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Full Length Research Paper

Vancomycin resistant coagulase-negative Staphylococcal isolates from HIV positive patients in the Limpopo Province, South Africa

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Coagulase-negative staphylococci (CNS) are a major cause of nosocomial infection, especially in critically ill and immunocompromised patients. CNS is usually a multi-drug resistant and glycopeptide antibiotics that have been considered to date, the drug of choice for treatment. The aim of this study was to characterize CNS with reduced susceptibility to glycopeptides isolated from the respiratory tract of HIV positive patients from the Limpopo Province in South Africa between 2007 and November 2008. A total of 185 sputum samples were collected from HIV positive drug naive patients and analyzed for antibiotics resistant profiles of the coagulase-negative staphylococci isolates. The isolates were tested for susceptibility to extracts from five medicinal plants commonly used in the Venda region of Limpopo Province. Of a total of 185 sputum samples investigated, 88 were positive for CNS and all of this displayed reduced susceptibility to glycopeptides. Species distribution was as follows: *Staphylococcus auricularis* (5), *Staphylococcus capitis* (6), *Staphylococcus hominis* (23), *Staphylococcus epidermidis* (36) and *Staphylococcus saprophyticus* (18). The incidence of oxacillin, linezolid, daptomycin, imipenem and meropenem resistance was 100%. Evaluation of plant extracts against representative of the isolates showed all the extracts from the bark of medicinal plants to have inhibitory effect on their growth. There is high prevalence of vancomycin resistant CNS among HIV positive patients who participated in this study. This prevalence is quite alarming as this is the only drug of choice since methicillin resistance among this group of organisms is equally quite common. The plant extracts demonstrated antibacterial activity as they inhibited the growth of the isolates they were tested for. Surveillance by anti-biotyping with attention to multi-resistant profile, and warning to clinicians, is necessary. Similarly, further investigation is needed to identify the chemical composition of the active antibacterial compounds in the plant extracts.

Key words: Coagulase-negative staphylococci, vancomycin, antibiotic resistance, plant extracts, HIV.

INTRODUCTION

The group of Gram positive bacteria identified as coagulase negative staphylococci (CNS), often harmless commensals, has become important pathogens in clinical laboratories around the world (Kloos and Bannerman, 1994; Cerca et al., 2005; Arciola et al., 2006; Bayram and Balci., 2006; Caierao et al., 2006). CNS as human

pathogens are usually associated with healthcare settings and occur in patients who are immnuocompromised or harboring indwelling polymer or metallic devices (Bisno, 1995; Brodie et al., 2000; Sohan et al., 2001; Bannerman, 2003). By 1985, there were 19 recognized species of CNS, 8 of which exhibited a possible association with human infections (Kloos and Jorgensen, 1985). At that time, the most commonly isolated of the pathogenic species were *Staphylococcus epidermidis* and *Staphylococcus haemolyticus.*

Over the last decade, the number of CNS species has grown to a total of 39, with half of these species isolated from human tissues and blood samples (von Eiff et al., 2002; Euzeby, 2007). CNS is ubiquitous in nature, residing on skin and mucous membranes (Costa et al., 2004). When exposed to medical devices, the CNS anchor themselves to a polymer surface via van der Waal's forces, hydrophobic interactions and polarity, ultimately forming a thick biofilm (Mack et al., 2006). Production of bio-film reduces the organism's susceptibility to specific antimicrobials which are highly active against planktonic cultures (John and Harvin, 2007). Wide spread use of antibiotics has provided a reservoir of antibiotic-resistant genes. Most invasive CNS strains that formed biofilms are resistant to multiple antibiotics, and more than 80% of these are methicillin resistant. In these strains, mecA genes are significantly abound. Genetic markers could help to discriminate between potential virulent and saprophytic strains of CNS (Piette and Verschraegen, 2009).

