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Detection of antibiotic resistant *Enterobacteriaceae* from dogs in North West University (South Africa) animal health hospital

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Bacteria belonging to the family *Enterobacteriaceae* are facultative anaerobic, Gram-negative, non-spore forming rod-shaped bacilli. Members of this heterogeneous group of bacteria do not only form part of the normal flora of humans and animals, but are also widely distributed in various environments such as water, soil and plants. Most members of the *Enterobacteriaceae* were previously considered to be harmless. However, there is evidence that some strains potentially cause diseases and pathological conditions such as diarrhoea, gastroenteritis, urinary tract infections and inflammatory bowel diseases in animals and humans. The aim of the present study was to isolate and determine the antibiotic resistant profiles of *Enterobacteriaceae* isolated from dogs that visited the North West University animal hospital. Fifteen (15) faecal samples were collected from the rectum of dogs that visited the Hospital, using sterile swabs and the samples were placed in transport media. The samples were immediately transported on ice to the laboratory for analysis. MacConkey agar with crystal violet was used for selective isolation of bacteria belonging to the family *Enterobacteriaceae*. Only isolates that satisfied the preliminary identification tests (Gram staining, triple sugar iron agar test, citrate agar test and oxidase test) and confirmatory identification test (API 20E) were retained for further analyses. Antibiotic susceptibility tests were performed on all positively confirmed isolates to determine their antibiotic resistant profiles against tetracycline (30 µg), ampicillin (10 µg), amoxicillin (10 µg), penicillin (10 µg), gentamycin (30 µg) and streptomycin (10 µg). A total of 120 isolates were positively identified as members of the *Enterobacteriaceae*. All the isolates were Gram negative rods and oxidase negative. A large proportion (92.5%) of these isolates fermented the sugars in the TSI agar with only a small proportion (23.3%) producing hydrogen sulphide gas. However, a relatively larger proportion of these isolates (62.5%) produced gas from the fermentation of sugars. On characterizing these isolates for the ability to hydrolyze citrate, a large proportion (71.7%) were negative for this test. The API 20E test results indicated that bacteria species belonging to four main genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were indentified. A large proportion (50%) of these isolates were identified as *Escherichia coli* while 25, 15.8 and 9.2% were *Salmonella* spp., *Klebsiella* spp. and *Shigella* species, respectively. Isolates from all the samples were most often, resistant to penicillin, ampicillin, tetracycline and amoxicillin while very little resistance was observed against gentamycin and streptomycin. The MDR phenotypes PG-AP-A-T, PG-AP-A-T-S, PG-AP-A, PG-A-T and PG-AP-A-T-GM-S were dominant in isolates from samples analyzed. Although a large proportion of the isolates were resistant to three or more antibiotics, a cause for concern was the fact that some isolates were resistant to all antibiotics screened. The identification of multiple antibiotic resistance among the isolates ignites the need to establish appropriate testing procedures before the administration of drugs to animals, thus reducing the possibility of the development and transfer of antibiotic resistant genes between animals and humans.

Key words: *Enterobacteriaceae*, multiple antibiotic resistance, multi drug resistance (MDR) phenotypes.

INTRODUCTION

In developing and developed countries, humans have a strong relationship with pets such as cats and dogs (Robertson et al., 2000). These animals live as companions in households where they contribute to the social, physical and emotional development of children and the well-being of their owners (Jennings, 1997). Companion animals such as dogs and cats are given certain privileges like spending time on the furniture (Wieler et al., 2011). Despite the fact that pets are significantly beneficial to the society, there are a number of health hazards associated with owning a pet (Manian, 2003; Damborg et al., 2009). Moreover, the number of human patients that are highly exposed to these health hazards is on the increase considering the increase in intensive care provided to these companion animals (Bush et al., 2011; Wieler et al., 2011).

