10.9)
Nalidixic acid	29(52.7)	12(21.8)	14(25.5)
Nitrofurantoin	0(0)	21(38.2)	34(61.8)
Cephalothin	30(54.6)	20(36.4)	5(9.1)

[a]No= number of isolates; R[a]= resistant; I[b]= intermediate resistance; S[c]= susceptible.

(54.6%) and nalidixic acid 29 (52.7%). Furthermore, the *Escherichia coli* isolates exhibited some level of intermediate resistances to all the antibiotics examined except vancomycin. Higher susceptibility ≥ 50% occurred for gentamicin (52.7%), ofloxacin (52.7%) and nitrofurantoin (61.8%).

Our result is comparable to that of other researchers. The high susceptibility of *Escherichia coli* isolates to gentamicin was consistent with reports by Adzitey *et al.* (2012a) and Adzitey *et al.* (2012c) who reported higher susceptibility of *Salmonella* and *Campylobacter* species isolated from ducks in Penang to gentamicin. A review by Adzitey *et al*. (2012c) on antibiotic resistant of duck *Campylobacter*, *Salmonella* and *L. monocytogenes* isolates also revealed higher susceptibility to gentamicin. Nonetheless, antibiotic resistances to tetracycline, streptomycin, chloramphenicol, sulfamethoxazole-trimethoprim, nalidixic acid, cephalothin and so on were either similar to or differ from this present study depending on the number and type of bacteria, species and/or serovar involved (Adzitey *et al*., 2012a; Adzitey *et al*., 2012c; Adzitey *et al*., 2012d). Zinnah *et al*. (2008) reported that 10 *Escherichia coli* isolated from duck cloacal swabs were resistant to ampicillin (90%), erythromycin (90%), nalidixic acid (90%), and tetracycline (70%). *Escherichia coli* isolated from human and environmental samples were resistant to tetracycline (81.4%), chloramphenicol (75.7%), gentamicin, (74.3%), ampicillin (72.9%), nalidixic acid (68.6%) and sulfamethoxazole-trimethoprim (62.9%) (Alhaji *et al*., 2007). Lim *et al.* (2009) observed that 47 *Escherichia coli* isolated from various public hospitals in Malaysia were resistant to ampicillin (77%), tetracycline (53%), nalidixic acid (28%), chloramphenicol (26%), and gentamicin (21%). Differences in production systems, sampling area, type and number of samples examined/analyzed account for the differences in the percentage antibiotic resistances observed by different authors.

The antimicrobial resistance profile and MAR index of the *Escherichia coli* isolates are presented in Table 2. All the *Escherichia coli* isolates except two were resistant to at least one antibiotic. The 55 *Escherichia coli* isolates also exhibited 23 different antibiogram patterns. The resistant pattern VA-S-C-OFX-SXT-TE-AMP-NA-KF was shown by seven *Escherichia coli* isolates and these isolates had the highest MAR index of 0.82. Of all the resistant patterns, VA-C-OFX-SXT-TE-AMP-NA-KF was the most common pattern and was exhibited by 9 *Escherichia coli* isolates. Resistant to 8 different antibiotics was also the most common and was exhibited by 13 *Escherichia coli* isolates. In general, 85% of the *Escherichia coli* isolates were resistant to ≥4 number of antibiotics with MAR index ranging from 0.36 to 0.82. Resistant of *Escherichia coli* or duck bacteria isolates to four or more antibiotics have been reported by other researchers (Alhaji *et al*., 2007; Lim *et al*., 2009; Sukhumungoon *et al*., 2011; Adzitey *et al*., 2012a; Adzitey *et al.*, 2012c; Adzitey *et al*., 2012d) and high MAR index suggests that the bacteria originated from sources with high exposure to the use of antibiotics (Krumperman, 1983).

