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ANTIBIOGRAM OF ENVIRONMENTAL ISOLATES OF ACINETOBACTER CALCOACETICUS *FROM NKONKOBE MUNICIPALITY, SOUTH AFRICA*

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ABSTRACT

The antibiogram of *Acinetobacter* isolates from freshwater and soil samples in Alice and Fort Beaufort towns in Nkonkobe Municipality, South Africa were assessed for their extended beta-lactamase (ESBL) spectrum. Eighty-six *Acinetobacter* isolates were obtained from the 50 samples of soil and 50 samples of water (25 in each location) analysed. The resistance of the Acinetobacter isolates ranged between 30-100% against penicillin G, ceftriazone, nitrofurantoin, erythromycin and augmentin, while 9% showed intermediate response to minocycline, and 10% were resistant to oxytetracycline. Both *Tet B* and *Tet 39* were detected in 66.7 % of the tetracycline resistant *Acinetobacter* isolates and in 44.4 % of the intermediately tetracycline resistant *Acinetobacter* isolates. An observation of 9.3% phenotypic expression of ESBLs was made while 3.5% were carrying the bla$_{CTX\text{-}M\text{-}1}$ gene; all of which were susceptible to the fluoroquinolone. The multiple antibiotic resistance (MAR) index of > 0.2 indicates that the isolates emerged from high-risk sources, in line with conventional standards. Commensal *Acinetobacter spp* in the environment have proven to be one of reservoirs for antibiotic resistance genes.

KEYWORDS:
Acinetobacter, Commensal Antibiogram, *TetB, Tet39,* ESBLs, opportunistic infection

INTRODUCTION

Acinetobacter species have been isolated from waterbodies, soil, sewage, fast foods and hospital fomites and are commensals in humans and animals [1, 2]. More than 30 species are known, but *Acinetobacter baumannii* is the most common and is of high clinical and sub-clinical importance [1]. *A. baumannii* are part of the normal bacterial skin flora but can cause various types of opportunistic infections among immunocompromised individuals [2]. Other species like i e *A. iwofii, A. junii, A. calcoaceticus, A. radioresistens, A. haemolyticus* [3] were regarded as true commensals and not harmful to immuno-competent humans and animals [4], but are now being implicated in infection in immunocompetent [1, , 5].

Resistance to antibiotics, serves as a major setback in the treatment of *Acinetobacter* infections [6]. The bacteria exhibit resistance to a wide range of antibiotics, from β-lactams including several antibiotics within the cephalosporin and carbapenem groups and to aminoglycosides and quinolones [7]. Zarakolu *et al*. [8] reported an incidence of 67% multiple antibiotic resistant *A. baumannii* strains, which will result in therapeutic failures [9].

Acinetobacter species may acquire novel resistance genes from possibly distantly related species; thus positioning them as important candidates for the evaluation of reservoirs of antibiotic resistance in the environment or even in human subjects [10]. The diverse uses of tetracycline have encouraged extensive studies into the resistance mechanisms. Several reports which encompass efflux- and ribosome-based resistance mechanisms relates also to first- and second-generation tetracyclines [11]. The acquisition of new genes has been recognized as a factor responsible for the emergence of the resistances, which have also been observed in isolates from aquatic sources, vegetables, sewage, and the hospital environment [2, 12-14].

Five tetracycline resistance genes have been identified in *Acinetobacter*; *tetA, tetB, tetH, tet39* and *tetM*. Resistance to tetracycline, cephalosporins and some other antibiotics are enzyme mediated, where the production of extended-spectrum β-lactamases (ESBLs) is one group. ESBLs are known for their indispensable mechanism of resistance to several different types of antibiotics, especially the β-lactam, broad spectrum cephalosporins and carbapenem antibiotics in Gram-negative bacteria. Naas *et al*. [15] reported the presence of PER-1 type ESBLs in *A. baumannii* and Nagano *et al*. [28] the occurrence of CTX-M-2

type ESBLs. The presence of CTX-M ESBLs makes the bacteria resistant to cefotaxime and sometimes to ceftazidime. The enzyme, of CTX-M ESBLs enhances the ability of the bacteria to resist high profile extended spectrum antibiotics like carbapenem [16]. The CTX-M β-lactamases are plasmid-borne. Also, VEB-1 type ESBLs has been found in *A. baumannii* where it is chromosomally borne on integron1 similar to those in *Pseudomonas aeruginosa* [32]. The integron determines the source and methods of dissemination among *A. baumannii*. [32, 33]. VEB-1 type ESBLs has been reported in many isolates from hospital environments including France, Belgium and Argentina.