Bacteria belonging to the family *Enterobacteriaceae* are facultative anaerobic, Gram negative, non-spore forming rod-shaped bacilli (Ghotaslou et al., 2009; Ateba and Setona, 2011). Within this family are members of the genus *Escherichia, Shigella, Salmonella, Proteus, Yersinia, Klebsiella, Erwinia, Enterobacter, Citrobacter, Providencia, Hafnia, Morganella, Edwardsiella* and *Serratia* (Blood and Curtis, 1995). This heterogeneous group of bacteria does not only form part of the normal flora of humans and animals, but are also widely distributed in various environments such as water, soil and plants (Lima-Bittencourt et al., 2007). The presence of these bacterial species in the gastrointestinal tract of humans and companion animals play an imperative role in maintaining both the normal digestive and immune functions of the hosts (Hall, 2004). In addition, these bacteria species have also been found to participate in metabolic activities that save energy and absorbable nutrients as well as protect the colonized host against invasion by foreign microbes (Guarner, 2006).

Despite the fact that most members of the *Enterobacteriaceae* were previously considered to be harmless, it is evident that some strains potentially cause diseases and pathological conditions such as diarrhoea, gastroenteritis, urinary tract infections and inflammatory bowel diseases in humans, and companion animals (Nakazato et al., 2004; Greiner et al., 2007; Costa et al., 2008; Suchodolski et al., 2010). It is therefore important to determine the occurrence of these bacterial species in companion animals in a country like South Africa where individuals keep them as pets. The NWU hospital provides veterinary health services to companion animals of individuals who live in the Mafikeng area. The aim of the study was to isolate and determine the antibiotic resistant profiles of *Enterobacteriaceae* isolated from dogs that visited the NWU animal hospital.

MATERIALS AND METHODS

Area of the study

This study was conducted in the North West University Mafikeng Campus, North-West Province, South Africa. Fifteen faecal samples were collected from the rectum of dogs that visited the North West University Animal Hospital, using sterile swabs and the samples were placed in transport media. The samples were immediately transported on ice to the laboratory for analysis.

Laboratory analysis

Selective isolation of Enterobacteriaceae

Rectal swabs obtained from animals were washed in 5 ml of 2% peptone water and then homogenized by vortexing. Ten fold serial dilutions were prepared using the homogenized mixture of faecal sample and a sterile peptone. Aliquots of 100 µl from each dilution were spread-plated on MacConkey agar that contains crystal violet for selective isolation of bacteria belonging to the family *Enterobacteriaceae*.

Bacterial identification

Gram staining

All presumptive isolates were subjected to Gram staining reaction using standard methods (Cruikshank et al., 1975). *Enterobacteriaceae* are Gram negative rod-shaped bacteria, hence all isolates that satisfied this criterion were subjected to preliminary biochemical identification tests.

Preliminary biochemical identification tests for Enterobacteriaceae

Triple sugar iron agar test: Triple sugar iron (TSI) agar (Biolab) obtained from Merck, SA, was used to distinguish members of the family *Enterobacteriaceae* from other Gram-negative bacteria based on the ability of the organisms to metabolize the three sugars: glucose; sucrose; and lactose at concentrations of 1, 0.1 and 0.1% respectively (Prescott et al., 2002). The test was performed as previously recommended (United States Pharmacopeial Convention; Inc., 2001). In performing the test, the media was prepared and aliquots of 5 ml were poured in sterile bottles. The media was sterilized and bottles kept in slanting positions in order to obtain a slant and butt when media solidified. All isolates were subjected to the test streaking the isolates on TSI agar slant and also stab inoculating into the butt using a sterile pin. The inoculated bottles were incubated at 37°C for 24 h. After incubation, the isolates were evaluated for the ability to ferment the sugars present with or without the production of acid, gas and hydrogen sulphide (H_2S). Results were recorded and analyzed as previously recommended (Forbes and Weissfeld, 1998).

Oxidase test: The oxidase test was performed using the oxidase test reagent from Pro-Lab Diagnostics- United Kingdom. The oxidase test is based on the principle that tetramethyl-p-phenylenediamine is oxidised by bacterial cytochrome in the presence of atmospheric oxygen to form purple coloured compound. In performing the test, a single colony was placed on Whatman filter paper (Whatman International Ltd, Maidstone, England). A drop of the oxidase test reagents was added on the paper. The two were mixed using sterile wire loop and the results were read within 30 seconds. The results were recorded based on colour change in which the formation of a purple colour was reported as a positive result and vice versa. However, bacteria belonging to the

