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Original Article **Open Access**

Phenotypic and molecular detection of methicillin-resistance and virulence factors in coagulase-negative staphylococci isolated from patients with urinary tract infections in southeastern Nigeria

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Abstract:

Background: The emergence of antibiotic resistance among coagulase-negative staphylococci (CoNS) is an important factor in nosocomial infections outcomes in the healthcare system. This study aimed to phenotypically detect methicillin resistance and genotypically detect *mec*A gene and other virulence markers in coagulase-negative staphylococci isolated from patients with suspected urinary tract infection (UTI) in Alex Ekwueme Federal University Teaching Hospital (AE-FUTHA), Abakaliki, Ebonyi State, Nigeria.
Methodology: A total of 704 patients (adults and children) with clinical features suggestive of UTI, attending the Alex-Ekwueme Federal University Teaching Hospital, were randomly selected for the study. Urine samples were collected from the patients, cultured in mannitol salt agar (MSA) and CoNS identified using conventional microbiological methods. Genotypic confirmation of CoNS was done for 15 randomly selected isolates using the 16S rRNA gene amplification and Big Dye terminator sequencing. Phenotypic detection of methicillin resistance was done using cefoxitin disc diffusion test and confirmed by PCR amplification of a 533-base pair *mec*A gene fragment. Data were analyzed using SPSS version 27.0, and Chi-square test was used to determine association between qualitative variables obtained in this study.
Results: A total of 50 CoNS were isolated from the samples of 704 patients with suspected UTI, 29 (58.0%) of which were phenotypically methicillin-resistant-CoNS and included all the *mec*A gene positive isolates. Of the 15 confirmed CoNS by 16S rRNA sequencing, 4 were *Staphylococcus condimenti*, 4 were *Staphylococcus gallinarum*, 3 were *Staphylococcus simulans*, 3 were *Staphylococcus haemolyticus* and 1 was *Staphylococcus sciuri.*
Conclusion: The study recorded a high rate of methicillin resistance among CoNS isolates, indicating that there is need for consistent monitoring and evaluation of antibiotic use in hospital settings to curtail the concerning increase in prevalence of *mec*A gene-positive CoNS isolates.

Keywords: methicillin-resistance; coagulase-negative staphylococci; *mec*A; 16S rRNA; sequencing

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Détection phénotypique et moléculaire des facteurs de résistance à la méthicilline et de virulence chez les staphylocoques à coagulase négative isolés de patients atteints d'infections des voies urinaires dans le sud-est du Nigéria

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Résumé:

Contexte: L'émergence d'une résistance aux antibiotiques chez les staphylocoques à coagulase négative (CoNS) est un facteur important dans les résultats des infections nosocomiales dans le système de santé. Cette étude visait à détecter phénotypiquement la résistance à la méthicilline et à détecter génotypiquement le gène mecA et d'autres marqueurs de virulence chez les staphylocoques à coagulase négative isolés de patients suspectés d'infection des voies urinaires (IVU) à l'hôpital universitaire fédéral Alex Ekwueme (AE-FUTHA), Abakaliki, État d'Ebonyi, Nigéria.
Méthodologie: Au total, 704 patients (adultes et enfants) présentant des caractéristiques cliniques suggérant une infection urinaire, fréquentant l'hôpital universitaire fédéral Alex-Ekwueme, ont été sélectionnés au hasard pour l'étude. Des échantillons d'urine ont été prélevés sur les patients, cultivés dans de la gélose au mannitol et au sel (MSA) et les CoNS ont été identifiés à l'aide de méthodes microbiologiques conventionnelles. Français La confirmation génotypique de la CoNS a été effectuée pour 15 isolats sélectionnés au hasard en utilisant l'amplification du gène de l'ARNr 16S et le séquençage du terminateur Big Dye. La détection phénotypique de la résistance à la méthicilline a été effectuée en utilisant le test de diffusion sur disque de céfoxitine et confirmée par amplification PCR d'un fragment du gène mecA de 533 paires de bases. Les données ont été analysées à l'aide de SPSS version 27.0, et le test du Chi carré a été utilisé pour déterminer l'association entre les variables qualitatives obtenues dans cette étude.
Résultats: Un total de 50 CoNS ont été isolés à partir des échantillons de 704 patients suspectés d'infection urinaire, dont 29 (58,0%) étaient des CoNS phénotypiquement résistants à la méthicilline et comprenaient tous les isolats positifs pour le gène mecA. Parmi les 15 CoNS confirmés par séquençage de l'ARNr 16S, 4 étaient des *Staphylococcus condimenti*, 4 des *Staphylococcus gallinarum*, 3 des *Staphylococcus simulans*, 3 des *Staphylococcus haemolyticus* et 1 des *Staphylococcus sciuri*.
Conclusion: L'étude a enregistré un taux élevé de résistance à la méthicilline parmi les isolats de CoNS, ce qui indique qu'il est nécessaire de surveiller et d'évaluer régulièrement l'utilisation des antibiotiques en milieu hospitalier pour limiter l'augmentation inquiétante de la prévalence des isolats de CoNS positifs au gène mecA.

