



RESEARCH PAPER

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Study of multidrug-resistant clinical isolates of *Staphylococcus* coagulase negative from Assir region of Saudi Arabia

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*Microbiology Department, College of Medicine. King Khalid University, Saudi Arabia***Key words:** Coagulase-negative; Gram positive; Mec A gene, Multidrug resistance MDR, PCR.<http://dx.doi.org/10.12692/ijb/10.2.156-165>

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Abstract

Staphylococcus Coagulase negative bacteria are gram-positive and spherical cells in clusters. They commonly occur as skin commensals, occasionally cause nosocomial or community acquired infections. Numerous coagulase-negative staphylococci appear commonly on human skin, of these species, *Staphylococcus epidermidis* and *S. hominis* are the most abundant. While *S. epidermidis* tends to colonize the upper part of the body, *S. hominis* tends to colonize in areas with numerous apocrine glands, such as axillae and the pubic region. In this study, a total of 70 nasal swabs from isolates at Assir Central Hospital General Lab during the period of Sep. 2014- April 2015. The samples were tested by bactech, culture media, antibiotics sensitivity using diffusion disc test (MIC) and molecular polymerase chain reaction (PCR) for detection of the Mec A gene. Drugs found to be resistant to all patients were penicillin 100%, 60-70% to; erythromycin, ampicillin, cefoxime, carbinicillin, methotrexate and cefaclor. Drugs that showed resistance of 30-50% were; tetracyclin, fucidin, augmentin, gentamycin and ciprofloxacin. Whereas cotrimexazole and vancomycine were sensitive to all patients. All isolates were Mec A gene negative. In contrast to *S. aureus*, the coagulase-negative Staphylococci (CoNS) have been the subject of considerably less investigation, Normal commensals, however, CoNS species 15 are capable of causing infectious keratitis, sepsis of patients in neonatal intensive care units, and are now being recognized as a cause of gastroenteritis This study showed that, Staphylococcus coagulase negative bacteria were pathogen associated with community acquired and nosocomial infections. The frequency of staphylococcus multi-drugs resistance is rising.

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Introduction

Due to the high frequency, significant clinical impact, and cost of coagulase-negative staphylococcal infections, a great deal of effort continues in the investigation of the epidemiology, prevention, and treatment of coagulase-negative staphylococcal infection. The most important test used to distinguish *S. aureus* from other staphylococci is the production of coagulase, which nonenzymatically binds to prothrombin, forming a complex that initiates the polymerization of fibrin. *Staphylococcus epidermidis* and *S. hominis* are the most abundant among coagulase-negative staphylococcal infections.

S. epidermidis and a number of other species of coagulase-negative staphylococci are normal commensals of the skin, anterior nares, and ear canals of humans. Their large numbers and ubiquitous distribution result in frequent contamination of specimens collected from or through the skin, making these organisms among the most frequently isolated in the clinical laboratory. In the past, they were rarely the cause of significant infections, but with the increasing use of implanted catheters and prosthetic devices, they have emerged as important agents of hospital-acquired infections. Immunosuppressed or neutropenic patients and premature infants have been particularly affected. Organisms may contaminate prosthetic devices during implantation, seed the device during a subsequent bacteremia, or gain access to the lumina of shunts and catheters when they are temporarily disconnected or manipulated. The outcome of the bacterial contamination is determined by the ability of the microbe to attach to the surface of the foreign body and to multiply there. Initial adherence is facilitated by the hydrophobic nature of the synthetic polymers used in medical devices and the natural hydrophobic nature of many coagulase-negative staphylococci. Following attachment, some strains produce an extracellular polysaccharide slime or biofilm. This biofilm provides additional adhesion, completely covers the bacteria, and serves as a mechanical barrier to antimicrobial agents and host defense mechanisms; it is also believed to enhance nutrition

of the microbes by functioning as an ion-exchange resin. Strains able to produce the polysaccharide biofilm are more likely to colonize intravenous catheters but have no known advantage in adherence to human tissues such as heart valves. The resistance of many coagulase-negative staphylococci to multiple antimicrobial agents contributes further to their persistence in the body. Infections are generally low grade, but unless controlled, they can proceed to serious tissue damage or a fatal outcome.