Table 2. Antibiotic resistance pattern and multiple antibiotic resistance index of duck *E. coli* isolates

Antibiotic resistant pattern	MAR index	Number of *Escherichia coli* isolates
VA	0.09	2
VA-S-TE	0.27	4
VA-SXT-TE	0.27	1
VA-TE-NA	0.27	1
VA-S-SXT-TE	0.36	1
VA-S-TE-AMP	0.36	3
VA-S-TE-NA	0.36	2
VA-S-TE-KF	0.36	3
VA-S-C-SXT-AMP	0.46	1
VA-S-SXT-TE-AMP	0.46	1
VA-S-SXT-TE-NA	0.46	1
VA-S-TE-AMP-KF	0.46	2
VA-C-SXT-TE-AMP	0.46	1
VA-S-C-SXT-TE-AMP	0.55	3
VA-S-SXT-TE-AMP-KF	0.55	1
VA-S-C-SXT-TE-AMP-KF	0.64	3
VA-S-SXT-TE-AMP-NA-KF	0.64	2
VA-C-OFX-SXT-TE-AMP-NA	0.64	3
VA-CN-C-OFX-SXT-AMP-NA-KF	0.73	1
VA-S-C-OFX-SXT-TE-AMP-NA	0.73	2
VA-S-C-OFX-TE-AMP-NA-KF	0.73	1
VA-C-OFX-SXT-TE-AMP-NA-KF	0.73	9
VA-S-C-OFX-SXT-TE-AMP-NA-KF	0.82	7

VA= Vancomycin; CN= Gentamicin; S= Streptomycin; C= Chloramphenicol; OFX= Ofloxacin; SXT= Sulfamethoxazole-trimethoprim; TE= Tetracycline; AMP= Ampicillin; NA= Nalidixic Acid; F= Nitrofurantoin; KF= Cephalothin.

The use of antibiotics in human disease and animal treatment is responsible for the spread of antibiotics resistant genes among bacterial population (Towner, 2000). Besides that, the exposure of ducks to antibiotics during feeding will increase the antibiotic

Table 3. Plasmid DNA sizes and antibiotic resistant patterns of duck *Escherichia coli* isolates

