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Antibiotic Resistance Patterns and Virulence Factors of Coagulase Negative *Staphylococcus* Associated with Urinary Tract Infections in Bulawayo Province, Zimbabwe

Joshua Mbanga[1*], Sithabile Masuku[2] and Silence Luphahla[2]

[1]Department of Applied Biology and Biochemistry, National University of Science and Technology, P.O.Box AC939 Ascot, Bulawayo, Zimbabwe.
[2]Premier Diagnostic Laboratories, P.O.Box 2025 Bulawayo, Zimbabwe.

Authors' contributions

This work was carried out in collaboration between all authors. Author JM was responsible for the project design, was the principle supervisor and carried out experimental work on the project. Authors SM and SL carried out experimental work on the project and assisted author JM in aspects of the project relating to laboratory work. All authors contributed to writing of this article. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the antibiotic resistance patterns and virulence factors of coagulase negative *Staphylococcus* (CoNS) associated with urinary tract infections (UTIs). The virulence factors assayed for were the *atl* E and *ica* AB genes. The prevalence of the antibiotic resistance gene, *mec* A, was also determined.
Place and Duration of Study: Southern Pathology Clinical Laboratories and the National University of Science and Technology microbiology department, between December 2012 and March 2015.
Methods: A total of 754 urine samples were analyzed for bacteria by standard procedures. From

**Corresponding author: Email: joshmbanga@gmail.com, joshua.mbanga@nust.ac.zw;*

these, 126 isolates were positively identified as CoNS. Antimicrobial susceptibility testing of the isolated CoNS was done using the disc diffusion method.The polymerase chain reaction (PCR) was also carried out to detect the presence of the *mec* A, *ica* AB and *atl* E genes.

Results: Antibiogram profiles showed that CoNS had high prevalences of resistance to nalidixic acid (88.1%), cotrimoxazole (72.2%) and oxacillin (69.8%).There were however high prevalences of sensitivity to nitrofurantoin (79.4%) and gentamycin (68.3%). A total of 106 (84%) isolates were resistant to three or more antibiotics and 12 multi-drug resistance patterns were observed. The most common pattern (resistance to nalidixic acid, ampicillin, oxacillin, tetracycline and cotrimoxazole) was exhibited by 33 isolates. A total of 40 CoNS isolates were then used to determine the prevalence of the *mec* A, *ica* AB and *atl* E genes. PCR results showed that most isolates 25/40 (62.5%) were positive for the *mec* A gene. The *ica* AB and *atl* E were detected in 32.5% and 25% of the isolates respectively. All isolates which were positive for both the *mec* A and *ica* AB genes showed resistance to multiple antibiotics.

Conclusion: There is emerging antibiotic resistance in CoNS that cause UTI's. The occurrence of both the *mec* A and *ica* AB genes in CoNS isolates may lead to an increase in antibiotic resistance.

Keywords: CoNS; antibiotic resistance; virulence genes; Zimbabwe.

1. INTRODUCTION

Coagulase-negative staphylococci (CoNS) have long been regarded as non-pathogenic but their important role as pathogens and their increasing incidence has been recognized and studied in recent years. As a group, the CoNS are among the most frequently isolated bacteria in clinical microbiology laboratories and are becoming increasingly important pathogens especially as causes of hospital acquired infections. These bacteria are normal inhabitants of the human skin and mucous membrane and were normally considered as contaminants when isolated [1]. Therefore, one of the major challenges of diagnostic work is to distinguish clinically significant CoNS from contaminant strains. CoNS have emerged as a major cause of urinary tract infections due to the combination of an increased number of hospitalised and immune-compromised patients and the high number of nosocomial UTI's associated with the use of urinary catheters [2,3]. The microbial etiology of UTI's is however still regarded as being well established and reasonably consistent with *Escherichia coli* remaining the predominant cause of all UTI's worldwide followed by CoNS [4].