S. hominis are small, usually 1–2 mm in diameter after 24 hours' incubation in blood agar media at 35°C, white or tan in colour. Occasional strains are resistant to novobiocin and may be confused with other resistant species (e.g. *S. saprophyticus*). It is one of only two species of *Staphylococcus* that display sensitivity to desferrioxamine, the other being *S. epidermidis*. Unlike *S. epidermidis*, *S. hominis* produces acid from trehalose, so the two tests together serve to identify the species. *S. hominis* is able to produce acid aerobically from glucose, fructose, sucrose, trehalose and glycerol. Some strains were also able to produce acid from turanose, lactose, and galactose, melezitose, mannitol, and mannose. The cell wall contains low amounts of teichoic acid and glutamic acid. The cell wall teichoic acid contains glycerol and glucosamine (Gilad J., 2007).

When grown in agar cultures, colonies are usually circular, 4.0 to 4.5 micrometers in diameter. Agar colonies usually have wide edges and an elevated center. They are commonly smooth yellow-orange pigmented in the center of the opaque colonies. They grow both in aerobic and anaerobic conditions, but tend to grow significantly less in the latter. The optimal growth temperature range was around 28 to 40°C, but good growth is still observed at 45°C, while no growth is observed at 15°C. *S. hominis* can be differentiated from staphylococci by its colony morphology and pigmentation patterns, predominant tetrad cell arrangement, poor growth in thioglycolate, low tolerance of NaCl, and carbohydrate reaction pattern. Each species is also significantly different in cell wall composition, lactic acid configuration,

temperature extremes of growth, coagulase activity, hemolysis acetylmethylcarbinol production, nitrate reduction, and phosphatase, DNase, and bacteriolytic activities. Similarities in these properties between *S. hominis* and several other species suggest there is a close relationship between *S. hominis* and *S. epidermidis*, *S. haemolyticus*, and *S. warneri* (Jiang S., 2012).

Most boils and superficial staphylococcal abscesses resolve spontaneously without antimicrobial therapy. Those that are more extensive, deeper, or in vital organs require a combination of surgical drainage and antimicrobics for optimal outcome. Penicillins and cephalosporins are active against *S. aureus* and Staph coagulase negative cell wall peptidoglycan and vary in their susceptibility to inactivation by staphylococcal lactamases. Although penicillin G is the treatment of choice for susceptible strains, the penicillinase-resistant penicillins (methicillin, nafcillin, oxacillin) and first-generation cephalosporins are more commonly used because of resistance.

The penicillinase is encoded by plasmid genes and acts by opening the lactam ring, making the drug unable to bind with its target. Alterations in the lactam target, the peptidoglycan transpeptidases (often called penicillin-binding proteins, or PBPs), is the basis for resistance to methicillin. These methicillin-resistant *S. aureus* and Staph coagulase negative (MRSA) strains are also resistant to the other penicillinase resistant penicillins such as oxacillin. The most common mechanism is the acquisition of a gene.

Material and methods

Collection of samples

A total of 70 cases of *Staphylococcus coagulase nrgative* were detected directly from nasal swab specimens using bacteriological methods from clinical isolates presented with variable nosocomial or community acquired infections such as respiratory infection, central nervous system infections, urogenital infection, musculoskeletal (joints) and skin infections which collected from Assir Central Hospital, Saudi Arabia during the period from Sep. 2014- April 2015.