Mots-clés: résistance à la méthicilline; staphylocoques à coagulase négative; *mec*A ; ARNr 16S; séquençage

Introduction:

Nosocomial infections are complicated by the fact that antibiotic resistance (AMR) has developed in coagulase-negative staphylococci (CoNS) population (1). A number of researchers have recently concentrated on the capacity of CoNS to produce different types of extracellular enzymes. Enzymes such as proteases, phospholipases, esterases, lipases, and elastases are part of this group (1,2). There are some enzymes that are toxins such as those in the enterotoxins, haemolysins, exfoliative toxins, and toxic shock syndrome toxin-1 (TSST-1) families (3,4). These enzymes and toxins are virulence factors of staphylococci responsible for invasion and death of nearby tissues (5). In addition, β-lactam antibiotics are ineffective against methicillin-resistant CoNS (MR-CoNS) (6), and over 80% of hospital isolates of MR-CoNS have been shown to be multi-drug resistant (MDR) (7). These pathogens infect hosts and survive by employing a wide variety of strategies.

According to Kumurya (7), the health-care system is facing an increasingly critical problem with AMR in CoNS population. The purpose of this study was to phenotypically identify methicillin-resistant coagulase-negative staphylococci (MR-CoNS) in patients with suspected urinary tract infections (UTIs) at the Alex-Ekwueme Federal University Teaching Hospital, Abakaliki, Nigeria, and to detect *mec*A gene and virulence markers in the isolates.

Materials and method:

Study period, participants and sample collection:

Between April 2018 and March 2019, 704 inpatients and outpatients suspected of urinary tract infection at the Alex Ekwueme Federal University Teaching Hospital (AE-FUT HA), Abakaliki, Ebonyi State, Nigeria, were consecutively selected for the study.

Clean-catch mid-stream urine samples were collected from the patients into sterile

universal containers and transported immediately to the microbiology laboratory for analysis.

Ethical approval:

Approval to conduct the study was obtained from the Ethical and Research Committee of the Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Ebonyi State, with approval number FETHA/REC/VOL 2/2019/260

Culture and isolation of CoNS bacteria:

Standard bacteriological examinations were carried out on the urine samples. Using aseptic technique, each urine sample was cultured on mannitol salt agar (MSA) and aerobically incubated at 37^{o}C for 24 hours. In order to obtain pure colonies and determine the haemolytic pattern of the bacteria, the presumed CoNS colonies were sub-cultured onto fresh MSA and 5% sheep blood agar (3).

Gram staining, mannitol fermentation, catalase, coagulase, deoxyribonuclease, haemolysin, ornithine decarboxylase, pyrrolidonyl arylamidase, and other conventional biochemical tests were then performed on the isolates to phenotypically identify them to species level.

Phenotypic screening for virulence and detection of methicillin resistance:

Using blood agar, Tween 80, and Tween 20 as substrates, the isolates were examined for their ability to produce haemolysin, lipase, and esterase. Phenotypic methicillin resistance was detected on isolated CoNS by the cefoxitin disc diffusion method.

