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ANTIBIOTIC RESISTANCE OF ESCHERICHIA COLI ISOLATED FROM CHICKEN IN MALAYSIA

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ABSTRACT. Colibacillosis is an important disease affecting the poultry industry in many countries, caused by the Avian Pathogenic *E. coli* (APEC): it manifests as various clinical signs. It contributes significantly to economic loss for poultry farmers as a result of high mortality and morbidity in poultry. To overcome this, antibiotics have been widely used to eliminate *E. coli* infection in poultry farms in recent years. Treatment with antibiotics has been considered as a vital regimen to control *E. coli* infection at the farm level for many years. However, high frequency of antibiotic resistance of *E. coli* isolates from chicken has become the centre of attention due to public health importance. The aim of the present study is to determine the multidrug resistant profiles of *E. coli* strains isolated from chicken. *E. coli* isolates obtained from clinical cases were re-identified and classified by conventional methods. Multidrug resistant profiles against 13 different antibiotics of 125 *E. coli* isolates were determined by using disk diffusion method according

to Clinical Laboratory Standard Institute (CLSI). Antibiogram revealed that 81.6% of the *E. coli* isolates showed multidrug resistant profiles to different antibiotics. Most of the *E. coli* isolates were highly resistant to erythromycin (52.8%), followed with tetracycline (52.0%), spectinomycin (39.2%), trimethoprim (38.4%) and flumequin (37.6%). Out of 125 isolates tested, 19.2% were resistant to more than eight antibiotics, with one isolates found to be multidrug resistant to most of antibiotics except polymyxin B. These findings also demonstrated that most of the isolates were susceptible to antibiotics commonly used for *E. coli* infections treatment in poultry with lowest resistant score against polymyxin B (92.8%) and colistin (92.0%). Moderate resistant profiles were observed towards amoxycilin (25.6%), apramycin (16%), kanamycin (8.8%) and streptomycin (8.0%). High percentage of multidrug resistance was found among the *E. coli* isolated from chicken as an indicator to more serious problems in animal health. Therefore, continuous surveillance of

antibiotic resistance profiles in chicken and other food animals is crucial to ensure food chain safety.

Keywords: *E. coli*, antibiotic resistant, antibiotic, chicken

INTRODUCTION

E. coli is one of the most important aetiological agents causing diseases in poultry which leads to significant economic losses related to high mortality, poor weight gain of infected chicken and poor carcass quality (Ewers *et al.*, 2004). The *E. coli* infection in poultry is usually considered as a secondary infection, which is triggered by various predisposing factors particularly environmental factors including poor ventilation, overcrowding and other biological predisposing factors such as viral or parasitic infections (Vandekerchove *et al.*, 2004). Appropriate farm management approaches have been suggested to overcome *E. coli* problems in poultry farm but several studies reveal that management approaches are not applicable to prevent incidence of *E. coli* infection in the farms. Thus, antibiotics have been introduced into animal populations mainly for disease treatment for many years, just after the first antibiotic, tetracycline, were introduced to human health. Antibiotics also have been used to control disease outbreaks including *E. coli* infection, thus reducing morbidity and mortality rates due to the infections. Even though antibiotics are highly recommended for therapeutic purpose, certain class of antibiotics were

also used for subtherapeutic reasons to prevent occurrence of disease outbreaks in farms. Emergence of multidrug resistant *E. coli* in food animals including chicken arise due to the improper use of antibiotics, thereby reducing the clinical efficacy to antibiotics commonly used in human and veterinary medicine (Wang *et al.*, 2013). Most of the time, antibiotics have been administered to the healthy animals, instead of sick animals. This is because it is easier to treat whole flock of animals rather than individually, and sometimes usage of antibiotics did not follow the prescribed doses (Van Der Bogaard *et al.*, 2002). Unnecessary introduction of antibiotics to healthy animals induced those commensal bacteria strains to develop resistance against commonly used antibiotics. Usually, antibiotic resistant profiles were observed after a huge introduction of related antibiotics for therapeutic use in the human medicine and veterinary field. For example, resistance to tetracycline was observed after tetracycline was introduced in human medicine. However, later in the mid-1950s it was delivered in animal feed in order to enhance weight ratio in poultry. In Malaysia, multidrug resistant profiles of *E. coli* isolated from chicken against various antibiotics have been reported which involved chicken samples from poultry farms and diagnostic samples in veterinary laboratory which were limited to certain geographical locations (Geidam *et al.*, 2012; Khoo *et al.*, 2013). Concrete details on antibiotic resistant profiles of *E. coli* isolated from chicken in the country