Clinical data including the inpatient and outpatient categories and patient's demographic data were collected. Then from each patient had a specimen collected from the nares with a dry, unmoistened swab. The tip of the collection swab was inserted approximately 1 in. (2.56 cm) into the nares and rolled five times in each nostril. Collected specimens were transported and stored at room temperature. Cultures were inoculated and specimens were stained and visualized microscopically. Each sample was examined using the procedure described below:

Microbiological tests

The cultures were carried out on blood agar. The plates were incubated for 24 to 48 hrs. at 35 C° and examined for growth. After incubation each plate was examined to observe the characters of colonial morphology, and the effect of the organism on culture media. The colonies appeared as medium to large, smooth, entire, slightly raised, translucent, most colonies pigmented creamy yellow, most colonies showed beta-hemolysis. Confirmation of *Staphylococcus* species were conducted using microscopic examination of gram stained film, catalase testing using hydrogen peroxide solution and coagulase testing according to the working steps of Kayser, Medical Microbiology © 2005 Thieme. coagulase test is demonstrated by incubating staphylococci in plasma; this produces a fibrin clot within hours. A dense emulsion of *S. aureus* cells in water also clumps immediately on mixing with plasma due to direct binding of fibrinogen to a factor on the cell surface. Kirby-Bauer and Stokes' method (disc diffusion method).

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the NCCLS. The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here. NCCLS is an international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators etc.

It promotes accurate antimicrobial susceptibility testing (AST) and appropriate reporting by developing standard reference methods. MIC's and the results have corroborated with data NCCLS. NCCLS is approved by FDA-USA and recommended by WHO (World Health Organization),

Preparation of Müller-Hinton agar culture plates

An even distribution of the dissolved and mixed *Staphylococcus hominis* culture colonies in normal saline (1 colony/1 ml of NaCl) was spread on Müller-Hinton agar plates. The inoculated disks were incubated at 30 to 35°C for 24 hours or longer (up to 72 hrs). The antibiotics disc filter paper was applied over the growing culture colonies again incubated at 30 to 35°C.

Preparation of antibiotics dried filter paper discs

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. The loop used for delivering the antibiotics is made of 20 gauge wire and has a diameter of 2 mm. This delivers 0.005 ml of antibiotics to each disc.

Reading plates and interpreting results

After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth which reflects the minimum inhibitory concentration (MIC). The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest millimeter, using slide calipers or a ruler. Minimum inhibitory concentration breakpoints of the NCCLS M100-S12: Performance standards for antimicrobial susceptibility testing: Results were reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

Zone diameter interpretive criteria

The antimicrobial agents suggested for routine testing of *Staph. spp.* Each zone size is interpreted by

reference to the zone diameter interpretative standards and equivalent minimum inhibitory concentration breakpoints for *Staph. spp.* The breakpoints for *S. aureus* are different from those for coagulase-negative staphylococci (CoNS). The zone diameter interpretative standards and equivalent minimum inhibitory concentration (MIC) breakpoints for CoNS *Staph. spp.* for oxacillin sensitivity pattern in CoNS: Susceptible pattern; minimum inhibitory diameter was more than 18 mm and the minimum antimicrobial concentration was less than 0.25mg/ml to be considered as sensitive antibiotic while for resistant antibiotic MIC was less than 17 mm and the minimum antimicrobial concentration was more than 0.5 mg/ml. Polymerase Chain reaction (PCR).

A nasal specimen is collected and transported to the laboratory using the recommended swab with Liquid Stuart Medium. For testing, the swab was placed in sample buffer. The specimen is concentrated and lysed. An aliquot of the lysate is added to PCR reagents which contain the species-specific primers used to amplify the genetic target, if present (Pasko C., *et al.*, 2012). A few colonies were picked from blood agar; suspended in 200µl of a lysis buffer containing 10mM Tris-HCl buffer (pH 8.0), 50mM NaCl, lysostaphin (10µg/ml), achromopeptidase (100µg/ml), and RNase (100µg/ml); incubated at 30°C for 45min; boiled for 5min; and then diluted by the addition of 400µl of TE (10mM Tris-HCl [pH 8.0], 1mM EDTA). For the PCR, 1µl of lysate was added as a template to 24µl of a reaction mixture containing 10mM Tris-HCl (pH 9.0), 1.5mM MgCl₂, 50mM KCl, 0.1% Triton X-100, 0.2mM each deoxynucleoside triphosphate, and 0.75 U of Supertaq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom). *Mec A* DNA was amplified with the primers 5'-GTT GTA GTT GTC GGG TTT GG-3' and 5'-CTT CCA CAT ACC ATC TTC TTT AAC-3' (20µM) (Wielders C. L. C., 2002). These primers were designed on the basis of the *mec A* sequence (Gen Bank accession no. X52593) (Kilic A, 2011).