Molecular detection of CoNS isolates and 16S rRNA gene sequencing:

Molecular analysis (16S rRNA amplification sequencing and *mec*A gene amplification) was performed on 15 randomly selected CoNS isolates

DNA extraction:

The DNA miniprep (Zymo, catalogue number: D6005) was used for DNA extraction. A ZR bashing™ lysis tube was used to mix 2ml of bacterial cell broth with 750ml of lysis solution. The mixture was then centrifuged in a microcentrifuge at > 10,000 x g for 1 min. After transferring 400 µL of supernatant to a collection tube with an orange-top Zymo-Spin™ IV spin filter, it was spun at 7,000 x g for 1 minute. Next, 1,200 µL of DNA binding buffer was added to the mixture following filtration. After 800 µL of the mixture was spun in a collection tube at 10,000 x g for one minute, it was moved to a Zymo-Spin™ IIC column. The gathered flow was then discarded, and the last process was repeated.

The Zymo-Spin™ IIC column was mixed with 200 µL of DNA Pre-Wash buffer using a fresh collection tube, centrifuged at 10,000 x g for 1 minute, and the mixture subsequently applied. After a minute of spinning at 10,000 x g, the Zymo-Spin™ IIC column was washed with 500 µL of bacterial/fungal DNA wash buffer. Then, 100 µL of DNA elution buffer was mixed directly with the Zymo-Spin™ IIC column matrix after its transfer to a fresh 1.5 µL microcentrifuge tube. To extract the DNA, the mixture was spun in a centrifuge at 10,000xg for 30 seconds.

PCR amplification of 16S rRNA gene:

The following reagents were used to prepare the 16S rRNA gene PCR sequencing cocktail; 10 µL of 5x Go Taq colorless reaction, 1 µL of 10 mM of dNTPs mix, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 pmol each of the forward primer (27F: AGAGTTTGATCMTGGCTCAG) and reverse primer (1525R: AAGGAGGT GWTCC ARCCGCA), and 0.3 units of Taq DNA polymerase (Promega, USA) mixed with 8 µL of DNA template to achieve 42 µL solution.

The PCR assay was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR cycling condition consisting of an initial denaturation at 94°C for 5 min; followed by 30 cycles consisting of 94°C for 30secs, annealing of primer at 56^{o}C for 30secs and 72°C for one and half minutes, and a final termination at 72°C for 10 mins and chilled at 4^{o}C.

PCR amplification of mecA gene:

The *mec*A-specific primer pairs used to amplify a 533-base pair fragment were; forward 5′-AAAATCGATGGTAAAGGTTGGC-3′ and reverse 5′-AGTTCTGGAGTACCGGATTTGC-3′ (8). A volume of 1 µL of the prepared DNA (0.5 µg) was incorporated into a final volume of 25 µL PCR mixture, which consisted of 10 µL of 2× Master Mix (Ampliqon, Denmark), containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L dNTP, and 1.25 IU Taq DNA polymerase (Ampliqon Co., Denmark), along with 0.7 µL of 0.8 µmol/L of each primer and 12.6 µL of sterile distilled water.

The thermal cycling condition for PCR included an initial step at 95 °C for 3 minutes, followed by 33 cycles at 94°C for 1 minute, 53°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 6 minutes.

Agarose gel electrophoresis of PCR amplicons:

The agarose powder and 100 mL of 1 x TAE were mixed in a microwavable flask. Microwaving the mixture for one to 3 minutes dissolved the agarose completely. Ten µL of EZ vision DNA stain was added once the agarose solution had cooled to around 50^{o}C. After positioning the well comb on a gel tray, the agarose was carefully added. In order to ensure that the freshly poured gel solidified, it was allowed to sit at room temperature for 20-30 minutes or cooled to 4°C for 10-15 minutes.

The loading buffer was applied to all DNA samples and PCR products. The gel elect-

rophoresis box was used to transfer the set agarose gel. The gel box was filled to the top with 1xTAE (or TBE) after a molecular weight ladder was loaded into the first lane. A voltage range of 80 to 150 V was applied to the samples for about one to one and a half hours after they were delicately placed into the additional gel wells. The power was turned off and the electrodes were disconnected from the power source after the gel was carefully removed from the gel box.

The PCR amplicons were observed with the aid of the UV transilluminator. The gel band was compared to that of a hyper ladder1 (molecular weight ladder) to determine the sizes of the PCR amplicons. The amplicon has a size of about 1200 base pairs for 16S rRNA gene and 533 base pairs for *mec*A gene.