The worldwide increase in antibiotic resistance is a public health concern. The frequency of oxacillin resistance in CoNS strains has increased substantially over recent decades [5] and CoNS are typically resistant to multiple drug classes [6]. Increasing resistance of CoNS to commonly used antibiotics like glycopeptides, aminoglycosides and macrolides has been reported in different countries around the world [7,8]. Knowledge of the antibiotic susceptibility patterns of microorganisms is very important as it reduces unnecessary expenses, reduces development of resistance to useful and life saving antibiotics and also minimizes many side effects. The susceptibility patterns tend to vary from one country to another and within a country as antibiotic prescribing patterns and strains vary.

The pathogenesis of CoNS species comes from the production of an impressive repertoire of virulence factors [9]. These are properties coded for by specific genes and they enable microorganisms to establish themselves on or within a host. Some of these virulent genes include the *ica* gene (intercellular adhesion - operon *ica*) involved in biofilm formation and the *atl* E gene, which encodes the vitronectin-binding cell surface protein involved in primary attachment. The *mec* A gene controls the synthesis of the additional penicillin-binding protein PBP2a and is responsible for the resistance of some species to methicillin [10]. The *ica* gene is considered to be the main marker of virulence in CoNS. It has been found to be the most amplified gene in virulent strains of CoNS.

In this study, we obtained data on the antibiotic resistance patterns of clinical CoNS isolates associated with UTI's. We also investigated the occurrence of the *ica* AB, *atl* E and *mec* A genes and examined the correlation if any with multiple resistance to antibiotics. Despite the increasing incidence of CoNS, little work has been done to characterize them genotypically in Africa especially in Southern Africa. This study will therefore add to the available literature on CoNS.

2. MATERIALS AND METHODS

2.1 Sample Collection

In total, 126 CoNS strains were used in this study. Between the months of December 2012 to May 2013, 518 urine samples were obtained from patients visiting a leading diagnostic Medical Laboratory in Bulawayo. These samples were from symptomatic patients being tested for UTI's. Of the 518 urines only 86 were found to be positive for CoNS. Over a period of three months (October - December 2014) 40 residual CoNS isolates were obtained from the same diagnostic Medical Laboratory, these were from 236 patients with suspected UTI cases. Only one mid stream urine specimen per patient was included in the study.

2.2 Isolation and Identification of CoNS

The urine samples were cultured on Cysteine Lysine Electrolyte Deficient (CLED) agar and Blood agar plates (Oxoid, England) and incubated aerobically at 37ºC for 24 hours. Identification and evaluation of cultures was done visually and then followed by Gram staining and further biochemical tests. Biochemical tests carried out included the catalase and coagulase tests. The tube coagulase test was used to detect CoNS. The microbiology work was done mostly at Southern Pathology Clinical Laboratories.

2.3 Antimicrobial Susceptibility Testing

The disc diffusion method was used to determine antibiotic susceptibility of the isolates on Mueller Hinton agar (Oxoid, UK). Each isolate was tested for antibiotic susceptibility using a panel of the following antibiotics: Nitrofurantoin (50µg), Ampicillin (25µg), Tetracycline (30µg), Nalidixic Acid (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Oxacillin (5µg) and Gentamycin (10µg). All antibiotic disks were from Oxoid, United Kingdom. The plates were incubated at 37ºC for 24 hr, and inhibition zones were measured. Interpretation of results followed criteria recommended by the Clinical Laboratory Standard Institute [11].

2.4 DNA Isolation

Bacterial strains were subcultured at 37℃ overnight in Luria-Bertani (LB) broth (Oxoid, Basingstoke, Hampshire, UK) and genomic DNA was extracted using a standard Phenol-Chloroform method [12]. To check for purity, DNA was run along a 1% ethidium bromide stained agarose gel (Sigma-Aldrich, St Louis, USA) with a 1kb DNA ladder (Thermo Scientific, USA) in TBE buffer for 1hr at 100V and then viewed using a Uvipro-Silver Gel Documentation System (Uvitec, UK). The concentration of DNA was estimated by comparing the band light intensity to the band intensity on the 1kb ladder on the Uvipro-Silver Gel Documentation System. DNA concentration of samples ranged from 75 ng / 0.5 µg – 100 ng / 0.5 µg.