is useful for better implementation of antibiotics usage for effective control of disease caused by pathogenic *E. coli* in poultry. Therefore, the aim of the present study is to determine the multidrug resistant profiles of *E. coli* strains isolated from chicken in Malaysia.

MATERIAL AND METHODS

Preparation of *E. coli* cultures

A total of 125 *E. coli* isolates were used in this study. The collection of *E. coli* stock cultures at the Veterinary Research Institute gene bank was used in this study with prior approval from the Department of Veterinary Services. All the isolates were selected from cases submitted for diagnostic purposes for *E. coli* infection, in broiler and village chicken with findings of profuse growth on agar medium. Visceral organ samples were inoculated onto 5% blood agar and MacConkey agar for selective isolation of Gram-negative bacteria. All the plates were incubated at 37°C for 18 to 24 hours. Well isolated presumptive *E. coli* colonies were subcultured on blood agar to obtain pure growth for further identification. Presumptive *E. coli* colonies were greyish in colour, small to medium sizes on blood agar plates, while they ferment lactose on MacConkey agar by producing bright pink colonies. The presumptive colonies were confirmed by conventional biochemical tests including Triple Sugar Iron (TSI), Indole, Methyl red, Simmons citrate,

motility, urease and decarboxylase tests such as ornithine, lysine, arginine and malonate broth according to standard protocols (Quinn *et al.*, 1994). The isolates were further classified into serogroups by using classical serological slide agglutination method according to Kauffmann-White scheme against commercially available specific antisera. The isolates were kept in maintenance medium until further tests were conducted.

Antibiotic sensitivity tests

The disk diffusion method was used to determine the susceptibility of *E. coli* isolates to several antibiotics of veterinary significance on Mueller Hinton agar. Thirteen different antibiotics which are typical for treatment of *E. coli* infections were selected based on the OIE List of Antimicrobial Agents of Veterinary Importance, 2014. The antibiotics with the following concentrations were used in this study: erythromycin (15 µg), tetracycline (30 µg), kanamycin (30 µg), colistin (10 µg), ceftiofur (30 µg), polymyxin B (300 µg), spectinomycin (25 µg), amoxicillin (30 µg), gentamicin (10 µg), flumequin (30 µg), trimethoprim (5 µg), streptomycin (10 µg) and apramycin (15 µg). Zones of inhibition were measured to the nearest millimeter using a ruler and reported either as sensitive (S), intermediate resistant (I) or resistant (R) on the basis set by the Clinical Standard Laboratory Institute (CLSI), 2012.

RESULTS

Biochemical profiles of *E. coli* isolated from chicken

In this experiment, several biochemical profiles showed reliable characteristics among *E. coli* isolates, with most of the isolates (>90%) showing similar expected *E. coli* reactions in biochemical profiles including citrate, lysine, urease, triple sugar iron (TSI) and methyl red. All 125 *E. coli* isolates tested (100%) were able to ferment lactose and were negative for malonate test, and these findings are similar to expected *E. coli* profiles. However, two isolates were negative for indole test, and one isolate was positive for citrate test; invariably these are different from expected *E. coli* reactions. The number of isolates with negative methyl red reaction was higher, with 16/125 (12.8%) isolates showing contradictory reactions with *E. coli* isolates. Generally, *E. coli* isolates showed Triple Sugar Iron (TSI) reaction to both acid butt and slant, with production of gas but no hydrogen sulphides. However, one isolate (0.83%) was found to show acid butt and slant reaction with no gas production observed. Most of the *E. coli* strains isolated from chicken usually showed variety in motility, arginine and ornithine reaction, with a small number of isolates showing differences in lysine reaction. Consequently, as reported in previous studies, contradictory reactions of biochemical properties by certain *E. coli* strains may occur (Bettelheim, 1994).