Purification of 16S rRNA gene amplicon:

The PCR reagents were removed by ethanol-amplified fragment removal following gel integrity. In a new sterile 1.5 µl tube from Eppendorf, 7.6 µL of Na acetate 3M and 240 µl of 95% ethanol were added to around 40 µL of PCR amplified result. After the mixture was thoroughly mixed by vortexing, it was kept at -20°C for at least 30 minutes to guarantee proper storage. Before being centrifuged for 15 minutes at 7500 g and 4°C, the pellet was rinsed with 150 µL of 70% ethanol and mixed after being inverted onto the trash once.

Centrifugation was then done for 10 minutes at 13,000 g and 4°C to remove the supernatant. After turning the tube upside down and placing it in the trash, the supernatant was collected again. Once it had dried out in the fume hood for 10-15 minutes at room temperature, it was mixed with 20 µL of sterile distilled water and kept at -20^{o}C until sequencing. Once again, the presence of the purified product was verified by electrophoresis of the isolated fragment on 1.5% agarose gel at 110 V for about an hour.

16S rRNA gene sequencing:

Utilizing the Big Dye terminator v3.1 cycle sequencing kit, the instructions of the Applied Biosystems Genetic Analyzer 3130xl sequencer was followed to sequence the amplified fragments. Bio-Edit and MEGA 6 were employed for all phylogenetic analysis. The 16S rRNA gene sequences of all 1,1820 strains currently available in the Type Strain Genomic Server (TYGS) database were BLASTed (9) against each of the sequences obtained. The genomes of CoNS isolates were used to extract them using RNA primer (10). This was utilized as a stand-in for each genome bitscore to identify the most closely related type strains, and then utilized the Genome BLAST Distance Phylogeny method (GBDP), with the 'coverage' algorithm and the d5 distance calculation to determine exact distances (11).

Data analysis:

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 27.0. Association between qualitative variables was done using Chi-square test with $p<0.05$ considered statistically significant.

Results:

Of the total of 704 participants (196 males and 508 females) with suspected UTI whose mid-stream urine samples were cultured, 29 (4.1%) grew CoNS. Tables 1 and 2 show the distribution of CoNS isolates among the patients with respect to sex and age. There was no significant difference in the frequency of CoNS isolation from urine with respect to sex (OR=1.384, p=0.4038) or age group (OR =0.83, p=1.000).

Out of the 65 male patients below 18 years of age in the study, 4 (6.2%) were positive for CoNS isolates while only 1 (1.3%) of the 75 female patients below 18 years of age was positive (OR=4.852, p=0.1830). Six (4.6%) of the 131 adult male patients (18-84 years) were positive for CoNS while 18 (4.2%) of the 433 adult female patients (18-84 years) were positive for CoNS (OR=1.107, p= 0.8071).

Table 1: Frequency of coagulase-negative staphylococci isolation from urine of selected patients with respect to sex

Sex	Number positive for CoNS (%)	Number negative for CoNS (%)	Total	x^2	OR (95% CI)	*p* value
Male	10 (5.1)	186 (94.9)	196	0.5462	1.384 (0.03-3.03)	0.4038
Female	19 (3.7)	489 (96.3)	508			
Total	**29 (4.1)**	**675 (95.9)**	**704**			

CoNS = Coagulase negative staphylococci; OR = Odd ratio; CI – Confidence interval

Table 2: Frequency of coagulase-negative staphylococci isolation from urine of selected patients with respect to age group

Age group (years)	Number positive for CoNS (%)	Number negative for CoNS (%)	Total	x^2	OR (95% CI	*p* value
<18	5 (3.6)	135 (96.4)	140	0.016	0.83 (0.31-2.23)	1.000
18-84	24 (4.3)	540 (95.7)	564			
Total	**29 (4.1)**	**675 (95.9)**	**704**			

CoNS = Coagulase negative staphylococci; OR = Odd ratio; CI – Confidence interval

Table 3: Reactions of different CoNS isolates to haemolysis, lipase production and esterase

Age group (years)	No of isolates	Haemolysis Production		Lipase Production	Esterase Fermentation
		Weak/delayed	Strong		
Children (< 18)	5	1 (20.0)	4 (80.0)	5 (100.0)	5 (100.0)
Adults (18-84)	24	4 (16.7)	20 (83.3)	24 (100.0)	24 (100.0)
Total	**29**	**5 (17.2)**	**24 (82.7)**	**29 (100.0)**	**29 (100.0)**