This study evaluates the antibiogram characteristics of environmental *Acinetobacter* strains isolated from the Nkonkobe Municipality as well as the presence of *tetA, tetB, tetH, tetM* and *tet39* genes in their genomes and their ESBLs status

MATERIALS AND METHODS

Study Location and samples collection. Nkonkobe Municipality is a highly populated black settlements of the Eastern Cape Province, South Africa, with a population of about 128 658 where only about 20% resides in urban settlements, mostly in Alice and Fort Beaufort towns. Twenty-five samples each of soil and water in Alice and another twenty-five samples each of water and soil samples were collected from Fort Beaufort. Sand-loamy soil samples of about 15 g from the surface to a depth of 20 cm [36] were collected aseptically into sample bottles while about 1 litre of surface water per sampling point was collected and transported to the laboratory under ice. A measure of 10% (w/v) soil suspension was made and shaken for 15 min on a rotary shaker [17] in preparation of 1 g soil in 10 L of sterile distilled water for preliminary isolation.

Primary Isolation. Primary isolation was done following Culbreath *et al.* [38] with modification as follow. Five mL aliquots of water and the prepared soil suspensions respectively were inoculated into 10 mL sterile double strength nutrient broth and incubated at 37 ^{o}C for 24 hours. Thereafter the broth cultures were aseptically streaked onto CHROMagar™ Acinetobacter (Chromagar, Paris, France) for initial isolation of *Acinetobacter sp.* colonies which appears as large red colonies while other Gram-negative bacteria, Gram-positive bacteria and yeasts are inhibited. Occasionally, *Stenotrophomonas maltophilia* may grow on this medium, but with much smaller colonies than *Acinetobacter* species.

Characterization of the isolates. The presumptive Acinetobacter colonies from the CHROMagar™ plates were randomly sub-cultured on fresh plates, purified on nutrient agar plates and Gram stained. The Gram-negative rods were further characterized for oxidase production, using the oxidase test kit (Sigma-Aldrich, Germany). Oxidase negative isolates was analysed with API 20 NE (Bio'Merieux). Multiplex PCR based on species-specific gyrB primers of Higgin et al. , was used to confirm the identification (Table 1).

Antibiotic Susceptibility Test (AST). The phenotypic antibiotic testing was done according to CLSI guidelines [40, 41]. Thirteen standard antibiotics (MAST Diagnostics, Merseyside, United Kingdom) were employed in this assay including penicillin G (11U), imipenem (30 µg), meropenem (30 µg), amoxicillin - clavulanic acid (20 µg +16µg), trimethoprim - sulphamethoxazole (1.25 µg + 23.75 µg), nalidixic acid (5 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg),

TABLE 1
Multiplex PCR based on species-specific gyrB primers

Primer	Sequence (5′-3′)	Species	Amplicon size (bps)
gyrB-2	CTTACGACGCGTCATTTCAC	*A. calcoaceticus* and GS3 reverse sequencing primer	
D14	GACAACAGTTATAAGGTTTCAGGTG	*A. calcoaceticus*	428
D19	CCGCTATCTGTATCCGCAGTA	*A. calcoaceticus*	428
D16	GATAACAGCTATAAAGTTTCAGGTGGT	GS3	194
D8	CAAAAACGTACAGTTGTACCACTGC	GS3	194
Sp2F	GTTCCTGATCCGAAATTCTCG	*A. baumannii*	1,194
Sp4F	CACGCCGTAAGAGTGCATTA	*A. baumannii* and GS13TU	1,194
Sp4R	AACGGAGCTTGTCAGGGTTA	*A. baumannii* and GS13TU	

ceftriaxone (30 µg), cefotaxime (30 µg), augmentin (30 µg), erythromycin (10 µg), chloramphenicol (30 µg), minocycline (10 µg), oxytetracycline (10 µg) and test strips (Oxoid Diagnostics, England).

Multiple Antibiotic Resistance Index (MARI). The MARI was calculated as the ratio of the number of the antibiotics to which resistance occurred by the isolates (a) to the total number of antibiotics to which the isolates were exposed (b), i.e: MARI= a/b

Phenotypic Extended Spectrum Beta-Lactamase (ESBLs) activity. The double disc synergy test (DDST) for phenotypic assessment of ESBLs production was employed in line with the protocol of Bradford [43]. An amoxicillin-clavulanate disc was placed at the center and the four 3rd generation cephalosporins which includes ceftazidime, cefotaxime, ceftriaxone, cefpodoxime (30 µg each) were placed at distance of 15 mm from the centre and incubated for 24 hours at 37 ºC. The observation of synergistic inhibition zone between cluvulanic acid bearing disc and any of the third generation cephalosporins was interpreted as positive. Those without such synergy were interpreted as negative.

PCR-based Assessment of Tetracycline Resistance and ESBLs genes. Extended spectrum β-lactamases' genes and tetracycline resistance genes were assessed using the primers in Tab 2 and 3 at the appropriate PCR conditions stated in the respective tables.