Data Analysis

Clinical and Laboratory data were recorded in special formats and analyzed using statistical computer program (SPSS).

Ethical considerations

All patients with active lesions who took part in this research project had given informed consent. All the risks had been explained to them by the principal investigator before the beginning of the programme. The procedures followed in this study were in accordance with the ethical standards of the responsible committee on human experimentation (King Khalid University, Saudi Arabia) and in keeping with the Helsinki Declaration of 1964, as revised in 2000. Human rights were fully fulfilled.

Results and discussion

This study include 70 Staphylococcal coagulase negative samples collected from patient in different departments of Assir Hospital.

The colonies that appeared as medium to large, smooth, entire, slightly raised, translucent, most colonies pigmented white color, most colonies showed beta-hemolysis. Confirmation of Staphylococcus species were performed by coagulase test. PCR for all isolates were Meg A gene negative. Demographic characteristic of the patients: Around 80% of the patients were Saudi, 10% were Arab but not Saudi and

10% were from other nationalities. 30% of patients' age range from 1 to more than 50 years, 60% from 16 to 50 years and finally 10% of them were more than 50 years old. Two third of the patients were male (60%). Concerning marital status of patients, 60% were married. All females were non-pregnant . Table 1.

All samples taken from patients were nasal swabs. The hospital departments from where samples were collected are shown in Table 2.

Some antibiotics: Penicillin, erythromycin, ampicillin, cefixime, carbimicillin, cotrimexazole, amikacine, vancomycine, methotrexate, cefaclor, tetracyclin, fucidin, augmentin, gentamycin and ciprofloxacin were tested to find out their sensitivity to Staphylococcus coagulase bacteria . Drugs found to be resistant to all patients were penicillin 100%, 60-70% to; erythromycin, ampicillin, cefixime, carbimicillin, methotrexate and cefaclor. Drugs that showed resistance of 30-50% were; tetracyclin, fucidin, augmentin, gentamycin and ciprofloxacin. Whereas cotrimexazole and vancomycine were sensitive to all patients. Table 3. The distribution of different cases Staphylococcal coagulase negative with gender Fig. 1.

Table 1. Demographic characteristic.

Sex istribution	No. of patient	Percent %
Male	46	65.7
Female	24	34.3
Age in years	No. of pt	Percent%
0-15	33	47.2
16- 50	19	27.1
51&more	18	25.7
Organism found	No. of patient	Percent%
Staphylococcus epidermidis	59	84.3
staphylococcus hominis	10	14.3
Staphylococcus capitis	1	01.4
Nationality	No. of patients	Percent%
Saudi	56	80
Non Saudi	14	20

The interpretation of cultures that grow coagulase-negative staphylococci is grown with difficulty. In most cases, the finding is attributable to skin contamination, although it can indicate infection when a patient has implanted devices,

or has defenses that are otherwise compromised. The presence of at least moderate numbers of organisms or the repeated isolation of a strain with the same antibiogram argues for infection over skin contamination.

Table 2. Study cases versus hospital departments.