Table 4: The phylogenetic tree showing the genetic relationship of the 15 CoNS isolates

S/N	% pair wise identity (Similarity index)	CoNS strain
1	90.80	*Staphylococcus condimenti* strain CIP105760
2	91.70	*Staphylococcus condimenti* strain DSM 11674
3	92.90	*Staphylococcus condimenti* strain FAN 4249
4	90.9	*Staphylococcus condimenti* strain CIP105760
5	92.9	*Staphylococcus simulans* strain GZ202
6	94.9	*Staphylococcus simulans* strain GZ202
7	95.00	*Staphylococcus simulans* strain MS8-25
8	90.30	*Staphylococcus gallinarum* strain F4
9	92.10	*Staphylococcus haemolyticus* strain SHKS1
10	89.90	*Staphylococcus haemolyticus* strain M1-1
11	93.70	*Staphylococcus gallinarum* strain VIIII
12	90.7	*Staphylococcus gallinarum* strain OTM101-2
13	84.60	*Staphylococcus haemolyticus* strain M1-1
14	93.4	*Staphylococcus gallinarum* strain ABR
15	93.1	*Staphylococcus. sciuri* strain LZH-22

CoNS = Coagulase negative staphylococci

Table 3 shows the reactions of the different CoNS isolates to haemolysis, lipase production and esterase activity. All 29 CoNS isolates were positive for lipase, esterase and haemolysis, with 5 (17.2%) being weakly haemolytic. Table 4 show the similarity index of the various isolates. Among the 15 CoNS isolates, 4 *S. condimenti* strains, 4 strains of *S. gallinarum*, 3 strains of *S. simulans*, 3 strains of *S. haemolyticus* and 1 strain of *S. sciuri* were observed.

Fig 1 shows the frequency of phenotypic methicillin resistance among the CoNS isolates with respect to sex and age of the patients from whom the isolates were recovered. Of the 29 CoNS isolates, 16 (55.2%) were MR-CoNS, while 13 (44.8%) were methicillin-sensitive-CoNS. The frequencies of MR-CoNS isolation in the male and female children were 1 (3.5%) and 0 (0%) respectively, and 3 (10.3%) and 12 (41.4%) for adult male and female respectively.

Fig 2 confirms that all the isolates are of bacterial origin by amplification of the 16S rRNA gene (1200bp). Fig 3 is the gel image of amplification of *mec*A gene at 150bp. All selected CoNS isolates expressed the *mec*A gene which renders these organisms resistant to β-lactam antibiotic.