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Table 1. List of antibiotics used during the study. The concentrations used as well as inhibition zone measurements in (mm) considered resistant (R), intermediate (I), and susceptible (S) are shown according to NCCLS (1999)

Group	Antibiotic	Abbreviation	Disc conc.	R	I	S
Aminoglycosides	Streptomycin	S	10 µg[a]	≤11	12-14	≥15
	Gentamycin	GM	30 µg[b]	≤12	13-16	≥17
Beta- lactams	Ampicillin	AP	10 µg[a]	≤11	12-14	≥15
	Penicillin	PG	10 µg[a]	≤11	12-21	≥22
	Amoxycillin	A	10 µg[a]	≤11	12-21	≥22
Tetracyclines	Tetracycline	T	30 µg[b]	≤14	15-18	≥19

The superscripts [a] and [c] indicate the concentrations of the discs according to the standard method as stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom.

family *Enterobacteriaceae* are oxidase-negative and the results obtained for all the isolates are shown in the Appendix Tables.

Simmons citrate utilization test: In performing the test, isolates from a pure colony were streaked on the slant and stab inoculated into the butt of Simmons citrate agar (Fluka, Biochemika) using a sterile pin. The inoculated cultures were incubated at 37°C for 24 h. After incubation a colour change from green to blue was recorded as a positive reaction and vice versa (Brenner, 1984).

Confirmatory biochemical tests for *Enterobacteriaceae*

Analytical Profile Index (API 20E)

Presumptive species confirmation was done using the API 20E test. The API 20E is a standardized test kit intended to facilitate the identification of bacteria belonging to the *Enterobacteriaceae*. The test was performed following the manufacturer's protocol (BioMerieux, France). In performing the test, the microtubes were inoculated with bacterial suspensions. After inoculation, the test strips were incubated at 37°C for 24 h. The results were read with or without the addition of reagents. Results were interpreted using the manual provided by the manufacturer and indices generated were used to determine identities of the isolates with the API web software.

Antibiotic susceptibility tests

Antibiotic susceptibility tests were performed on all positively confirmed isolates to determine their antibiotic resistant profiles using the Kirby-Bauer disc diffusion technique (Kirby-Bauer et al., 1966). The antibiotics tested are shown in Table 1 and the test was performed as recommended by National Committee for Clinical Standards (NCCLS, 2000). Bacterial suspensions were prepared using fresh cultures and aliquots of 100 µl from each suspension were spread-plated on Muller-Hinton agar (Merck) plates.

The antibiotic discs were placed on the inoculated plates using a sterile needle and the plates were incubated aerobically at 37°C for 24 h. The isolates were classified as susceptible, intermediate resistant and resistant by measuring the diameter of the zone of inhibition and comparing them with standard reference values (Table 1). Table 1 presents the details of antibiotics used in the study.

RESULTS

The detection of *Enterobacteriaceae* in animal samples

Fifteen faecal samples collected from the rectum of dogs that visited the North West University animal hospital were analyzed for the presence of bacteria species belonging to the family *Enterobacteriaceae*. A summary of the isolates that satisfied both the preliminary and confirmatory identification characteristics for *Enterobacteriaceae* are shown in Table 2. As shown in Table 2, all the isolates were Gram-negative rods and oxidase negative. A large proportion (92.5%) of these isolates fermented the sugars in the TSI agar with only a small proportion (23.3%) producing hydrogen sulphide gas. However, a relatively larger proportion of these isolates (62.5%) produced gas from the fermentation of sugars. On characterizing these isolates for the ability to hydrolyze citrate, a large proportion (71.7%) were negative. The API 20E test results indicated that bacteria species belonging to four main genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were indentified. A large proportion (50%) of these isolates were identified as *Escherichia coli* while 25, 15.8 and 9.2% were *Salmonella* spp., *Klebsiella* spp. and *Shigella* species, respectively.