2.5 PCR for Detection of *mec* A, *ica* AB and *atl* E Genes

Single PCR was done to amplify the different genes. All PCR work was done at the National University of Science and Technology (NUST). Due to financial limitations, only the 40 CoNS isolated in 2014 were subjected to *mec* A, *ica* AB and *atl* E PCR using primers described previously [13]. Similar reaction conditions were used for all 3 genes.

A volume of 1.2 µl of DNA sample was mixed with all the necessary components for amplification in a 0.2 ml PCR tube (Perkin-Elmer, USA) in a 10 µl reaction. The reaction mixture included 1 µl PCR buffer (Thermo Scientific, USA), 0.4 µl deoxynucleotide triphosphate (dNTPs) with a concentration of 10 mM, 0.4 µl magnesium chloride of concentration 2.5 mM, 0.2 µl reverse primer, 0.2 µl forward primer both of concentrations 0.5 mM and 1.2 µl Taq DNA polymerase (Thermo Scientific, USA). This was then topped up to 10 µl using nuclease free water. Negative controls comprised of a water control. An Applied Biosystems GeneAmp® PCR System 9700 was used for the PCR thermal cycling conditions. The PCR profile was as follows: denaturation at 98ºC for 3 minutes, 35 cycles {denaturation at 94 for 1 minute, annealing at 60 for 45 seconds, extension at 72 for 45 seconds} and then a final extension at 72 for 10 minutes. The final products were run on a 1% ethidium bromide stained gel with a 100 bp ladder (Thermo scientific, USA) in TBE buffer for 1hr at 100V and then viewed using a Uvipro-Silver Gel Documentation System (Uvitec, UK).

3. RESULTS AND DISCUSSION

A total of 754 urine samples were obtained from patients with suspected UTI's at a leading diagnostic laboratory in Bulawayo and 126 (16.7%) were positive for coagulase negative *Staphylococcus*. All 126 isolates were identified

and confirmed to be CoNS through culturing and biochemical tests. The isolated CoNS strains were subjected to the disc diffusion assay which was a modification of the Kirby-Bauer method [14]. The CoNS were assayed against a panel of eight antibiotics and the results obtained are shown in Table 1. A high level of antibiotic resistance was observed, as many isolates exhibited resistance to more than one antibiotic. Most CoNS isolates were, however, sensitive to nitrofurantoin, gentamicin and ciprofloxacin (Table 1).

The prevalence of antibiotic resistance phenotypes of 106 (84%) of the CoNS isolates is presented in Table 2. These 106 isolates were resistant to at least 3 antibiotics, that is multi drug resistance (MDR), and 12 different antibiotic resistance patterns were observed. The most common resistance pattern, exhibited by 33 isolates was resistance to nalidixic acid, ampicillin, oxacillin, tetracycline and cotrimoxazole (pattern I) and the least common resistance pattern was pattern J exhibited by only 2 isolates (Table 2).

In our study the overall prevalence of CoNS was found to be 16.7%, this prevalence rate is similar to those from previous studies [15,16], which reported prevalences of 16.5% and 16.1% respectively. Coagulase negative Staphylococci have emerged in recent years as pathogens in a growing number of serious nosocomial infections as well as community based infections including UTI's. Hospitals and communities have been struggling with increasing numbers of multi drug resistant methicillin resistant coagulase negative staphylococci (MRCoNS). Treatment is especially difficult due to biofilm formation and frequent antibiotic resistance. However, virulence mechanisms of these important opportunistic pathogens have remained poorly characterized.

