Emerging Antibiotic Resistant Nosocomial Infection – Coagulase Negative Staphylococci Isolated From Patients in General Hospitals Within Suburban Areas of Delta State, Nigeria.

[1]OJO, S.K.S. [2]Esumeh, F.I. [1]Osanyinlusi, S.A [3]Ojerinde, A.O. and [3]Uzairue, L.I.

*[*1]Department of Microbiology, Federal University Oye-Ekiti, Ekiti State, Nigeria; P.M.B. 373, Oye-Ekiti. [2]Department of Microbiology, Ambrose Alli University, Ekpoma, Edo State, Nigeria. [3]Medical Centre, Federal University Oye-Ekiti, Ekiti State, Nigeria.*

Phone: +2348067966393

Abstract: Emerging infectious diseases especially with the coagulase negative staphylococci (CoNS) is becoming prevalent and alarming in our health institutions and as community acquired infections. This study aimed at isolating antibiotic resistant and methicillin-resistant coagulase negative staphylococci from various clinical sites of patients at different tertiary hospitals. 200 clinical samples were obtained using sterile cotton swabs and plated on freshly prepared nutrient and mannitol salt agar using standard microbiological procedures and biochemical identification. β-lactamase production and antimicrobial susceptibility test were carried out using standard microbial cells and CLSI standards. Quantitative PCR (qPCR) assay were performed on the antibiotic resistant isolates using the required PCR conditions of time and temperature and the appropriate reagent mixture. Analyzed samples yielded 77 *Staphylococcus* spp. with 12(15.6%) being coagulase-negative staphylococci, and 3 β-lactamase producing coagulase-negative staphylococci. The antibiotic resistant profile showed that 5 isolates of CoNS were methicillin-resistant (MR) to oxacillin and other antibiotics while 7 isolates of CoNS were only resistant to aminoglycosides and fluoroquinolones. Quantified *mec*A products in this study were expressed in 5 (100%) of 5 Methicillin Resistant Coagulase Negative Staphylococci (MRCoNS) isolates indicating an alarming trend in the emerging infectious coagulase negative staphylococci, while 3 of 5 MRCoNS expressed *sea* (enterotoxin) gene. This calls for urgent clinical attention to curtail the spread of this emerging infection either as a nosocomial or community-acquired infection.

Key words: Coagulase negative staphylococci, antibiotic resistance, *mec*A genes, *sea* genes, emerging infectious disease

Introduction

Emerging opportunistic pathogens are increasingly demanding for attention. Clinical microbiologists and public health officials need comprehensive knowledge of the local antimicrobial susceptibility pattern of the bacterial pathogens, especially among Coagulase Negative Staphylococci (CoNS) infection. Like MRSA, CoNS do not only have high rate of methicillin resistance but are also resistant to other multiple antibiotics (Yameen *et al*., 2010).

Coagulase-negative *Staphylococcus* (CoNS) is an important component of the normal skin flora, which is usually non-pathogenic but cancause infections in immune-compromised individuals. They are opportunistic pathogens associated with community-acquired and nosocomial infection. From early 1980s, in clinical settings, *Staphylococcus epidermidis* has also emerged as an important pathogen in foreign body infections (Gamal *et al*., 2010; Yameen *et al*., 2010; Bashir *et al*., 2007). Most developed countries have reported an increase in colonization and infection in hospitalized patients by CoNS while there are scanty data on infections caused by CoNS in developing countries (Akinjogunla and Enabulele, 2010; Bashir *et al*., 2007).

*Corresponding author:
stephen.ojo@fuoye.edu.ng; ****[1]Ojo, S.K.S***

More than 30 species are recognized, but few are commonly incriminated in human infections. *S. epidermidis* accounts for about 75% of all clinical isolates, probably reflecting its preponderance in the normal skin flora. Other species include *S. haemolyticus, S. hominis S. capitis and S. xylosus*, *S. schleifieri*, *S. saprophyticus* (causal agent of urinary tract infections inimmunocompetent women), *S. lugdunensis* (implicated in sepsis), and *S. haemolyticus* which hasbeen associated with endocarditis and osteomyelitis (Azih and Enabulele, 2013; Bashir *et al*., 2007; Greenwood *et al*., 2002). Evidence indicates that pathogenicity might be related to the production of an extracellular polysaccharide known as slime that permits these microorganisms to adhere to smooth plastic surfaces, colonizing catheters, prosthetic heart valves, and joint prosthesis (Azih and Enabulele, 2013) and tissue surfaces. Many CoNS also produce several lipases, proteases and other exoenzymes which possibly contribute to the persistence of CoNS in the host and may degrade host tissues (Otto, 2004). The purpose of this study was to determine the antibiotic resistance pattern of this emerging CoNS and the molecular detection of *mec*A and *sea* genes frequently isolated in MRSA.

