ANTIMICROBIAL RESISTANCE IN VETERINARY CLINICAL ISOLATES OF *Escherichia Coli* FROM NORTHERN REGION OF PENINSULAR MALAYSIA

SHAHAZA O.[1]*, MOHD. AZIZUL O.[1], ZAKIRAH S.[1], MUHAMMAD AZIM FIKRI A.G.[2], SYAMSYUL A.[1] AND MASWATI M.A.[1]

1 Regional Veterinary Laboratory of Bukit Tengah, 14007 Bukit Mertajam, Penang
2 Faculty of Medicine & Health Sciences, Universiti Putra Malaysia
* Corresponding author:shahaza@dvs.gov.my

ABSTRACT. Increased emergence in microbial resistance to antibiotics is a growing problem globally. A retrospective investigation was made of antimicrobial resistance in a total of 2,345 *Escherichia coli* isolates from clinical cases submitted to Regional Veterinary Laboratory of Bukit Tengah, Penang, Malaysia between January 2000 and December 2015. Analysis using WHONET 5.6 revealed that resistance to ampicillin, norfloxacin and aminoglycosides was detected in isolates from poultry and pigs more often than ruminants. Resistant to colistin was higher in ruminants compared to other animal groups. Norfloxacin susceptibility was very high in isolates from ruminants, pet birds (82.2%) and zoo animals (88.6%). Isolates from pet birds had higher resistant rate to all of the antimicrobials tested compared to zoo and companion animals. Multidrug-resistant (MDR) *E. coli* isolates (29.25%, 684/2345) were detected in this study. Further efforts, for instance, national monitoring and surveillance, are required to quantify the exposure of animals to antimicrobial agents and resistant pathogenic or commensal bacteria in the animals and also its environment. Such useful information indeed will assist the prudent use of antimicrobial agents in veterinary practice; therefore, suppress the emergence of antimicrobial resistance in animals.

Keywords: WHONET, antimicrobial resistance, *Escherichia coli*, animal

INTRODUCTION

Escherichia coli had been used as indicator bacteria for antimicrobial resistance monitoring and surveillance programmes in developed countries such as Denmark (DANMAP), United States of America (NARMS) and Japan (JVARM). In addition, many researchers had reported the prevalence of this indicator bacteria resistant to antimicrobials in clinical isolate in human (Kumar *et al.*, 2014; Nkang *et al.*, 2009), healthy livestock (Usui *et al.*, 2014), wildlife (Navarro-Gonzalez *et al.*, 2013) and animal based products (Rasheed *et al.*, 2014). In Malaysia, a few works revealed presence of multidrug resistant *E. coli* from pigs (Ho *et al.*, 2013), ducks (Adzitey *et al.*, 2013) and surface of chicken eggs (Aw *et al.*, 2015). Nevertheless, data on the antimicrobial resistance from the veterinary clinical isolates are sparse in Malaysia. This information is needed to formulate guidelines for the empirical treatment while awaiting the culture and sensitivity reports. Furthermore, this statistics is required for risk assessments of the efficacy of antimicrobials in treating animal diseases, and both potential animal health and public health consequences. The present study was carried out using the WHONET 5.6 software. The software is freely downloadable, Windows-based database software with a special focus on the analysis of the antimicrobial susceptibility test results. To date, this software is used by clinical, public health, veterinary, and food laboratories in over 90 countries, to support the local and national surveillance programs (Ghosh *et al*, 2013). The Regional Veterinary Laboratory of Bukit Tengah (RVLBT) is the first laboratory under Department of Veterinary Services (DVS), Malaysia to employ the WHONET 5.6 program to collect, collates and analyzes the antimicrobial susceptibility data of all veterinary clinical isolates. The objective of this study was to assess variation in resistance among clinical *Escherichia coli* isolates from seven different groups of animals to nine antimicrobials of importance within the veterinary settings.