Resistance profiles of the CoNS strains against the 8 antibiotics are presented in Table 1. The resistance to oxacillin was 69.8 %, gentamycin, 31.7%, nitrofurantoin, 19%, ciprofloxacin, 33.3% and cotrimoxazole, 72.2%. However higher percentage resistances have been reported using the same antibiotics forinstance in one study CoNS were 100% resistant to gentamycin and had the following resistance rates; nitrofurantoin 90.2%, ciprofloxacin 56.4% and cotrimoxazole 67.3% [17]. A possible reason for the higher resistance rates might be due to the fact that the strains that were used in the study [17] were isolated from urines obtained entirely from catheter tips. Catheter- related species have been reported to be more resistant to antibiotics than non -catheter related species [17-19]. Another study [20] also reported higher percentage resistances. In their study the resistance to gentamycin was 90%, cotrimoxazole, 68% and ciprofloxacin, 59%. However the isolates used in their study were all collected from patients who were hospitalized. According to literature, CoNS strains of nosocomial origin are more resistant to antibiotics compared to community based strains [3]. Most of the isolates used in our study were community acquired and not from hospitalized patients.

The resistance to gentamycin (31.7%) observed in our study was similar to the mean resistance (29.5%) observed in another study on 745 CoNS isolates in China [5]. However the low resistance to ciprofloxacin (33.3%) observed in our study varied from their mean resistance of 52.8%. In a different study in which they analyzed trends in antibiotic resistance in CoNS in the United States from 1999 to 2012, they found that resistance to ciprofloxacin increased from 58.3% in 1999 to 68.4% in 2012 [8], both these figures are higher that the resistance rates for ciprofloxacin in our study. These differences could relate to differences in antibiotic prescribing patterns which tend to vary in different parts of the world.

Table 1. Antibiotic susceptibility profiles of CoNS isolates from urinary tract infections

Antibiotic (conc. µg)	Resistant no. (%)	Intermediate no. (%)	Susceptible no. (%)
Ciprofloxacin (5)	42 (33.3)	0 (0)	84 (66.7)
Gentamycin (10)	40 (31.7)	0 (0)	86 (68.3)
Nitrofurantoin (50)	24 (19)	2 (1.6)	100 (79.4)
Cotrimoxazole (25)	91 (72.2)	1 (0.8)	34 (27)
Ampicillin (25)	45 (35.7)	0 (0)	81 (64.3)
Tetracycline (30)	79 (62.7)	2 (1.6)	45 (35.7)
Oxacillin (5)	88 (69.8)	1 (0.8)	37 (29.4)
Nalidixic acid (30)	111 (88.1)	1 (0.8)	14 (11.1)

In our study the percentage prevalence of MRCoNS was found to be 69.8% (Table 1). This percentage prevalence is in agreement with many studies, including those done in Africa. A Study done in Nigeria reported a 66% prevalence of MRCoNS [21]. Prevalences of 72% [22] and 69.5% [23] were also reported in two studies done in Turkey. These results re-affirm that CoNS are indeed highly resistant to the antibiotic oxacillin.

Table 2. Antimicrobial resistance patterns of the CoNS isolates

Pattern	Number of isolates	Resistance pattern
A	3	Na, Amp, Ox
B	5	Cot, Ox, Gent
C	4	Cot, Ox, Ni
D	7	Cot, Ox, Cip, Gent
E	7	Cot, Ox, Cip,Ni, Gent
F	18	Na, Amp, Ox, Te
G	3	Na, Amp, Ox, Cip
H	12	Na, Ox, Te, Cot
I	33	Na, Amp, Ox, Te, Cot
J	2	Amp, Na, Ox, Te, Gent
K	3	Ox, Ni, Te, Co, Na
L	9	Amp, Na, Cot, Cip, Ox, Te

**Amp: Ampicillin; Na: Nalidixic acid; Ox: Oxacillin; Cip: Ciprofloxacin; Cot: Cotrimoxazole; Gent: Gentamycin; Ni: Nitrofurantoin; Te: Tetracycline*

Eighty four percent of CoNS isolates showed resistance to 3 or more antibiotics and 12 MDR patterns were observed. Patterns F, H and I were the most common patterns (Table 2). The results of our study suggest that multiple drug resistance is widespread among local CoNS isolates associated with uti's. These observations are in agreement with the reports of several studies [5,8,24] on increasing antibiotic resistance in staphylococci.

Forty CoNS isolates, corresponding to the last 40 isolates to be collected in 2014, were used to assay for the prevalence of the *mec* A, *ica* AB and *atl* E genes. Simple PCR was used to detect the three assayed genes using specific primers (Figs. 1 and 2).