TABLE 2
Primers for detection of CTX-M 1 VEB Extended spectrum beta-lactamase genes in Acinetobacter spp

Primer	Primer Sequence	PCR conditions	Size bp	Target genes	Primer name	Sequence 5'→3'	PCR conditions	size (bp)
CTX-M-1f	5' GACGATGTCACTGGCTGAGC -3'	initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C and extension at 72°C for 1 min, and a final extension at 72°C for 3 min	490	*tetA*	*tetA-1*	GTAATTCTGAGCACTGTCGC	Denaturation for 3 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C. This was concluded with a final extension within 10 min at 72°C	957
CTX-M-1r	5'-AGCCGCCGACGCTAATACA -3'				*tetA-2*	CTGCCTGGACAACATTGCTT		
VEB-f	F:5'-ACGGTAATTTAACCAGATAGG-3'	denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 46ºC for 1 min and extension at 72ºC followed by a further extension at 72ºC for 10 min	970	*tetB*	*tetB-1*	CTCAGTATTCCAAGCCTTTG	Initial denaturation of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by final extension of 10 mins at 72°C	415
VEB-r	R:5'-ACCCGCCATTGCCTATGAGCC-3'				*tetB-2*	ACTCCCCTGAGCTTGAGGGG		
tetH-1	ATACTGCTGATCACCGTATAGATG	initial denaturation at 94°C for 5 mins, which was followed by 30 cycles consisting of 94°C for 30 s, annealing at 65ºC for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 7 min	1175	*tet39*	*tet39-1*	CTCCTTCTCTATTGTGGCTA	Initial denaturation of 3 mins at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by final extension of 10 mins at 72°C.	701
tetH-2	TCCCAATAAGCGACGC				*tet39-2*	CACTAATACCTCTGGACATCA		
tetM-1	GTTAAATAGTGTTCTTGGAG	initial denaturation at 94°C for 5 mins, which was followed by 30 cycles consisting of 94°C for 30 s, annealing at 64oC for 30 s, and extension at 72°C for 30 s. It was concluded with a final extension at 72°C for 7 min.	700					
tetM-2	CTAAGATATGGCTCTAACAA							

TABLE 3
Results of Acinetobacter isolates identified (N=sample size)

Location/ Species	Alice (N=50) No (%)	Fort Beaufort(N=50) No (%)	Total
A. calcoaceticus	32 (41.0)	46 (59.0)	78
A. *haemolyticus*	-	8 (100)	8
Total	32	54	86

RESULTS

After identification API and PCR primers in Tab 1), eighty-six *Acinetobacter* isolates were further assessed. All isolates from Alice town samples belonged to the *Acinetobacter calcoaceticus* complex, which in turn formed 41 % of the total *Acinetobacter* isolates in this study. About 85 % (59 % of total) of the Fort Beaufort isolates were *Acinetobacter calcoaceticus*, while the remaining 15% were *Acinetobacter haemolyticus* (Table 3).

Very high resistance were observed to penicillin G (100 %), nitrofurantoin (90 %), ceftriaxone (44.4%), erythromycin (20 %), imipenem (20 %), meropenem (10%). Similarly, 9 isolates were resistant to oxytetracycline, out of which 8 were intermediate in response to minocycline (Table 4). All the bacterial isolates showed high level MAR index (>0.2) ranging from 0.22-0.67 (Figure 1).

TABLE 4
Antibiogram Characteristics of the Acinetobacter isolates (N=86)

Antibiotics	S (%)	I (%)	R (%)
Penicillin G	0	0	100
Ceftriazone	56.6	0	44.4
Meropenem	80	10	10
Imipenem	70	10	20
Nalidixic Acid	85	5	10
Ciprofloxacin	100	0	0
Ofloxacin	100	0	0
Levofloxacin	100	0	0
Erythromycin	50	20	30
Chloramphenicol	80	10	10
Augmentin	70	0	30
Nitrofurantoin	10	0	90
Cotrimoxazole	80	0	20
Minocycline	91	9	0
Oxytetracycline.	90	0	10

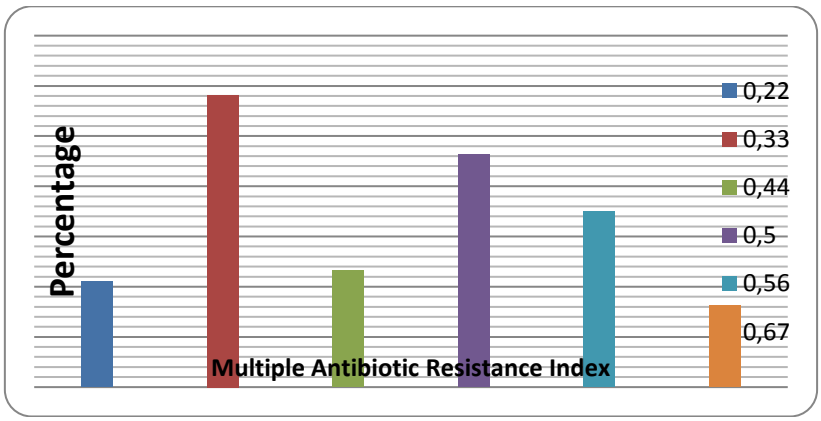

FIGURE 1
Multiple Antibiotic Resistant Index and the corresponding percentage of *Acinetobacter* Isolates

TABLE 5
Occurrence of tetracycline resistance genes in the phenotypically resistant isolates.