Percentage antibiotic resistance of *Enterobacteriaceae* isolated

A total of 120 isolates positively identified as members of the *Enterobacteriaceae* were subjected to antibiotic susceptibility tests. The proportion of isolates resistant to a particular antibiotic was determined and results expressed as percentages. Table 3 indicates the percentage

Table 2. Proportion of isolates from different samples that satisfied both preliminary and confirmatory identification characteristics for *Enterobacteriaceae.*

Sample no.	Gram staining		Oxidase		TSI			Citrate Utilization		API 20E
	+ve	-ve	+ve	-ve	Sugar fermentation	H_2S	Gas	+ve	-ve	
DAH1		8		8	8	0	8	1	7	8 (*Escherichia coli*)
DAH2		8		8	8	6	8	3	5	6 (*Salmonella* spp.) 2 (*Escherichia coli*)
DAH3		8		8	8	0	7	1	7	8 (*Escherichia coli*)
DAH4		8		8	8	7	8	4	4	7 (*Salmonella* spp.) 1 (*Klebsiella* spp.)
DAH5		8		8	8	1	8	1	7	1 (*Salmonella* spp.) 2 (*Escherichia coli*) 5 (*Shigella* spp.)
DAH6		8		8	7	0	4	1	7	4 (*Escherichia coli*) 4 (*Klebsiella* spp.)
DAH7		8		8	8	2	3	3	5	1(*Salmonella* spp.) 6 (*Shigella* spp.)
DAH8		8		8	8	0	8	8	0	8 (*Escherichia coli*)
DAH9		8		8	8	0	1	1	7	8 (*Escherichia coli*)
DAH10		8		8	4	0	3	2	6	8 (*Escherichia coli*)
DAH11		8		8	8	4	6	1	7	4 (*Salmonella* spp.) 4 (*Klebsiella* spp.)
DAH12		8		8	8	2	0	2	6	1(*Salmonella* spp.) 6 (*Escherichia coli*)
DAH13		8		8	8	1	4	3	5	1 (*Klebsiella* spp.) 7 (*Escherichia coli*)
DAH14		8		8	8	3	3	1	7	2(*Salmonella* spp.) 4 (*Klebsiella* spp.)
DAH15		8		8	8	2	4	2	6	3 (*Salmonella* spp.) 5 (*Klebsiella* spp.)
Total		120		120	111	28	75	34	86	

+ve=Positive; -ve=negative

of antibiotic resistant profiles of isolates tested. As shown in the table, isolates from all the samples were most often resistant to penicillin, ampicillin, tetracycline and amoxicillin.

However, very little resistance was observed against gentamycin and streptomycin.

MDR phenotypes of *Enterobacteriaceae* isolated

The predominant multiple antibiotic resistant phenotypes of isolates obtained are shown in Table 4. The MAR phenotypes PG-AP-A-T and PG-AP-A-T-S were dominant in isolates from samples 2 (DAH2) and 4 (DAH4) and were obtained at percentages of 62.5% each. Moreover, phenotypes PG-AP-A and PG-A-T were also obtained at 50%, respectively amongst isolates from samples 6 (DAH6) and 8 (DAH8).

Table 3. Percentage of antibiotic resistance of *Enterobacteriaceae* isolated.

Sample No		PG	AP	T	A	GM	S
DAH1	NR	2	3	7	3	2	2
	%R	25	37.5	87.5	37.5	25	25
DAH2	NR	8	5	5	5	0	5
	%R	100	62.5	62.5	62.5	0	62.5
DAH3	NR	8	8	8	8	3	4
	%R	100	100	100	100	37.5	50
DAH4	NR	5	5	8	5	0	0
	%R	62.5	62.5	100	62.5	0	0
DAH5	NR	0	0	8	0	0	0
	%R	0	0	100	0	0	0
DAH6	NR	4	4	1	4	0	1
	%R	50	50	12.5	50	0	12.5
DAH7	NR	8	3	2	3	2	2
	%R	100	37.5	25	37.5	25	25
DAH8	NR	8	4	5	0	0	0
	%R	100	50	62.5	0	0	0
DAH9	NR	7	2	2	3	2	0
	%R	87.5	25	25	37.5	25	0
DAH10	NR	8	2	4	7	0	0
	%R	100	25	50	87.5	0	0
DAH11	NR	2	0	8	2	0	0
	%R	25	0	100	25	0	0
DAH12	NR	7	0	5	0	0	0
	%R	87.5	0	62.5	0	0	0
DAH13	NR	4	3	4	7	1	0
	%R	50	37.5	50	87.5	12.5	0
DAH14	NR	5	2	7	4	1	0
	%R	62.5	25	87.5	50	12.5	0
DAH15	NR	1	2	8	2	2	0
	%R	12.5	25	100	25	25	0

PG (Penicillin), Ap (Ampicillin), A (Amoxycillin), T (Tetracycline), GM (Gentamycin), S (Streptomycin).