MATERIALS AND METHODS

The conventional methods were employed for the isolation and identification of *Escherichia coli* from organs of animals that were received in Regional Veterinary Laboratory Bukit Tengah, Penang, for routine culture in January 2000 to July 2016. Antimicrobial Susceptibility Testing (AST) was performed by Disc Diffusion method using antimicrobial disc of ampicillin (10 µg), tetracycline (30 µg), doxycycline (30 µg), streptomycin (10 µg), kanamycin (30 µg), neomycin (30 µg), gentamycin (10 µg), norfloxacin (10 µg) and colistin (10 µg). Raw data expressed in millimeters (mm) were then sorted into three categories; resistant, intermediate and susceptible by established clinical breakpoints. The breakpoints (in millimeter, mm) for ampicillin (14-16), gentamicin (13-14), kanamycin (14-17), neomycin (13-14), streptomycin (12-14), norfloxacin (13-16), colistin (S>=11), doxycycline (11-13) and tetracycline (12-14) were in accordance with CLSI guidelines. It is a routine practice for the RVLBT to include the *E.coli* ATCC® 25922 as quality control while performing the AST. The susceptibility data were entered manually into the WHONET 5.6 and analysed by groups; namely, poultry, pigs, small ruminants (sheep, goats), large ruminants (cattle, buffalo), pet birds, companion animals and zoo animals. Isolates from chicken, duck, turkey and geese were grouped into 'poultry'. Isolates from cat, dog, rabbit, and horses were included under the 'companion animal' category. The isolate were classified as resistant if susceptibility testing resulted in intermediate or resistant classifications. Trends in resistance to each antimicrobials and the multidrug-resistant (MDR) were compared among the seven animal groups.

RESULTS

A total of 2,345 clinical *Escherichia coli* isolates were analysed for the years 2000 to 2015. The isolates were from poultry (n=1660, 70.80%), pet birds (n=51, 2.17%), pigs (n=45, 1.92%), small ruminants (n=423, 18.03%), large ruminants (n=87, 3.71%), companion animals (n=41, 1.75%) and zoo animals (n=38, 1.62%). The number of isolates subjected to AST in 2003 (n=394) was the highest within the 15-year study period. The number of isolates subjected to AST fluctuated throughout 2004 to 2015 with the lowest in 2012 (n=6). For the past three years (2012-2015), the number of isolates tested for the nine antimicrobials were less than 20 per year (Figure 1). The highest ampicillin resistance rate were noted in poultry (92.7%), followed by pigs (91.7%), pet birds (87.2%) and companion animals (80%). The ampicillin resistance rate in large ruminants (51.3%) was lower than small ruminants (62.5%). In zoo animals, 66.7% of the isolates were non-susceptible to ampicillin resistance. This study confirmed the presence of fluoroquinolone-resistant *E. coli* in all animal groups. The lowest norfloxacin resistance rate was in large ruminants (8.7%). In contrast, high percentage of isolates from poultry (61.9%) and pigs (55.3%) were resistant to norfloxacin. Continuous monitoring of fluoroquinolones resistance and its use in animals, an especially food-producing animal is necessary to prevent spread of fluoroquinolone-resistance. *Escherichia coli* isolated from poultry had very high levels of resistance to tetracycline (91.6%) and doxycycline (86.4%). However, among all species, isolates from pigs had the highest resistance rate to tetracycline (97.1%) and doxycycline (91.7%), (Figure 2). Isolates from companion animals had the lowest resistance rate to tetracycline and streptomycin which were 42.9% and 33.3%, respectively. Streptomycin resistance percentage was very high in poultry (87.7%), pigs (86%) and pet birds (78.2%). Meanwhile, lowest resistant to three out of four aminoglycoside; kanamycin (22.2%), neomycin (31.6%) and gentamicin (11.7%) were noted among isolates from zoo animals. More than half of the isolates from poultry (73.4%), pigs (66.6%) and pet birds (55.3%) were non-susceptible to kanamycin. In companion animals the colistin resistant rate was 26.7%, which is the lowest among the animal groups. The highest colistin resistance was noted in large ruminants (65.1%), followed by small ruminants (63.8%), pigs (55.2%) and pet birds (47.9%). Colistin resistance rate in poultry and zoo animals were around 36%.

The WHONET analytical tools facilitate in clustering the isolates into 101 resistance phenotypes. The most common resistance profile was 'ATDCSKNG' (6.8%, 160/2345). A total of 684 isolates had multidrug resistance (MDR) phenotypes which were from poultry (80.26%, 549/684), small ruminants (12.28%, 84/684), pet birds (2.78%, 19/684), large ruminants (1.90%, 13/684), pigs (13, (13/684), companion animals (0.58%, 4/684) and zoo animals (0.29%, 2/684). Findings in clinical *E. coli* isolates from poultry echoes similar message by Kit *et al*., 2015, where in their study, 8.8% of *E. coli* isolated from surface of chicken egg and carrying trays were resistant to against 10 different antimicrobials. Five hundred nineteen *E. coli* isolates were identified as "Possible extensively drug-resistant (XDR)" with 69.6% (n=361) of the organism were isolated from chicken. In addition, there were isolates (0.1%, 2/2435) from poultry (chicken, n=1) and small ruminants (goat, n=1) reported as both 'Possible XDR' and 'Possible Pandrug resistant (PDR)'. Multidrug resistant patterns of the *E. coli* isolates with