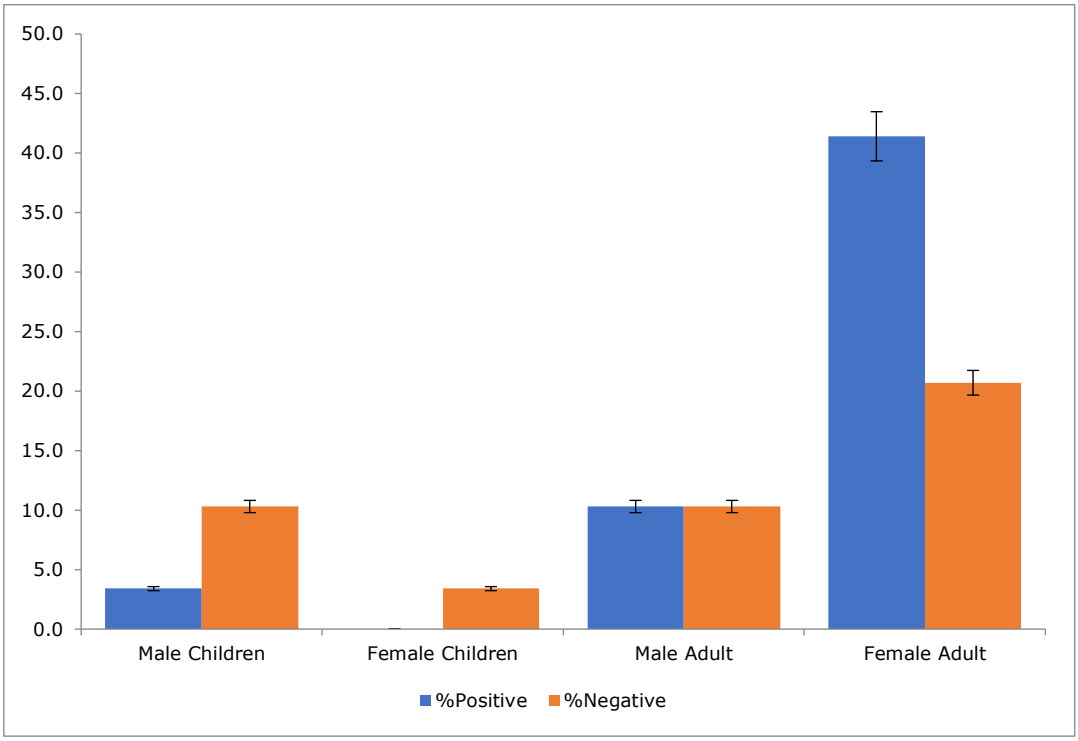

Fig 1: Frequency of methicillin resistance among coagulase negative staphylococci with respect to age and sex