Table 4. The predominant MAR phenotypes for *Enterobacteriaceae* isolated.

Sample no.	Phenotype	No observed	Percentage
DAH1	PG-AP-A-T-GM-S	2	25
	PG-AP-AT-GM	1	12.5
DAH2	PG-AP-A-T-S	5	62.5
DAH3	PG-AP-A-T-GM-S	3	37.5
DAH4	PG-AP-A-T	5	62.5
DAH6	PG-AP-A	4	50
DAH7	PG-AP-A-T-GM-S	1	12.5
DAH8	PG-A-T	4	50
DAH9	PG-AP-A	1	12.5

DAH=Dog Animal Health; NT=Number Tested.

The phenotype PG-AP-A-T-GM-S was obtained at 25% and 37.5% from samples 1 (DAH1) and 3 (DAH3), respectively. Although a large proportion of isolates were resistant to three or more antibiotics, a major preoccupation was the fact that some isolates were resistant to all antibiotics screened.

DISCUSSION

The main objective of this study was to selectively isolate bacteria belonging to the family *enterobacteriaceae* from faecal samples obtained from dogs that visited the NWU animal hospital in Mafikeng, North-West Province, South Africa. These isolates may cause gastrointestinal infections in these animals, may be self-limiting in some instances and may progress to more severe forms of complications. Generally, bacteria belonging to four genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were successfully isolated and their identities confirmed using both preliminary and confirmatory tests. These isolates were not identified at strain level. However, they belong to strains that are highly pathogenic to animals and even humans who interact with them. Bacteria that belong to the genera isolated have been found to be easily transmitted from animals to humans.

Another objective of the study was to determine the antibiotic resistance profiles of the isolates against a panel of six antimicrobial agents. The main reason was due to the fact that the animal hospital provides health care services to pets of residents of the Mafikeng area. However, the hospital is not equipped with a microbiology diagnostic unit that isolates and screens microbes for antibiotic resistant determinants. This usually results in prolonged treatment of infections in dogs and cats brought to the hospital.

Recently, companion animals such as dogs and cats live in close contact with their owners than was the case some time ago; they have increasingly gained the status of a family member in some urban households (Blouin, 2008). They spend time on furniture at home or close face-to-fur contact. Due to increasing intensive care

provided to the animals, the human population is also exposed to risks such as the acquisition of antibiotic resistant strains (Hossain et al., 2004; Sidjabat et al., 2006; Umber and Bender, 2009). With this reality, several studies have been carried out to determine the antibiotic resistant profiles of microbes in general and *Enterobacteriaceae* species in particular from companion animals (Walther et al., 2008; Murphy et al., 2009; Umber and Bender, 2009). The increase of antimicrobial resistance in these pathogens is most often accompanied by severe complications in both humans and companion animals (Alekshun and Levy, 2006; Weese, 2008).

The frequencies of resistance to penicillin, ampicillin, amoxicillin and tetracycline were generally high among *Enterobacteriaceae* isolated from dogs. Similar observations had been reported (Sáenz et al., 2001; Costa et al., 2008). Tetracycline and β-lactams are generally used in animal medicine as observed. Moreover, tetracycline is the drug of choice for the treatment of bacterial infection and growth promotion, but its extensive use has contributed to the emergence of resistance (Mulamattathil et al., 2000; Prescott et al., 2002; Threlfall, 2002; Choudhary, 2004; Falsafi et al., 2009). On the contrary, resistance to gentamycin and streptomycin was low for these isolates. These drugs are really used on animals in the clinic.

In conclusion, the identification of multiple antibiotic resistance among the isolates ignites the need to establish appropriate testing procedures. This is motivated from the fact that bacterial that harbour antibiotic resistance determinants can be easily transferred from companion animals and the owners.

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