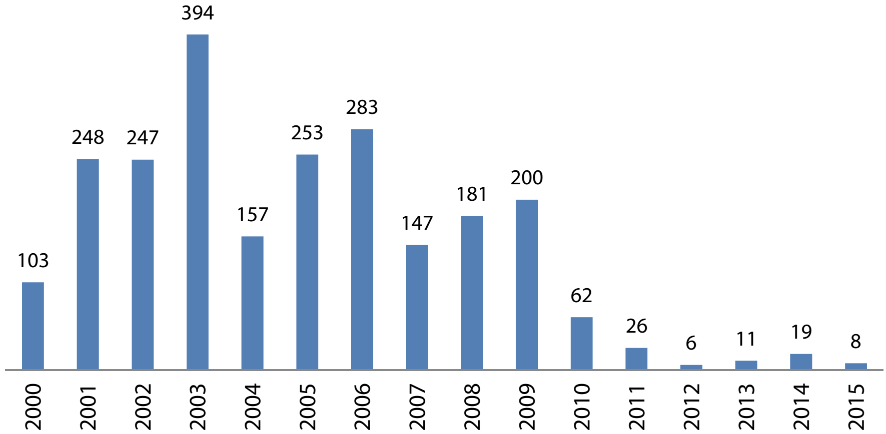
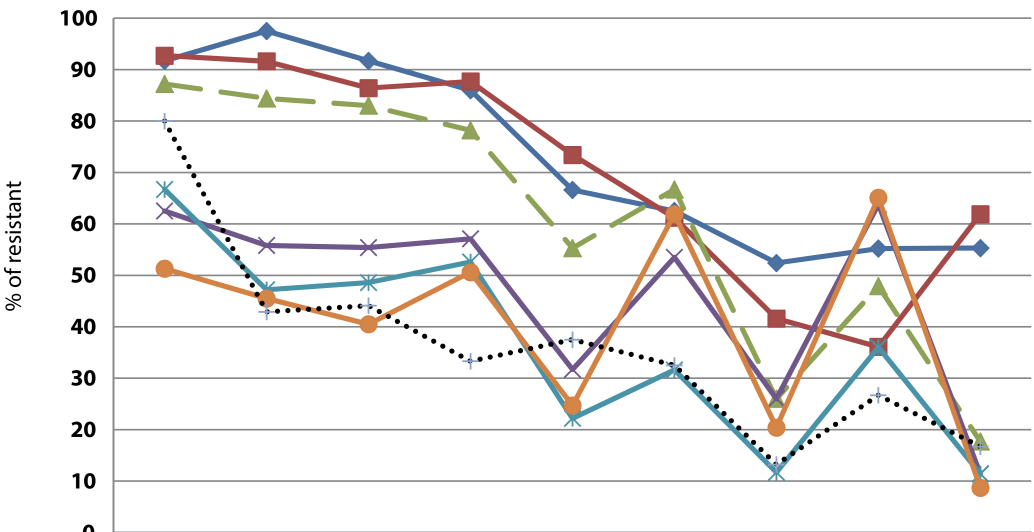


Figure 1. Number of *Escherichia coli* isolated from clinical specimens subjected to antimicrobial susceptibility testing between January 2000 and December 2015.



	Ampicillin	Tetracycline	Doxycycline	Streptomycin	Kanamycin	Neomycin	Gentamicin	Colistin	Norfloxacin
Pigs	91.7	97.5	91.7	86	66.6	62.5	52.4	55.2	55.3
Poultry	92.7	91.6	86.4	87.7	73.4	61.2	41.6	36.1	61.9
Pet birds	87.2	84.4	83	78.2	55.3	66.7	26	47.9	17.7
Small ruminants	62.5	55.8	55.4	57.1	31.7	53.5	26	63.8	11.4
Zoo animals	66.7	47.2	48.6	52.6	22.2	31.6	11.7	36	11.4
Large ruminants	51.3	45.5	40.5	50.6	24.7	61.9	20.4	65.1	8.7
Companion animals	80	42.9	44.1	33.3	37.5	32.5	13.2	26.7	16.7

MDR: Multidrug Resistance, A: Ampicillin, T:Tetracycline, D:Doxycycline, C:Colistin, S: Streptomycin, K: Kanamycin, N: Neomycin, G: Gentamycin. a) Possible Extensively drug-resistant (XDR).