Materials and Methods

Clinical Specimens and Collection of Samples

Two hundred (200) swab samples from infected sites of surgical dressings, wounds and burns patients admitted in the hospital for over a two weeks were collected from different hospitals within Delta State (Central Hospital, Agbor; General Hospital, Asaba; General Hospital, Obiaruku; Central Hospital, Warri and General Hospital, Ekpan), and transported to the laboratory using ice packs. The cotton swabs were allowed to assume room temperature before been applied to freshly prepared nutrient agar and mannitol salt agar (oxoid) and were incubated at 37^{o}C for 24h.

Isolation and Identification

Colonies growing on nutrient agar plates were subcultured on freshly prepared plates of mannitol salt agar (MSA) and incubated again at 35^{o}C. Primary characterization of isolates was based on Gram stain, cellular morphology and cultural characteristics, growth on nutrient agar and DNase agar, fermentation of mannitol, and production of catalase and coagulase based on standard microbiological procedures.

Standardization of Microbial Cell Suspension

Each of the 24 hours old pure culture was prepared to 0.5McFarland standard using CLSI (2008) standards.

β-lactamase Assay

Strips of starch paper measuring 4cm x 7cm were cut and disinfected with 70% ethanol. These were then soaked for 10min in a solution of benzyl penicillin dissolved in phosphate buffer. The soaked strips were spread evenly and placed on sterile Petri dishes. Young culture (24hr old) of the test isolates grown on nutrient agar were then inoculated on the surface of the paper over an area of 2-3mm. Each test paper was then used to test the staphylococcal isolates at a time with the inocula placed at least 1.5cm apart. The Petri dishes were then incubated for 30min at 37^{o}C, after which the plate was flooded with Gram's iodine solution, which was immediately drained off. This caused the starch paper to turn uniformly blue-black within 30seconds of application. Colonies with decolourized zones were indicative of β-lactamase production. Results were read within 5min, as black background tends to decolourize, making interpretations more difficult (Ako-Nai *et al*., 2005).

Antimicrobial Resistance Profile

The antimicrobial susceptibility profiles of the isolates were determined using broth dilution and disk diffusion method as described by the Clinical Laboratory Standards Institute (CLSI, 2008). Inocula were prepared by diluting overnight cultures in sterile NaCl (0.9%) suspension and then marched with the 0.5McFarland turbidity index, equivalent to 10^{5}cfu/ml. Bacterial suspensions were plated on to Mueller Hinton Agar, then the commercially available antibiotic discs were placed on the seeded plates, and the plates incubated overnight at 37^{o}C. Sensitivity, intermediate and resistance were assessed by presence/absence of zone of complete growth inhibition around each disk; according to CLSI (2008) reference standards. Reference type *S. aureus* strain (ATCC 25923) was used as positive control.

PCR Amplification of Antibiotic Resistant (*mec*A) and enterotoxin (*sea*) genes using qPCR

Quantitative PCR (qPCR) assay was set up to detect presence of antibiotic resistant and enterotoxin genes among CoNS strains using the protocol described by Grisold *et al*. (2002) with some modification. Chromosomal DNA was extracted and used as PCR template using the manufacturer's manual. The assay was performed using Hybaid OmniGene thermal cycler (Model no: TR3SM2). All strains were subjected to *mec*A and *sea* genes PCR primers using an initial denaturation process at 95^{o}C for 2 minutes for 1 cycle. Another denaturation process at 95^{o}C for 30 seconds, followed by annealing at 65^{o}C for 30 seconds and an extension at 72^{o}C for 1 minute in 40 cycles. The final extension temperature of 72^{o}C ran for 5 minutes in 1 cycle while the holding temperature of 10^{o}C was held for about 120 minutes in 1 cycle. The PCR products obtained were subjected to quantification in a Thermomax Microplate reader (Molecular Devices) at a wavelength of 405 and 450nm. The reading of the samples for quantification was done within 10 seconds and the data was analyzed using MYASSAYS software. Quality control strains included was *S. aureus* ATCC 25923.