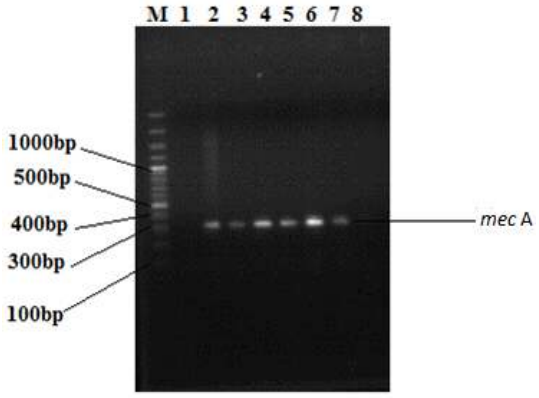

Fig. 1. *mec* A gene

Lane M indicates the lane containing the marker, GeneRuler 100 bp Plus DNA Ladder (Thermo scientific, USA), while lane 1 indicates the negative control. Other lanes show positive amplification of 6 isolates

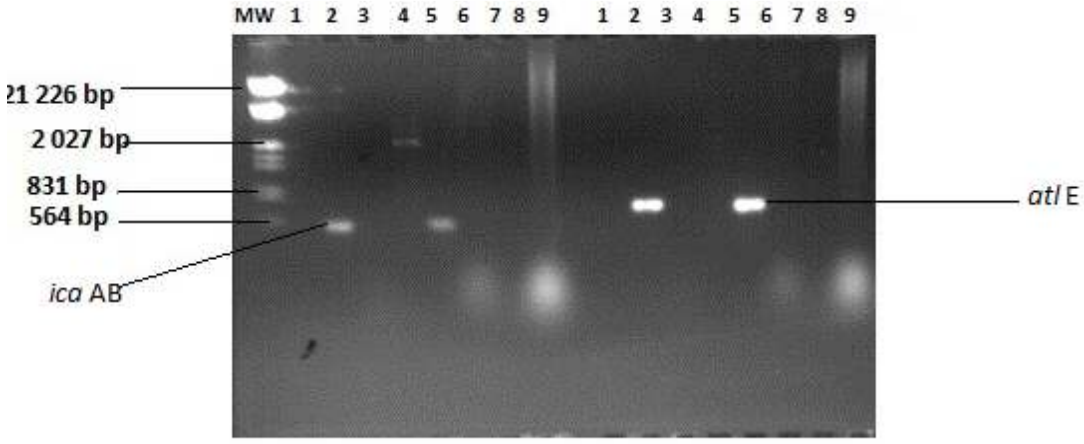

Fig. 2. *ica* AB & *atl* E amplification

Lane MW indicates the lane containing the marker, GeneRuler 1kb DNA Ladder (Roche, USA), while lane 1 indicates the negative control. Other lanes 2 & 5 show positive amplification of the ica AB (546 bp) and lanes 2, 6 (right side) positive amplification of atl E (682 bp)

The *mec* A gene is used as an indicator of methicillin/oxacillin resistance. Of the 29 isolates that were oxacillin resistant using the disc diffusion assay, 25 were positive for the *mec* A gene (Table 3). The prevalences of the 2 assayed virulence genes were determined to be 32.5% (*ica* AB) and 25% (*atl* E) (Table 3).

Table 3. Prevalence of the *mec* A, *ica* AB and the *atl* E genes for 40 CoNS isolates

CoNS isolate	*mec* A	*ica* AB	*atl* E
1	-	-	-
2	-	-	+
3	+	-	+
4	+	+	-
5	-	-	-
6	+	+	+
7	-	-	-
8	+	-	+
9	+	-	-
10	-	-	-
11	+	-	-
12	+	+	+
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	+	-	-
19	+	-	-
20	+	+	+
21	+	+	-
22	-	-	+
23	+	-	-
24	+	-	-
25	+	-	+
26	+	-	+
26	+	-	-
27	+	+	-
28	+	+	-
29	+	-	-
30	-	-	-
31	+	+	-
32	+	-	-
33	+	-	-
34	+	+	-
35	-	+	-
36	-	-	-
37	+	+	-
38	-	+	+
39	-	+	-
40	+	-	-
Total	25/40 (62.5%)	13/40 (32.5%)	10/40 (25%)