Figure 2. Percentage of *Escherichia coli* resistant to nine antimicrobials agents between January 2000 and December 2015 in Regional Veterinary Laboratory of Bukit Tengah, Penang, Malaysia.

Table 1. Multidrug resistant phenotypes of *Escherichia coli* isolated from veterinary clinical samples in Regional Veterinary Laboratory, Bukit Tengah, Penang.

No. of classes nonsusceptible	MDR patterns	No. of isolates							
		% Isolates	Pet birds	Poultry	Pigs	Large ruminants	Small ruminants	Zoo animals	Companion animals
4	ATDCSKNG[a)]	6.8	4	117	5	5	28	0	1
	ATDCSKN [a)]	3.3	7	62	1	1	5	1	0
	ATDCS N [a)]	1.2	0	10	0	3	15	0	0
3	ATDSKNG [a)]	4.5	3	99	2	0	1	0	0
	ATDSKN	3.6	2	80	0	0	3	0	0
	ATDS	2	2	43	0	0	2	0	1
	ATDSKG [a)]	1.1	0	25	1	0	0	0	0
	ATDSK	1	0	24	0	0	0	0	0

the occurrence rate equal to or more than one percent were summarized in Table 1. Resistance profile analysis revealed that only 1.7% (40/2345) of the isolates was susceptible to all of the antimicrobial agents tested. Adam *et al.* (2008) reported that the development of resistance in *E. coli* was due to alteration in gene expression (epigenetic inheritance) rather than by direct inheritance of a mutated gene. The rate of adaptive mutations in *E. coli* is 10^{-5} per genome per generation, which might implicates the evolution of antibiotic resistance (Perfeito *et al*., 2007). Resistant bacteria and the resistance genes they carry are selectively amplified by antibiotic exposure. Harvey *et al*., 2009 had observed that the percentage of tetracycline resistance genes in fecal flora of conventionally raised feetlot steers was significantly higher than that in fecal samples from antimicrobial-free cattle.

CONCLUSION

This present study found that resistance rate to antimicrobial agents from aminopenicillin, fluoroquinolone, aminoglycoside and tetracycline classes in poultry and pigs were higher compared the other animal groups, possibly due to usage of antimicrobial as feed additives. Feeding antimicrobial at subtherapeutic level of aforementioned classes had been practised in poultry and swine industries. It was added in poultry and pigs feed ration to increase the feed utilisation and average weight gain (Van Lunen, 2003), and also inhibit growth of pathogenic bacteria (Niewold, 2007). There were strong and significant correlation between the strains from animals (especially poultry and pigs) and humans for resistance to multiple drugs especially ampicillin, aminoglycoside and fluoroquinolone (Vieira *et al*., 2011).

According to Rahimi (2013), abusive use of antimicrobials is considered the most vital selecting force to antimicrobial resistance of bacteria resulting in poor response to treatment. Therefore, prudent use of antimicrobial agents should be practised in veterinary medicine. Identifying the bacteria susceptibility is essential in making a reasoned and sensible choice of an antibiotic for treatment of bacterial diseases. This will help practitioners to optimse prescribed treatment and increases the probability of the treatment being as successful as expected. This practice also helps in reducing the length of antimicrobial therapy to the shortest possible time to reduce selection pressure for resistance. There is little published information on antimicrobial usage and trends in antimicrobial resistance in veterinary settings in Malaysia. Hence, this knowledge gap has to be addressed; the occurrence of resistance in healthy livestock should be explored. It is necessary to evaluate the association of antimicrobial resistance with the misuse or overuse of antimicrobials in animals especially livestock. Future work should also include sampling on processing plants and markets to locate the source of antimicrobial resistance. This data can be called upon to establish recommendations of use of antimicrobial agents. A sound surveillance and monitoring programme on antimicrobials at the national level should be established before the impact of any antimicrobial resistance intervention initiatives can be investigated.