Code of *Escherichia coli* isolates	Plasmid size (kb)							Antibiotic resistant pattern
E3	ND							VA
E6	55.5	41.7	21.4					VA-S-TE-KF
E8	3.0	2.5						VA-S-SXT-TE-AMP-NA-KF
E11	53.8							VA-S-TE-KF
E12	10.7	2.9						VA-S-SXT-TE-AMP-Na-KF
E15	48.6	21.6						VA-S-TE-KF
E17	75.4	20.8						VA
E19	ND							VA-S-SXT-TE-NA
E21	ND							VA-SXT-TE
E24	27.0	1.8	1.2					VA-S-TE-NA
E26	53.8	20.8	6.7					VA-S-TE
E28	35.9	22.6						VA-S-TE
E30	54.3	16.5	2.9	2.5	2.4			VA-S-TE
E31	34.5							VA-S-SXT-TE
E1R	79.1	28.1	3.4	2.5	2.4			VA-S-TE-AMP
E2R	3.9							VA-S-TE-AMP-KF
E3R	74.9	27.2						VA-S-TE-AMP-KF
E4R	80.5	21.3	4.1	2.4				VA-S-TE-AMP
E5R	76.5	20.1	3.9	2.4				VA-S-TE-AMP
E6R	77.0	36.8	7.5	4.1				VA-S-TE-NA
E7R	80.0	34.8	6.3	3.8				VA-TE-NA
E8R	81.5	58.2	36.3					VA-S-SXT-TE-AMP
E9R	57.9	40.3	19.0					VA-S-SXT-TE-AMP-KF
E10R	20.8	11.9	2.8					VA-S-TE
E1F	27.0	5.2	2.8	2.5	1.9			VA-S-C-SXT-AMP
E3F	58.4	38.8	20.2	5.4	2.1			VA-CN-C-OFX-SXT-AMP-NA-KF
E5F	56.9	40.3	16.2					VA-S-C-SXT-TE-AMP
E7F	53.8	42.3	12.3	7.3	3.9	3.1	1.8	VA-S-C-SXT-TE-AMP-KF
E8F	72.0	46.2	24.8					VA-S-C-SXT-TE-AMP
11F	72.5	47.3	27.5					VA-S-C-SXT-TE-AMP-KF
E15F	72.5	48.4						VA-S-C-SXT-TE-AMP-KF
E17F	74.1	58.8	24.8	14.4	7.3	4.0		VA-C-SXT-TE-AMP
E21F	58.6	41.6	18.6					VA-S-C-SXT-TE-AMP
E25F	3.0	2.4						VA-C-OFX-SXT-TE-AMP-Na-KF
E30F	ND							VA-S-C-OFX-SXT-TE-AMP-NA
E34F	20.9	2.9						VA-S-C-OFX-SXT-TE-AMP-NA-KF
E38F	16.0							VA-S-C-OFX-SXT-TE-AMP-NA
E1S	23.6	7.4	4.6	2.6				VA-C-OFX-SXT-TE-AMP-NA-KF
E2S	48.3	28.9	2.2					VA-C-OFX-SXT-TE-AMP-NA-KF
E3S	43.0	27.6	3.4	2.1				VA-S-C-OFX-TE-AMP-NA-KF
E5S	54.1	37.7	18.4					VA-S-C-OFX-SXT-TE-AMP-NA-KF
E6S	2.6							VA-S-C-OFX-SXT-TE-AMP-NA-KF
E7S	2.6							VA-C-OFX-SXT-TE-AMP-NA-KF
E8S	2.6							VA-C-OFX-SXT-TE-AMP-NA-KF
E9S	59.0	33.7	16.0					VA-S-C-OFX-SXT-TE-AMP-NA-KF
E41F	58.2	28.1	4.7					VA-S-C-OFX-SXT-TE-AMP-NA-KF
E44F	17.3							VA-S-C-OFX-SXT-TE-AMP-NA-KF
E47F	55.5	14.3						VA-C-OFX-SXT-TE-AMP-NA
E49F	46.7	28.1						VA-C-OFX-SXT-TE-AMP-NA
E51F	39.6	29.7	10.5	6.1	2.9			VA-C-OFX-SXT-TE-AMP-NA-KF
E10S	8.7	5.6	2.7					VA-C-OFX-SXT-TE-AMP-NA-KF
E12S	13.3							VA-C-OFX-SXT-TE-AMP-NA
E14S	2.7							VA-C-OFX-SXT-TE-AMP-NA-KF
E15S	24.8	8.8	2.8					VA-C-OFX-SXT-TE-AMP-NA-KF
E17S	62.6	45.1	20.5					VA-S-C-OFX-SXT-TE-AMP-NA-KF

VA= Vancomycin; CN= Gentamicin; S= Streptomycin; C= Chloramphenicol; OFX= Ofloxacin; SXT= Sulfamethoxazole-trimethoprim; TE= Tetracycline; AMP= Ampicillin; NA= Nalidixic Acid; F= Nitrofurantoin; KF= Cephalothin.

resistant potential of bacterial foodborne pathogens isolated from such sources. Pathogenic bacteria are also increasingly becoming resistant to antibiotics as a result of their ability to undergo genetic mutations which occur either in the deoxyribonucleic acid (DNA) of the bacteria chromosomes or plasmids (Ekwenye and Kazi, 2007; Adzitey *et al*., 2012e). Antibiotic resistance can diffuse and be transferred from one bacterium to the other during plasmid conjugation (Karmaker *et al*., 1991).