Statistical analysis. SPSS for Windows (version 20.0) software was used for the analysis. Categorical variables were compared by using Pearson Chi-square tests. P-values calculated at $P<0.05$ was considered statistically significant.

Results and Discussion

The exceeding increase in the emergence of multidrug resistance pathogens especially β-lactamase producing *Staphylococcus* species in the developing countries is attributable to indiscriminate use of antibiotics, complex socio-economic, behavioural antecedents and dissemination of drug-resistant pathogens in human medicine (Akinjogunla and Enabulele, 2010) and more importantly in the veterinary domain since humans have a direct association either as pet or for consumption. Thus, β-lactamase producing CoNS have posed a great threat to clinical practice in the administration of antibiotics in this part of the continent and needs urgent public health attention.

The isolation of 12 (15.6%) CoNS from 200 clinical wound and burns samples (Table 2) was in agreement with the findings of Mohammed *et al*. (2011)

who isolated 5 (11.1 %) CoNS isolate from 70 burn wounds patients; Yameen *et al*. (2010) also isolated 47 (16.61%) *S. epidermidis* (a CoNS) from 283 nasal samples.

Bashir *et al*. (2007) isolated 29 (33 %) *S. epidermidis* from wounds and burns patients and Gamal *et al*. (2010) also isolated 55 CoNS strains from 470 clinical specimens. The incidence of 118 (40.3 %) of 293 children being colonized with various species of CoNS was reported by Akinkunmi and Lamikanra (2010) while Ako-Nai *et al*. (2005) reported 56 CoNS isolates from 245 nasal and pus samples. Reports from Akinjogunla and Enabulele (2010) was partly in consonance with the results of this study revealing a high CoNS isolation of 21 (15.4%) from 136 patients with acute otitis media. However, Moran *et al*. (2006) had a low isolation rate of 3% for CoNS, which is in agreement with this study (Table 2).

It is noteworthy that this study has been able to establish the incidence of β-lactamase producing staphylococci and antibiotic resistant strains among the penicillin family (oxacillin, ampiclox and amoxicillin) used in this study, which is in accordance with CLSI (2008).

Multiple antibiotic resistance was also noted, which is corroborated by a previous study (Ojo *et al*., 2014). β-lactamase production in staphylococci isolates is an indication of antibiotic resistance to penicillin family because of the β-lactam ring present in those antibiotics and in most strains, the enzyme is encoded by plasmids but can also be found on the chromosome (Shakibaie *et al*., 2002).

Of the 28 β-lactamase (βL) producing isolates, 4 (14.3%) were β-lactamase (βL) producing CoNS in this study as earlier observed by Ojo *et al*. (2013a) reported obtaining 3 (20%) β-lactamase producing CoNS, which was also in agreement with other authors has reported by Akinjogunla and Enabulele (2010), that had 9 (42.9 %) CoNS β-lactamase producers; Ako-Nai *et al*. (2005) had 26 (46.4 %) βL CoNS strains (Table 2).

The rate of isolation from various sites of infection as reported in this study and by the afore-mentioned authors become paramount in creating hospital/health awareness of the possible spread either as a nosocomial infection or community-acquired infection.

Table1. Isolation rates of staphylococci from various sample sources

Sample source	Nos of sample collected	Nos with staphylococci	Isolation rate (%)
Head	20	9	11.7
Hand	60	16	20.8
Feet	15	9	11.7
Leg	25	14	18.2
Eye/Face	10	4	5.2
Ear	7	4	5.2
Mouth	8	2	2.6
Shoulder	10	3	2.6
Lap	45	16	20.8
Total	**200**	**77**	**98.8**

Table 2. Prevalence of CoNS and β-lactamase strains on wounds and burns from various sample sources of patients