It is an established fact that antimicrobial resistance development can potentially cause treatment failure in both human medicine and veterinary medicine. Using WHONET 5.6 software, this study found that, resistance to critically important antimicrobials was apparently high in clinical *E. coli* isolates from animals. The highest ampicillin resistance rate was noted in isolates from poultry. The isolates from large ruminant had the lowest ampicillin resistance rate. Resistance rate to tetracycline were higher compared to doxycycline (excluding the zoo and companion animals). Isolates from pet birds had higher resistance rate to all of the antimicrobials tested compared to zoo and companion animals. Gentamicin resistance rate was relatively lower compared to the other three aminoglycosides. Resistance to norfloxacin was present in isolates from all animal groups. The lowest resistance rate to norfloxacin was in ruminants, whilst isolates from poultry had the highest norfloxacin resistance rate. The most common resistance profile was 'ATDCSKNG' (6.8%, 160/2345). Isolates of *Escherichia coli* with the MDR (n=684), possible XDR (n= 519) and possible PDR (n=2) phenotypes were detected. Further efforts are required to assess the amount of exposure of animals to antimicrobial agents. The exposure of animals to resistant pathogenic or commensal bacteria in the environment should also be quantified. Comprehensive surveillance and monitoring systems at national level aimed at detecting occurrence and analysing changes in prevalence of antimicrobial resistance in animals will provide useful information for prudent use of antimicrobial agents in veterinary practice. Therefore, this will contribute in decelerating the emergence of resistance to clinically important antimicrobial agents.

REFERENCES

1. Adzitey F., Ali G.R.R., Huda N. and Ting S.L. (2013). Antibiotic resistance and plasmid profile of *Escherichia coli* isolated from ducks in Penang, Malaysia, *International Food Research Journal* **20(3)**: 1473-1478.
2. Aw Y.K., Loong M.J., Yue C.X., Lee K.M., Ng S.Y. and Lee S.M. (2015). Antimicrobial resistance of *Escherichia coli*, Salmonella and enterococci isolated from surface of conventional broiler eggs, "Kampung" chicken eggs and carrying trays from wet markets in Selangor, Malaysia. *Malaysian Journal of Microbiology,* **11(2)**: 109-115.
3. Ghosh A.N., Bhatta D.R., Ansari M.T., Tiwari H.K., Mathuria J.P., Gaur, A., Supram H.S. and Gokhale S. (2013). Application of WHONET in the Antimicrobial Resistance Surveillance of Uropathogens: A First User Experience from Nepal*, Clin Diagn Res.* **7(5):** 845–848.
4. Harvey R., Funk J., Wittum T.E. and Hoet A.E. (2009). A metagenomic approach for determining prevalence of tetracycline resistance genes in the fecal flora of conventionally raised feedlot steers and feedlot steers raised without antimicrobials. *Am. J. Vet. Res.* **70:** 198-202. doi:10.2460/ajvr.70.2.198
5. Ho W.S., Tan L.K., Ooi P.T., Yeo C.C. and Thong K.L. (2013). Prevalence and characterization of verotoxigenic *Escherichia coli* isolates from pigs in Malaysia, *BMC Veterinary Research*, **9**: 109.
6. Nkang A.O., Okonko I.O, Mejeha O.K, Adewale O.G., Udeze A.O., Fowotade A., Fajobi E.A., Adedeji A.O. and Babalola E.T. (2009). Assessment of antibiotics susceptibility profiles of some selected clinical isolates from laboratories in Nigeria, *Journal of Microbiology and Antimicrobials*, **1(2):** 019-026.
7. Perfeito L., Fernandes L., Mota C. and Gordo I. (2007). Adaptive Mutations in Bacteria: High Rate and Small Effects. *Science*, **317(5839)**: 813–815
8. Rahimi M. (2013). Antibioresistance profile of Avian pathogenic *Escherichia coli* isolates recovered from broiler chicken farms with colibacillosis in Kermanshah province, Iran, *Global Veterinaria* **10 (4)**: 447-452.
9. Rasheed M.U., Thajuddin N., Ahamed P., Teklemariam Z. and Jamil K. (2014). Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources, *Rev. Inst. Med. Trop. Sao Paulo,* **56(4):** 341-346.
10. Usui M., Ozawa S., Onozato H., Kuge R., Obata Y., Uemae T., Ngoc P.T., Heriyanto A., Chalemchaikit T., Makita K., Muramatsu Y. and Tamura Y. (2014). Antimicrobial Susceptibility of Indicator Bacteria Isolated from Chickens in Southeast Asian Countries (Vietnam, Indonesia and Thailand), *J. Vet. Med. Sci.* **76(5)**: 685-692.
11. Van Lunen T.A. (2003). Growth performance of pigs fed diets with and without tylosin phosphate supplementation and reared in a biosecure all-in-all-out housing system. *Can. Vet. J.* **44**: 571-576.
12. Vieira A.R., Collignon P., Aarestrup F.M., McEwen S.A., Hendriksen R.S., Hald T. (2011). Association between antimicrobial resistance in *Escherichia coli* isolates from food animals and blood stream isolates from humans in Europe: an ecological study. *Foodborne Pathog. Dis.* **8:** 1295-1301. doi:101089/fpd.2011.0950