S/N	Tetracycline Resistance Genes (from resistant isolates to oxytetracycline + intermeditate resistant isolates to minocycline)	Detection of genes	Percentage of the resistant isolates with genes
1	*TetA*	_	0
2	*TetB*	+	66.7
3	*TetH*	_	0
4	*TetM*	_	0
5	*Tet39*	+	44.4

ey: - (absent); + (present).

The tetracycline resistance genes were all assessed on genomic DNA, where six isolates were positive to *TetB*. The observed phenotypic expression reveals that 66.7 % (55/81) were positive for the gene. Similarly, the presence of recently described novel tetracycline genes, *Tet39* in 44.4 % (26/81) of the phenotypically resistant was observed (Table 5). Also, 12 of the isolates showed phenotypic extended spectrum beta-lactamases (ESBLs) activity. When the 12 phenotypic ESBLs positive isolates were assessed, three were positive for $bla_{CTX\text{-}M\text{-}1}$ genes, while none was positive for $bla_{\text{VEB-1}}$ gene. No difference was observed between the resistance in soil isolates and water isolates.

DISCUSSION AND CONCLUSIONS

Acinetobacter baumannii and *Acinetobacter haemolyticus* have been implicated in nosocomial infection [2] and have been nicknamed "Gram-negative MRSA" [18]. The wide range of resistance as demonstrated in this study and elsewhere is a cause for concern. The high incidence of resistance to β-lactam antibiotics (Table 5) from environmental samples corroborates with findings from the hospital environment [19]. High susceptibility of 91 % to minocycline by the isolates is justified because it has been reported as an effective alternative against strains resistant to doxycycline, tetracycline and imipenem [20, 21]. This is further corroborated by Bishburg and Bishburg [51] who reported that *Acinetobacter baumanni* exhibited 86.9% susceptibility to minocycline and 81% susceptibility to imipenem.

The resistance of the *Acinetobacter* species to the β-lactam antibiotics used in this study, including the cephalosporins and the carbapenems, might be due to the presence of Extended Spectrum Beta-Lactamase (ESBLs). Some $bla_{CTX\text{-}M}$ alleles are of special concern when their distribution in various geographical regions is considered. CTX-M-2 for instance is found in many places like Argentina in South America and Japan in Asia [23]. In most of these places, there were concomitant reports of fluoroquinolone resistance [24]. However, in this study, high fluoroquinolone susceptibility by the *Acinetobacter* isolates including the ESBLs producers were observed. This makes fluoroquinolone a drug of choice in clinical situations involving these isolates. An MAR index > 0.2 implies that isolates are from high-risk sources [25] which was the case in this study, probably from the wastewater leading to high antibiotic resistance selective pressure [25].

The observed isolates with *TetB* and *Tet39* justify the resistance observed. he *TetB* gene and *Tet*39 gene code for resistance to tetracycline and its derivatives have been demonstrated in *Acinetobacter* species [26]. *Tet39* positive *Acinetobacter* isolates has earlier been reported from water samples and is usually spread by horizontal transmission of plasmids [27], while *TetB* has been reported from clinical isolates of *A. baumannii* [28]. *TetB* genes are specifically important in conferring resistance to tetracycline and minocycline [11]. The *tet* gene mobility depends on where they reside, for example *Tet* efflux genes of some Gram-negative reside on transposons that are inserted into plasmids originated from mostly conjugative incompatibility groups [27].

These identified determinants are not only of concern in the Acinetobacter. Their gene transfer by any method to other bacteria are of great concern to human health. This emphasizes the need to adhere to strict rules of personal and general hygiene to reduce the risk of opportunistic infection by such difficult to control bacteria [9]. Cases of Acinetobacter infection have been traced to environment due to poor hygiene, especially hand hygiene.

The results of this study showed that the commensal *Acinetobacter* species present in the soil and water environment of Nkonkobe municipality, South Africa, were resistant to many conventional antibiotics. High MAR index and production of extended spectrum beta lactamase suggest their sources to be of potential threat to public health while the presence of tetracycline resistance genes and the $bla_{CTX\text{-}M\text{-}1}$ genes among the bacteria showed them as reservoirs for resistance genes transferable to other bacteria in the environment.

Conflict of Interest Statement: All authors have no conflict of interest

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