According to the results obtained, a total of 63/125 isolates (50.4%) were found to show contradictory reaction of *E. coli* in arginine, ornithine (60.8%) and motility (50.4%). Only 2/125 (1.6%) isolates produced hemolysis on blood agar, each one producing beta and alpha hemolysis reaction. The *E. coli* usually does not show hemolysis, and the colony is usually greyish white in colour, opaque, slightly moist or mucoid appearance with entire edges (Quinn *et al.*, 2004). Out of 125 *E. coli* isolates tested, only 24 isolates (19.2%) gave similar reactions compared with the reference strain *Escherichia coli* ATCC 25922 which was used as control in this experiment. Other isolates were found to show different profiles from reference strain mentioned above. Table 1 shows different biochemical profile results of *E. coli* isolates tested in this experiment.

Identification of *E. coli* isolated from chicken serogroups

The highest number of *E. coli* isolates identified in this study as non-typeable *E. coli* (ECU), was 92 out of 125 (72%) isolates which could not be assigned to any serogroups tested. Several studies previously revealed different findings on prevalence of *E. coli* serogroups identified, with some studies reporting a low prevalence of *E. coli* non-typeable strain. However, findings from the present study correlate with other studies showing high prevalence of non-typeable strain as shown by Ewers *et al.*, (2007), whereby 50.4% of

Table 1. Biochemical profiles of *E.coli* isolated from chicken

Biochemical test	<i>E.coli</i> reactions	No. of isolates showed positive results	Percentage (%)
Indole	+	118/125	94.4%
Methyl red	+	109/125	87.2%
Triple Sugar Iron	A/A with gas	119/125	95.2%
Motility	V	63/125	50.4%
Citrate	-	119/125	95.2%
Urease	-	119/125	95.2%
Arginine	V	63/125	50.4%
Ornithine	V	76/125	60.8%
Lysine	+	113/125	90.4%
Malonate broth	--	125/125	100%
Hemolysis on Blood agar	V	2/125	0.016%

Table 2. *E. coli* isolates and serogroups identified from local village and broiler chicken from 2010- 2015

Serogroups	Number of isolates
<i>E.coli</i> Untypeable (ECU)	92
<i>E.coli</i> O1:K1	17
<i>E.coli</i> O2:K1	3
<i>E.coli</i> O78:K8O	13
Total	125

E. coli isolates tested were assigned to non-typeable serogroups. However, the results may vary depending on the study, due to the differences of antisera used in the study and geographical locations, meaning that different countries may show some variability in results. It was observed from various reports that the trend of *E. coli* non-typeable prevalence was found to increase steadily from the year 2010 in Malaysia (Maswati *et al.*, 2011). Most of the *E. coli*

isolated from year 2010 was not able to be assigned to any serogroup compared to the previous years, therefore the prevalence of non-typeable isolates showed an increase since 2010, with only a small percentage of the isolates being able to be identified according to their serogroups by available antisera. However, the most predominant *E. coli* serogroups identified in this study were O1 (14%), followed by O78:K8O (11%) and O2 (3%). Table 2 shows the

summary of serogroups identified from *E. coli* originating from chicken, isolated from diagnostic cases received in VRI from the year 2004 until 2015 used in this study.

A high percentage of multidrug resistant profiles, that is 81.6% against different types of antibiotics tested was observed among *E. coli* isolated from chicken in this study. Out of 125 *E. coli* isolates tested, 102 isolates exhibited multiple antibiotic resistant profiles, while the remaining 23 (18.4%) isolates were found to be resistant against less than four types of antibiotics. Most of the isolates were highly resistant to erythromycin (52.8%), tetracycline (52.0%), spectinomycin