Sample site	Samples: Wounds					Samples: Burns					Total Isolation Rates of CoNS (%)
	No. of Isolates *S. aureus*	CoNS	Isolation Rates of CoNS (%)	β-lactamase *S. aureus*	CoNS	No. of Isolates *S. aureus*	CoNS	Isolation Rates of CoNS (%)	β-lactamase *S. aureus*	CoNS	
Head	8	1	1.4	3	0	0	0	0	0	0	1.4
Hand	14	0	0	10	0	2	0	0	1	0	0
Lap	13	1	1.4	2	1	2	0	0	1	0	1.4
Feet	7	2	2.9	1	1	0	0	0	0	0	2.9
Leg	8	4	5.8	1	0	1	1	12.5	1	1	18.3
Eye/Face	4	0	0	1	0	0	0	0	0	0	0
Mouth	1	1	1.4	0	0	0	0	0	0	0	1.4
Shoulder	1	0	0	0	0	1	1	12.5	1	1	12.5
Ear	3	1	1.4	2	0	0	0	0	0	0	1.4
Total	**59**	**10**	**14.3**	**20**	**2**	**6**	**2**	**25**	**4**	**2**	**39.3**

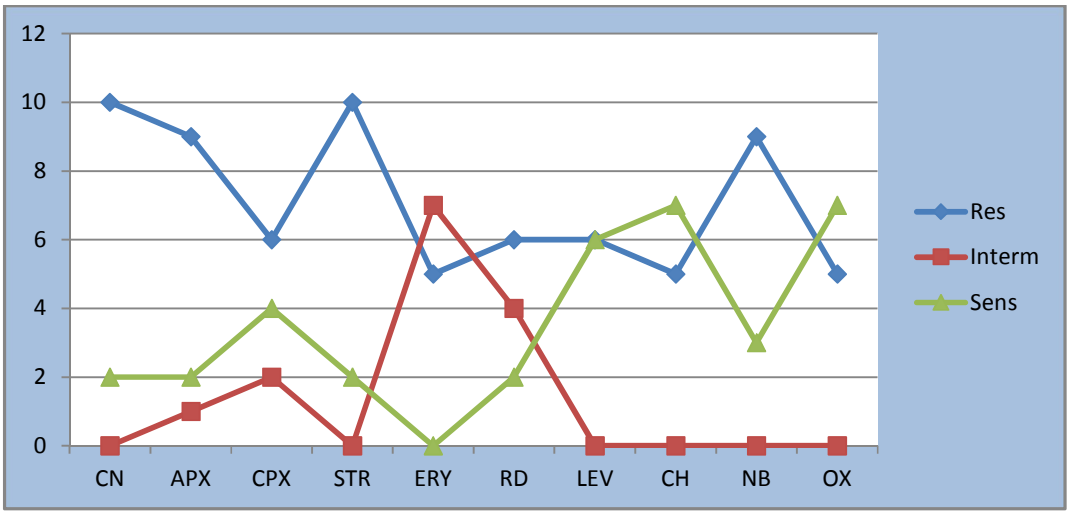

Fig. 1. Antibiotic Susceptibility Profile of CoNS strains {Key: CN-Gentamicin; APX-Ampiclox; CPX-Ciprofloxacin; STR-Streptomycin; ERY-Erythromycin; RD-Rifampin; LEV-Levofloxacin; CH- Chloramphenicol; NB- Norfloxacin; OX-Oxacillin; Res-Resistant; Sens-Sensitive; Interm-Intermediate (CLSI, 2008)}.

Resistance pattern observed among the CoNS isolates revealed that 10 isolates out of 12 were resistant to the aminoglycosides (gentamicin and streptomycin), between 6-8 isolates were resistant to fluoroquinolones while 5-9 isolates were resistant to the penicillin family (ampiclox and oxacillin) {Figure 1}. Similar trend was observed from Akinkunmi and Lamikanra (2010) with 13 of 149 CoNS isolates showing resistance to gentamicin, and 19-45 CoNS isolates showing resistance to Ciprofloxacin and Norfloxacin. Yahmeen *et al*. (2010) had a resistant profile of 50% ciprofloxacin and 21% gentamicin.