Presence (+) OR absence (-) of the expected amplicons for the mec A, ica AB and atlE genes

The *mec A* gene determines resistance to methicillin in Staphylococci species [25]. It encodes the low-affinity penicillin-binding protein PBP 2A. The detection of the *mec A* gene from the CoNS isolates was 62.5% (Table 3). This high prevalence was in agreement with a study in which they found 60% (23/38) of their total isolates to be *mec A* positive [26]. Another similar study reported that 57% of CoNS isolates harbored the *mec A* gene [20]. In our study, out of the 29 isolates that showed resistance to oxacillin, only 25 (86%) were positive for the *mec A* gene. Our findings are similar to a study in India, where they found that only 80% of the methicillin resistant *Staphylococcus* strains exhibited the *mec* A gene. In another study [27], out of 88 clinical strains that were resistant to methicillin, only 80 expressed the *mec* A gene. However many studies have reported that all isolates that are resistant to oxacillin tend to be positive for the *mec A* gene [9,18,28].

Biofilms produced by CoNS consist principally of a polysaccharide intercellular adhesin which is encoded by the *ica* operon. In this study, 32.5% (13/40) isolates were positive for the *ica* AB gene (Table 3). This result is consistent with other studies [29] who reported 33.3% amplification (10/30 isolates), and [20] who reported 32% (12/38 isolates) amplification of the *ica* AB gene.

Some studies have suggested that biofilm production may play a role in antibiotic resistance and that biofilm producing strains are more resistant to antibiotics than non-biofilm producing strains [30,31]. These assertions were supported by a study [20] that reported that 19 out of 59 CoNS isolates were positive for the *ica* AB gene and 15 of these were resistant to all the antibiotics used. In our study 9 out of 13 isolates that were positive for the *ica* AB gene showed resistance to almost all the antibiotics that were used (results not shown), however no direct relationship was found between the occurrence of the *ica* AB gene and resistance to antibiotics. Interestingly the 9 isolates that were positive for the *ica* AB gene and multi-drug resistant were also positive for the *mec* A gene (Table 3). These findings concur with the results of a study that reported that 58 clinical CoNS isolates that were positive for both the *ica* AB and *mec* A genes were resistant to multiple antibiotics [13]. In addition to the association of antibiotic resistance with isolates found to be positive for both the *ica* AB and *mec* A genes, the study also concluded that isolates that harboured both genes appeared to be the best distinguishing factor between infectious and contaminating strains [13].

The *atl* E gene is involved in primary attachment during the formation of biofilm, and also in vitronectin binding activity *in vitro*. In this study, the *atl* E gene had a prevalence of 25% (Table 3). This is in contrast to other studies that have reported the ubiquitous amplification of the *atl* E gene in both clinical and contaminating CoNS strains [13,32]. The *atl* E gene occurred with either the *mec* A or *ica* AB in all 10 isolates in which it was detected suggesting its products also play a role in virulence.

4. CONCLUSION

Results obtained in our study suggest that CoNS that are associated with UTI's are becoming resistant to multiple antibiotics that are commonly used against them. The possibility of using the presence of the *mec* A and *ica* AB genes as one of the criteria for distinguishing clinical from contaminating CoNS strains should be investigated further. Antibiotic susceptibility studies using antibiotics that are still relatively uncommon in Zimbabwe like vancomycin, teicoplanin, and oxazolidinones should be considered as resistance to these drugs has already been reported in other parts of the world. There is need for continued surveillance to determine the extent of emerging resistance in CoNS inorder to advance strategies to reduce inappropriate use of antibiotics and to allow for policies to be established for adequate and rational use of antibiotics.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The authors obtained all necessary ethical approval from Institutional Ethical Committee. This study was sanctioned by the NUST ethical committee and no names were recorded during the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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