(39.2%), trimethorpin (38.4%) and flumequin (37.6%). The majority of isolates were resistant to at least one antibiotic (99.2%), while only one isolate was found to be susceptible to all antibiotics tested. In this study, a total of 24 (19.2%) isolates were resistant to more than eight types of antibiotics, while only one isolate was found to be resistant to all antibiotics used in this study. High frequencies of isolates were susceptible to polymyxin B (92.8%) and colistin (92.0%). However, ten isolates (8.0%) showed moderate sensitivity towards colistin, with none of the isolates being resistant to this particular antibiotic. Most of the *E. coli* isolates also were highly susceptible to ceftiofur with the

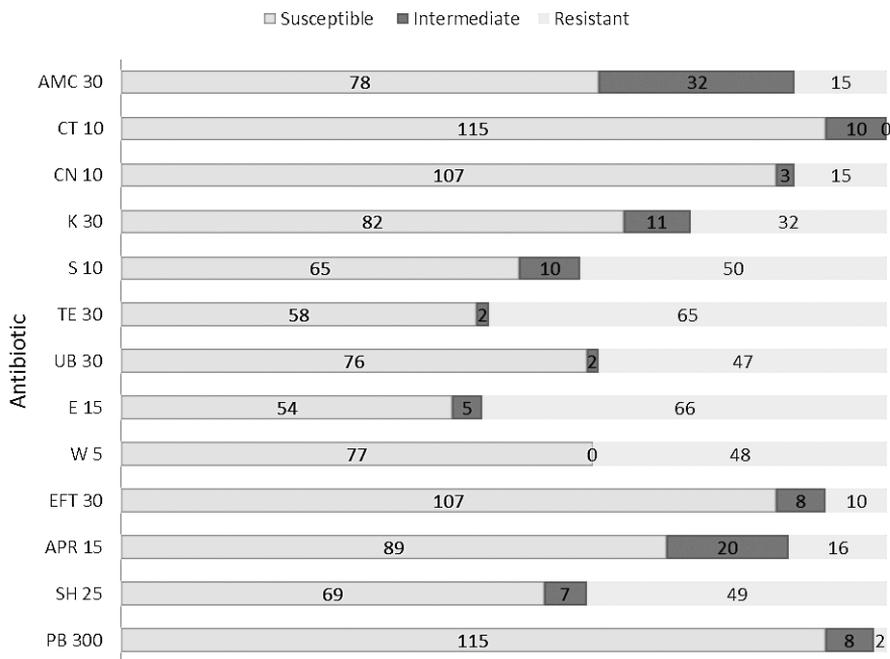


Figure 1: Antibiotic susceptibility of *E. coli* isolated from chicken.

lowest resistance score (8.0%), followed by gentamicin, apramycin and kanamycin. Moderate sensitivity profile was observed among isolates mostly towards amoxicillin and apramycin, with lesser extent towards kanamycin and streptomycin.

DISCUSSION

Conventional biochemical tests have been used since the last decade for identification of *E. coli*. To date, these methods have been used intensively in many laboratories for biotyping and subtyping purposes, in order to get clear characteristics and phenotypic profiles of *E. coli* isolates. However, most of the studies revealed that *E. coli* itself has diverse characters, making it difficult to biotype the *E. coli* strains by using conventional methods alone. Recently, due to the diversity of the *E. coli* group itself, no particular biochemical characteristic was specific to the *E. coli* group that can be used for *E. coli* strain identification (Sojka, 1965). This finding is in agreement with the previous findings which revealed that *E. coli* strains isolated from chicken with colibacillosis poses a variable biochemical characters (Raji *et al.*, 2007). Another finding also showed that various *E. coli* biotypes with diverse biochemical profiles were identified in poultry isolates (Kika *et al.*, 2013). Even though certain biochemical tests have been used widely for *E. coli* identification in many laboratories, but still no definite results can be obtained by conventional methods alone. Certain *E. coli* strains produced slightly different

biochemical profiles compared to expected *E. coli* reaction, and have been reported previously in several studies (Bettelheim, 1994). However, the serotyping scheme available has been accepted internationally as a method for choice for epidemiological investigation purposes, but a large number of fully characterized antisera is largely restricted to most of laboratories. Some biochemical reactions which are particular for *E. coli* have been used to differentiate among the strains as reported previously, with extensive studies on the development of biotyping schemes for *E. coli* based on the ability of strains to ferment dulcitol, raffinose and sorbose and to decarboxylase ornithine (Bettelheim, 1994). Those studies concluded that when the strains from a variety of sources were tested, then good discrimination was achieved, however when the sources were limited, the discrimination was not so good, suggesting that the strains may be related to each other.