Several authors reported that prolonged stay in the hospital has led to the acquisition of MRSA and MRCoNS among in-patients in different wards of the hospitals, (Ojo *et al*., 2014; Khalil and Al-Ruaily, 2008), which was in line with the report of this study. Five (41.7%) of 12 CoNS were oxacillin resistant isolates and considered as MRCoNS (Figure 2) indicating a high trend of antibiotic resistance pattern among the emerging opportunistic pathogens. In similar studies, Ayepola *et al*. (2014) reported an oxacillin resistance ranging from 11.5-100% among the various CoNS strains; Khan *et al*. (2014) reported a 93.6% oxacillin resistance among 178 CoNS strains and Ma *et al*. (2011) revealed a 79.1% oxacillin resistance.

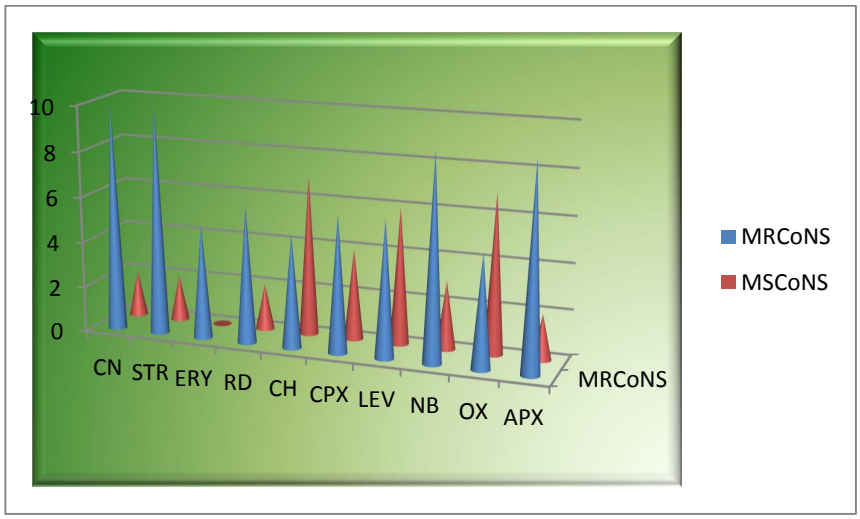

Figure 2. Comparison of Antibiotic Resistance Profile among MRCoNS and MSCoNS isolates

Table 3. Quantitative Determination of *mec*A and *sea* Genes from Methicillin Resistant CoNS isolates using qPCR

Sample ID/ Well no.	*sea* gene		*mecA* gene		Characterized isolate
	ABS	Gene Qty (nm)	ABS	Gene Qty (nm)	
W237/ A11	0.066	150.02	0.082	390.68	MRCoNS
W275/B6	0.056	0	0.061	74.81	MRCoNS
W380/B7	0.084	420.76	0.079	345.56	MRCoNS
W248/C12	0.016	0	0.123	1007.38	MRCoNS
W337/D8	0.08	360.6	0.061	74.81	MRCoNS
ATCC25923/C11	0.074	270.35	0.058	29.69	*S. aureus*

Key:ABS-Absorbance

It was discovered that most researches or medical attention is geared towards MRSA with an insignificant attention on antibiotic resistant coagulase negative staphylococci (especially MRCoNS). This study however brings to limelight the prevalence of this insignificant infection with fairly high enterotoxin gene and high methicillin-resistant (*mec*A) gene. Investigation has shown that several isolates that were declared as MRSA in routine practice are actually coagulase negative *Staphylococcus epidermidis* that are misidentified. *S. epidermidis* is part of normal flora of skin and contaminates clinical samples if sample collection is not done aseptically. Methicillin resistance is also a common phenomenon in *S. epidermidis* (Romeeza *et al*., 2009).

Interestingly, *mec*A products in this study were expressed in 5 (100%) of 5 MRCoNS isolates quantified indicating an alarming trend in the emerging infectious coagulase negative staphylococci, while 3 of 5 MRCoNS expressed *sea* gene (Table 3). Similar trends were reported by Jonas *et al*. (2002) who detected 64 *mec*A products from 64 oxacillin resistant CoNS. The *mec*A genesquantified from this study had a higher concentration as compared to *sea* genes, thus calling for urgent clinical and public health attention in health-care sectors.

In conclusion, the multi-resistant emerging pathogens among CoNS is becoming worrisome and should be of utmost clinical concern for further studies on the epidemiological investigation and comparative molecular pattern of the community acquired and nosocomial strains.

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