Name of department	No. of patients	Percentage %
OPD outpatient department	10	14.2
ER emergency room	3	4.2
*ICU intensive care units	14	20
NICU nursery intensive care unit	9	12.8
*FW female wards	6	8.5
Pw paediatric wards	2	2.8
PICU paediatric intensive care unit	18	25.6
*MW male wards	8	11.3

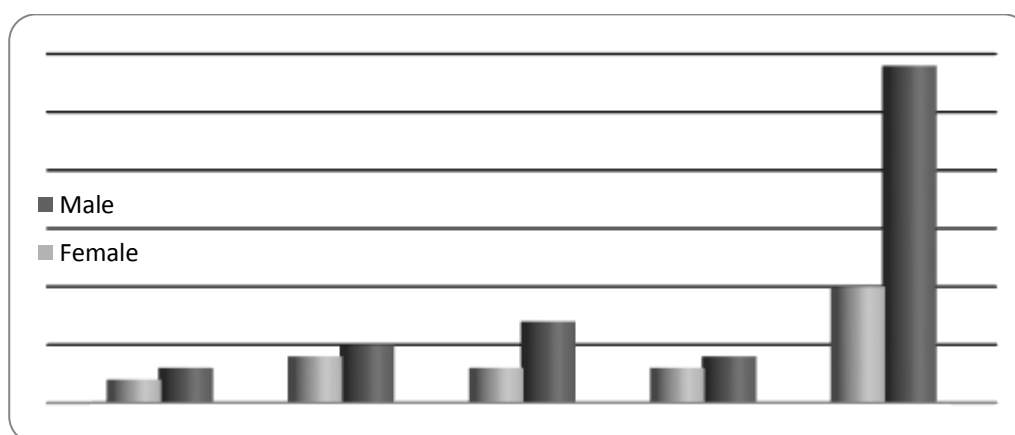
*Male wards includes medical , orthopedic and surgical

*Female wards includes surgical, obstetrical, medical

*Intensive care units (ICU) includes, cardiac care unit (CCU), burn (BU)

Table 3. Percentage of resistance of different drugs.

Name of the Drug	Resistance Percentage	Name of the drug	Resistance Percentage
Erythromycin	63	Ciprofloxacin	60
Ampicillin	69	Cefaclor	67
Cifoxine	68	Tetracyclin	60
Carbimicillin	70	Fucidin	54
Methotrexate	67	Augmentin	40
Gentamycin	53	Amikacine	2

**Fig. 1.** The distribution of different cases Staphylococcal coagulase negative with gender.

Sepses include road traffic accident, trauma and head injury.

URS: Upper respiratory tract infection and respiratory distress (RDS)

CVA: cerebro vascular accident

UTI: urinary tract infection

Most coagulase-negative staphylococci infection isolates in this study were from various intensive care units (ICU, PICU, CCU) which comprises around 54% of the study group. Many invasive devices were used for managing these cases in form of ventilators and indwelling catheters.

Most coagulase-negative staphylococci infections encountered were resistant to penicillin, and many were

also methicillin resistant. Eradication of coagulase-negative staphylococci from prosthetic devices and associated tissues with chemotherapy alone is very difficult unless the device is also removed.

Based on study of a total of 240 strains, all were resistant to lysozyme, some were slightly resistant to lysostaphin, 77% were susceptible to penicillin G, 97% to streptomycin, 93% to erythromycin, 64% to tetracycline, and 99% to novobiocin (Kloos W.E., 1998).

Several studies on antibiotics sensitivity profile in Saudi Arabia were conducted (Abdalla N.M., 2011). *Novobiosepticus* (SHN) subspecies was found recently. The name derives from the combination of *novobio*, pertaining to the property of novobiocin resistance, and *septicus*, pertaining to the ability to cause sepsis (Sohn A. H., 2001).

In addition, this new subspecies are resistant to nalidixic acid, penicillin G, oxacillin, kanamycin and streptomycin. They were also somewhat resistant to methicillin and gentamicin, and most of them were resistant to erythromycin, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole and ciprofloxacin, as well.

In addition, SHN is not reported to be isolated from the human skin (Palazzo I. C, 2008). The SHN is so similar to the original *S. hominis*, now called *S. hominis* subsp. *hominis*, that a Micro Scan system used by clinical microbiology laboratories can barely differentiate it from *S. hominis* cultures (d'Azevedo P. A., 2008). A zone of inhibition measuring ≤ 15 mm in Mueller-Hinton agar or ≤ 11 mm on Trypticase soya agar plates was considered indicative of novobiocin resistance (Bouchami O., 2001).