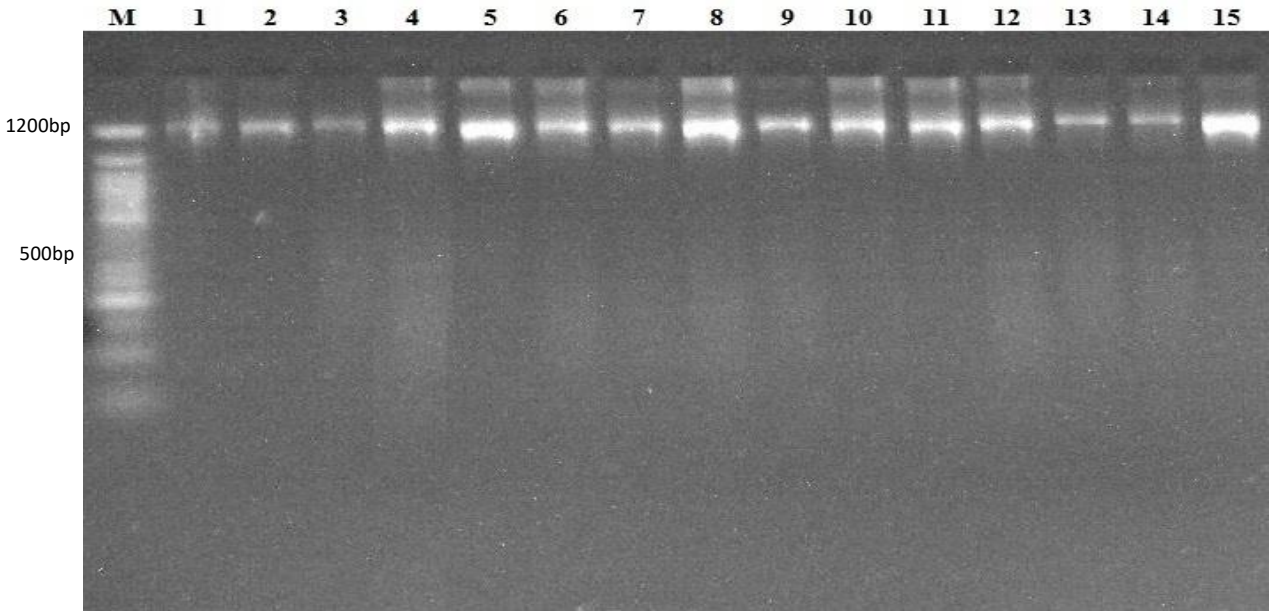

Fig 2: Amplification of bacteria 16S RNA gene region at 1200bp

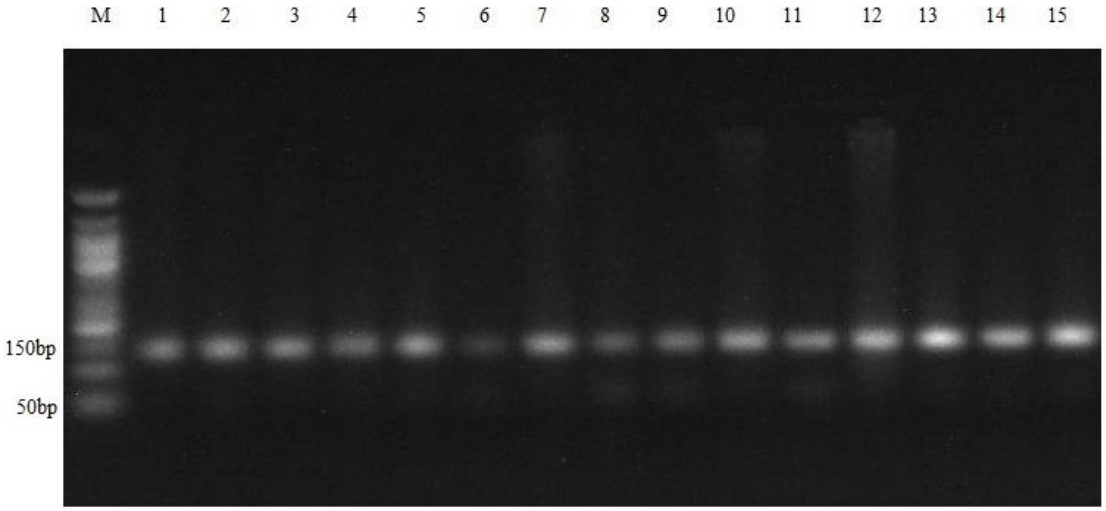

Fig 3: Gel image showing the amplification of *mec*A gene

Discussion:

Over the period of this study (April 2019–March 2019), CoNS were recovered from 29 of the 704 patients with suspected UTIs, recruited into the study. These isolates tested positive hydrolytic enzymes such as lipase, esterase, and haemolysin. While only 5 of the isolates (17.2%) showed mild haemolysis, all of them tested positive for lipase and esterase which can be considered virulence factors of CoNS. Sixteen (55.2%) of the CoNS isolates tested positive for methicillin-resistance as determined by the zone of inhibition to cefoxitin (30µg). This finding is consistent with previous studies that showed large proportion of CoNS isolates tested positive for methicillin resistance (12).

The 16S rRNA sequence analysis on 15 randomly selected isolates confirmed the isolates to be CoNS from the urine samples of the patients. The *mec*A gene was detected in all the 15 isolates, which agrees with a previous study in Japan that reported *mecA*-positive CoNS in 7.9% of the genitourinary tract isolates, although the CoNS isolates in the study were *Staphylococcus saprophyticus* (13). Methicillin resistance in staphylococci is conferred by the production of abnormal penicillin binding protein (PBP2a), that is encoded by *mec*A gene.

At genus-level phylogenetic analysis, 16S rRNA gene sequence analysis has been helpful, but at species-level, its usefulness has been called into question (14). In our study, 14 of the 15 CoNS had 16S rRNA sequence similarity of 90–99%. Four of the CoNS isolates were identified as *S. condimenti*, 4 as *S. gallinarum*, 3 as *S. simulans*, 3 as *S. haemolyticus* and 1 as *S. sciuri*, while only *S. haemolyticus* strain M1-1 had less than 90% (84.6%) sequence similarity. Our finding agrees with what has been previously established on the use of 16S rRNA sequencing for bacterial identification at genus and species levels (15). Using 16S rRNA gene sequencing, the clinical importance of the strain of CoNS species isolated from blood culture could be established, enabling optimal care of patients (16).

Conclusion:

The high carriage of *mec*A gene by the CoNS isolates in this study is a worrying development. This warrants continuous surveillance and review of the antimicrobial use as well as infection control, in order to contain emergence and spread of drug-resistant bacteria in our hospitals.

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Contributions of authors:

IJK was involved in acquisition of data, analysis and interpretation of data, drafting the manuscript and critically revising it for important intellectual content; IOO was involved in analysis and interpretation of data, drafting the manuscript or critically revising it for important intellectual content; CRI, IOO and CMO were involved in acquisition of data, drafting the manuscript and critically revising it for important intellectual content; IOO and SOA were involved in data analysis and inter-

pretation, drafting the manuscript and critically revising it critically for important intellectual content; IOO, UCA, EUU, SIO, OO, and PUM were involved in conception and design of the study, analysis and interpretation. All authors approved the final manuscript submitted for publication.

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Conflict of interests:

Authors declare no conflict of interests

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