Lactose was one of the first substances used to determine the biochemical properties of *E. coli*. It is a reliable method to distinguish *E. coli* from other enterobacteriaceae. In this study, all the *E. coli* isolates tested were able to ferment lactose, which is in agreement with findings reported previously (Tonu *et al.*, 2011). However, certain *E. coli* isolates that did not give a positive reaction has been reported previously (Rodriguez-Siek *et al.*, 2005). Rodriguez-Siek *et al.* (2005) reported that 0.19% *E. coli* isolates haemolysed blood agar, in contrast with the

recent findings with 1.67% of the isolates able to haemolysed blood agar. In another finding, Rodriguez- Siek *et al.* (2005b) also reported that none of the APEC strains tested showed hemolytic activity on blood agar.

Certain properties including utilization of citrate, Voges-paskeur and methyl red are not constant but liable to fundamental changes e.g. after storage for a considerable time. Other earliest properties of *E. coli* was its ability to form indole in a medium containing peptone in the presence of tryptophan. However, there were strains which failed to produce indole (Bettelheim, 1994). Typical *E. coli* strains do not grow on an ammonium substrate containing sodium citrate or known to produce Simmons citrate negative reactions, nevertheless strains which are 'citrate positive' also occur in small number of isolates. Therefore, it is not surprising to observe the contradictory reactions in this finding. Fermentation of malonate on the other hand, gave expected results as 100% of the isolates tested gave negative reaction. Fermentation of malonate is very useful to differentiate *E. coli* from strains of other groups of enterobacteriaceae, such as *Aerobacter*. According to previous reports, almost 69.5% of *E. coli* strains produced positive reaction of lysine decarboxylase, 4% arginine decarboxylase and 52% ornithine decarboxylase. However, these findings do not correlate with recent findings whereby there was a higher percentage of *E. coli* isolates in this study positive for the above reactions

as compared to previous reports. Some of the *E. coli* strains are motile organisms, so variety in motility results were observed in this experiment. Khaton *et al.*, (2008) reported that all the *E. coli* strains isolated from poultry in Bangladesh were motile.

However, strains of *E. coli* may become altered in their biochemical characteristics following subculture, whereby both losses and gain in biochemical activity following subculture can occur or when the bacteria was kept in room temperature for a certain period as described previously, but strains stored at -70°C or 4°C did not show any changes (Katouli *et al.*, 1990). Therefore, certain differences in the biochemical activity may relate to the storage condition of the isolates. As mentioned earlier, most of *E. coli* isolates used in this study were obtained from diagnostic cases and have been kept for various time periods from 1 to 5 years, in maintenance medium at room temperature. Besides, biochemical properties do not associate with their virulence properties, but are more correlated with the serogroups (Dho Moulin *et al.*, 1999). Based on the results obtained, it is concluded that no single feature is particularly characteristic of *E. coli*. Therefore, different tests are required for comparison of various reactions in detail for identification of *E. coli* in the laboratories.

Nowadays, usage of antibiotics is not restricted for therapeutic purposes only, but also widely used as feed additive and growth promoters in poultry farms.

Series of antibiotics such as amoxicillin, penicillin, trimethoprim, aminoglycosides, ciprofloxacin and semi synthetic antibiotics were suggested for treatment against *E. coli* infections, while antibiotics including tetracycline, trimethoprim, penicillin and bacitracin have been used as growth promoter and subtherapeutic purposes in livestock and poultry farms as well.