In this study, plasmid DNAs were detected in 93% (51/55) of the *Escherichia coli* isolates (Table 3). Thus 4 isolates or 7% of the *Escherichia coli* did not harbour plasmid DNAs. Furthermore, 17 *Escherichia coli* isolates (31%) harboured 3 plasmid DNAs, 11 isolates (20%) harboured 2 plasmid DNAs, 10 isolates (18%) harboured 1 plasmid DNA, 6 isolates (11%) harboured 4 plasmid DNAs, 5 isolates (9%) harboured 5 plasmid DNAs, 1 isolate (2%) each haarboured 6 and 7 plasmid DNAs. Plasmid DNA sizes vary among the *Escherichia coli* isolates. The largest plasmid DNA size was 81.5 kb and was detected in only one *Escherichia coli* isolate. Thirty three (33) *Escherichia coli* isolates harboured one or more plasmid DNAs that were >23.13 kb. Plasmid DNAs of the smallest size <2 kb were present in two isolates. There was no direct relationship between plasmid DNA sizes and antibiotic resistance patterns (Table 3). For instance, *Escherichia coli* (E3 and E17) with or without plasmid DNA(s) were resistant to only VA. Two *Escherichia coli* isolates (E2R and E3R) with the same resistant pattern (VA-S-TE-AMP-KF) had different plasmid DNA sizes. *Escherichia coli* isolates (E5S, E6S, E9S, E41F, and E44F) also exhibited the same resistant pattern but had different plasmid DNA sizes. Furthermore, *Escherichia coli* without plasmid DNA exhibited different resistant pattern of VA, VA-SXT-TE and VA-S-SXT-TE-NA. Certain plasmid sizes containing certain genes may be responsible for resistance to particular antibiotics (Adzitey *et al*., 2012a) and plasmid profile can be important for investigation of infectious disease and determination the source of bacteria infection (Olukoya and Oni, 1990).

Smith *et al.* (2003) showed that all *Escherichia coli* 0157:H7 isolated from healthy animals had plasmids of sizes ranging from 0.56 kb to >23.13 kb. They also stated that bacteria with plasmids of sizes >23.13 kb are considered as toxigenic strains. Bopp *et al*. (2003) also stated that bacteria having plasmid with molecular weight of 90 kb or larger is considered as toxigenic strains. Since some of the duck *Escherichia coli* isolates in this study had plasmids of sizes >23.13 kb, it can be suggested that some of the duck *Escherichia coli* isolates can be pathogenic. However, further work to identify the presence of

virulence genes is highly recommended to draw such a conclusion. Furthermore, plasmids are unstable and can easily change or be lost due to processes like mutation, conjugation, transformation and transduction for the exchange of genetic information making it difficult to reliable depend on plasmids for epidemiological studies. These characteristics also contribute to the ability of plasmids to transfer antibiotic resistant genes from one bacterium to another.

Conclusion

Escherichia coli isolates isolated from ducks exhibited high resistant to vancomycin, tetracycline, ampicillin, streptomycin and sulfamethoxazole-trimethoprim. While resistant to nitrofurantoin and gentamicin was not found or very low. With the exception of vancomycin, *Escherichia coli* isolates exhibited intermediate resistances to all the antibiotics, suggesting that more isolates can become resistant in the near future. Twenty three different antibiotic resistance patterns and multiple antibiotic resistant index of 0.09-0.82 were displayed by the 55 *Escherichia coli* isolates. Resistant to eight different antibiotics (13 isolates) was the commonest, followed by resistant to four antibiotics (9 isolates) and resistant to seven antibiotics (8 isolates). The smallest plasmid DNA size was 1.2 kb, while the largest plasmid DNA size was 81.5 kb. Plasmid DNAs were present in 93% of the duck *Escherichia coli* isolates. Plasmid sizes and antibiotic resistance patterns in this study suggested that the ability of *Escherichia coli* isolates to be resistant to antibiotics was mostly chromosomal mediated instead of plasmids. The use of antibiotics in the duck industry in Malaysia should be controlled to prevent the occurrence of multidrug resistant *Escherichia coli* isolates.

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