SHN causing bacteremia in hospitalized patients. Twenty-three isolates were from blood cultures, six were from catheters, one was from cerebrospinal fluid (CSF), one was from a wound, and one was from external ear fluid. 21 patients yielded an SHN-positive blood culture. Out of 21 isolates of SHN from blood, 21 (100%) were resistant to penicillin, oxacillin, and erythromycin while 20 (95.2%) were resistant to clindamycin and gentamicin, 19 (90.5%) were resistant to tetracycline, 6 (28.6%) were resistant to trimethoprim-sulfamethoxazole, 5 (23.8%) were resistant to chloramphenicol, and 2 (9.5%) were resistant to ciprofloxacin. All 21 isolates were resistant to nalidixic acid and susceptible to vancomycin. PCR analysis confirmed that all strains possessed a *mec A* gene homologue (Chaves F., 2005). Not all coagulase-negative staphylococcal infections (CONS) are identified to the species level. Molecular epidemiology (Katayama Y., 2003, Fitzgibbon J. E., 2001) was

successful in identifying emergence of *Staphylococcus hominis* strains expressing low level resistance to quinupristin/dalfopristin in Greece (Petinaki E., 2005). These findings are almost similar to the antibiotic sensitivity pattern in our study, as drugs found to be resistant to all patients were: Penicillin, erythromycin, ampicillin. The combined resistant to novobiocin and oxacillin is hypothesized to have originated from a simultaneous introduction of genes controlling the resistance of both of them. These genes were believed to have been acquired originally through heterologous DNA from a methicillin-resistant strain of one of the novobiocin-resistant species belonging to the *S. sciuri* or the *S. saprophyticus* groups (Garza-Gonzalez E., 2011). Spread of *S. spp.* (including MRSA) generally is through human-to-human contact, although recently some veterinarians have discovered the infection can be spread through pets, with environmental contamination. Cases of *S. spp.* Nosocomial infections have reported to be transported by polyester, the main material used in hospital curtains in hospitals across America (Donovan, S.T., 2013). An important and previously unrecognized means of community-associated MRSA colonization and transmission is during sexual contact (Crum-Cianflone, N.F., 2011).

Mec A gene is known associated factor of drug resistance for Oxacillin/Mithicillin drug as all isolates were *Mec A* gene negative, the resistance could be explained by the thick biofilm caused by this bacteria which guard against drug penetration (Toba F.A., 2011, Dumitrescu O., 2011).

Previous study on infants were believed to serve as reservoirs for the microorganism, and transmission takes place with contact between health workers and the infants. In addition, staphylococcal isolates from the nasopharynxes and hands of health care workers were shown to be genetically similar to those that colonize or cause disease in neonates (Sorlozano A., 2011). In other studies on neonatal CoNS infections that have demonstrated significant morbidity but a low rate of mortality, there were no deaths associated with SHN sepsis (Le Curr. Opin W. 2004).

In one study, 6 out of 13 (46%) neonates with clinically significant bacteremia had proven catheter-related bloodstream infections. CoNS account for a significant proportion of nosocomial bacteremia cases related to the insertion and maintenance of intravascular catheters. Coagulase-negative staphylococci (CoNS) are now recognized as a major cause of nosocomial infective endocarditis in coronary care units (CCU) (Kessler R. B., 1998).

Study done in Mexico which include Among the MR-CoNS strains studied, the most frequently isolated species were *Staphylococcus epidermidis* (n=26) and *Staphylococcus haemolyticus* (n=13). *Staphylococcus cohnii* (n=5), *Staphylococcus hominis* (n=3), *Staphylococcus sciuri* (n=1), *Staphylococcus pasteurii* (n=1) and the recently described species *Staphylococcus pettenkoferi* (n=1) were also identified (Garza-Gonzalez D. L., 2010).

Conclusion

Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. Appropriate antimicrobial drug use has unquestionable benefit, but physicians and the public frequently use these agents inappropriately..

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