Based on this finding, the high percentage of multidrug resistance (81.6%) in *E. coli* isolated from chicken was in agreement with other studies in other Asian countries. In Thailand, higher prevalence (100%) of multidrug resistance in broilers was reported (Mooljunttee et al., 2010). While in China, Dou et al. (2015) found that multidrug resistance in avian *E. coli* isolates was 80.3%, with high resistance to erythromycin and tetracycline. In Malaysia, previous studies reported that high multidrug resistant profiles towards different antibiotics were observed with the highest percentage reported (100%) in *E. coli* isolated from chicken in different geographical locations (Geidam et al., 2013; Ong et al., 2014). This finding also revealed that high frequencies of resistance against erythromycin (92.8%) and tetracycline (92.0%) were observed among isolates tested. Several studies also indicated high resistance rate of avian *E. coli* against erythromycin and tetracycline (Kazemnia et al., 2014; Wang et al., 2013; Zhang et al., 2012; Dou et al., 2015), and this situation is usually related to their subtherapeutic usage in Mycoplasma infections in poultry farms.

Erythromycin has been used in poultry farms as prevention and reduction of respiratory distress caused by *Mycoplasma synoviae* and *Mycoplasma gallicepticum* (Talebiyan et al., 2014). Several factors related with high resistance of *E. coli* towards tetracycline including other resistance mechanisms through presence of tetracycline alleles which is found in commensal *E. coli* as well. Miles et al. (2006) also reported that tetracycline resistance in avian *E. coli* isolates was mediated by presence of *tetB* and *tetD* gene, which usually acquired through horizontal transfer among *E. coli* isolates. Long term and frequent used of tetracycline for decades also leads to extremely severe tetracycline resistance (Dai et al., 2008). Meanwhile, cross contamination among antibiotics of the same class can also contribute to the high resistance rates to other antibiotics such as usage of tetracycline may cause high frequencies of resistance towards deoxycycline (Sharada et al., 2008). However, from the present study it was proposed that high resistance rate against erythromycin and tetracycline is not associated with the overuse or improper use of those antibiotics in poultry farms, as both antibiotics were not commonly used in poultry farms in the country. As described by Smith et al. (2007), usage patterns of antibiotics may not correlate with incidence of antibiotic resistance prevalence among avian *E. coli*.

The percentage of *E. coli* isolates resistant to quinolones (77.6%) observed in this study, was slightly lower compared

to studies in other countries (Moniri *et al.*, 2005; Rahimi, 2013; Allocati *et al.*, 2013). Resistance rate to quinolones were concomitantly related with consumption of quinolones in the farms (Moniri *et al.*, 2005). However, a previous study revealed that other resistance mechanisms including mutation of certain resistant genes were also found to be related with the high resistance rate to quinolones among *E. coli* isolates (Szmolka *et al.*, 2013).

Based on this study, most of the *E. coli* isolates found in chicken were highly susceptible to colistin, polymyxin B (92.0%) and ceftiofur. The results indicated a good choice for treatment of *E. coli* infections in the farms as both were clinically available polymyxin class antibiotics that has been effectively used for treatment of Gram-negative bacterial infections including *E. coli*. This finding is in agreement with several reports demonstrating that resistance to polymyxin class of antibiotics is very uncommon among *E. coli* isolates from different geographical regions (Gales *et al.*, 2011). Low resistance score of *E. coli* isolates (8.0%) towards certiofur in this study contradict with previous studies which reported that more than 80% of *E. coli* strains isolated from chicken were resistant to ceftiofur.

Meanwhile, the isolates which exhibited a high percentage of moderate susceptibility profiles towards amoxycilin (25.6%) and apramycin (16.0%) should be taken into consideration, as the profiles might develop into resistant profiles to

that particular antibiotic. The results of this study also indicates that resistance to both antibiotics may increase over time. Therefore, regular surveillance on antibiotic resistance is important in order to monitor any emergence of resistant profiles of *E. coli* in chicken.

In conclusion, our data demonstrated that there is high multiple resistance of *E. coli* strains isolated from chicken. Based on this finding, polymyxin B and colistin were recommended for use for treatment of *E. coli* infection in farms, as it was shown to be effective for disease control. However, establishment of guidelines for prudent use of antibiotics in farms with effective enforcement is required, thus occurrence of antibiotic resistance among *E. coli* in chicken can be managed in the future, so as to limit the flow of these elements of resistance into the human food chain.

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