Treatment of CNS infections has become increasingly difficult due to the high prevalence of antibiotic resistant strains. Widely used antibiotics including penicillins, particularly semi-synthetic penicillins, cephalosporins, macrolides, aminoglycosides and tetracyclines, have proven to be ineffective in inhibiting several prevalent species of CNS, thus necessitating the need for new and effective antimicrobials (Cerca et al., 2005; Arciola et al., 2006). CNS carries a wide variety of multi-drug resistance genes on plasmids which can be exchanged and spread among different species of staphylococci including *Staphylococcus aureus* and *Staphylococcus intermedius* (Neihart et al., 1998). The multi-resistant determinants can be transferred to new bacterial hosts as part of the large conjugative replicons which commonly encode resistance to some major antibiotics. Over the last two decades, the increasing incidence of methicillin-resistant staphylococci has caused significant clinical concerns worldwide. Methicillin resistance in CNS is also associated with resistance to several commonly used antimicrobial agents such as the macrolides, lincosamides, quinolines, trimethoprim-sulfamethoxazole and aminoglycosides. Glycopeptides have traditionally been considered as the drug of choice in the treatment of infections caused by these organisms. Since the first report of teicoplainin resistance in methicillin-resistance CNS in the USA and UK, the emergence of glycopeptides-resistant CNS in patients treated for long period with vancomycin has been increasingly documented (Aritaka et al., 1997; Smith et al., 1999; Natoli et al., 2009). With the increasing incidence of multi-drug resistant staphylococci and the emergence of resistance to glycopeptides in CNS, therapeutic options have become increasingly limited. Thus, there is a clear need for novel agents as alternatives in the treatment of infections caused by these organisms.

Medicinal plants have been used by humans as a source of relief from illness for thousands of years. They represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are sources of many potent and powerful drugs. The people of South Africa have a long history of traditional plant usage for the treatment of various diseases and aliments (Cunnigngham, 1993; Obi et al., 2003). In Limpopo Province, *Peltophorum africanum, Carissa edulis*, *Securidaca* spp. and *Combretum molle* are commonly used medicinal plants for different microbial infections and are claimed to have curative properties (Obi et al., 2003). If plants products are to be exploited as medicines for treatment of CNS, then isolation and characterization of their active molecules are essential. Most important are problems which follow development of methods for their preservation, stabilization, formulation, delivery and patentability rights. All of this requires enormous development costs and years of patient work. But it's very clear that there are active compounds that could be developed into new generations of modern drugs (Theo et al., 2009).

In this study, we ascertained the antibiograms of CNS isolates and also investigated the antimicrobial properties of the leaf and bark of the extracts of these plants against CNS isolates from HIV drug naive positive individuals.

MATERIALS AND METHODS

Ethical considerations

The study protocol was approved by the Health, Safety and Research Ethics Committee of the University of Venda, South Africa. Approval to collect samples for the study was provided by the Limpopo Provincial Department of Health, South Africa.

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Permission was also obtained from the authorities of the health establishments from where study participants were recruited. Potential study participants provided signed informed consent before the collection of demographic data and blood samples.

Study population and sample collection

A total of 185 sputum samples collected from HIV-1 positive patients in Limpopo Province, South Africa between December 2007 and November 2008 were screened for the presence of coagulase-negative staphylococci. The study population consisted of 135 females and the rest were males. The age range was between 16 and 56 years with a mean of 37 years. The WHO stage of the disease was not taken neither were the clinical conditions of the patients known. Also, their viral load and CD4 count were not taken at the time of sample collection.

Media and culture conditions

All sputum samples were first inoculated onto blood agar (Oxoid) plates. The plates were incubated at 37°C for 24 to 48 h. All suspected staphylococcal isolates were again inoculated onto manitol salt agar (Oxoid) and plates were incubated at 37°C for 24 to 48 h.

Coagulase test

Slide coagulase test of all the isolates were performed by emulsifying few pure colonies of staphylococci from blood agar on undiluted plasma. Tube coagulase tests were performed by diluting the plasma in freshly prepared normal saline (1:6). Three to four pure colonies were emulsified in 1 ml of diluted plasma and the tubes were incubated at 37°C. Readings were taken at 1, 2, 3 and 4 h and further incubated overnight at room temperature to ensure they were coagulase negative (Baird, 1996; de Mattos et al., 2003).

Biochemical and antimicrobial sensitivity tests

All CNS isolates from the blood agar plates were Gram stained and the Gram positive isolates were identified on positive 29 combo microscan plates (MicroScan Germany). This automated system uses computer software to analyze combined biochemical and antibiotic susceptibility profiles. This robotic system scan uses fluorimetric and photometric readings to record fluorescence, turbidity and colorimetric signals which indicate relative growth in each 96 well plates. The plates were inoculated with 24 h growth culture and incubated at 37°C for 24 h. The plates were then read in the MicroScan machine that was connected to a computer. Antibiotic resistance was determined according to CLSI (Clinical and Laboratory Standards Institute) standards.

Preparation of crude plant extracts

Roots or leaves of plants were washed in distilled water, chopped into small pieces and allowed to dry in an incubator for two weeks. Dried material was ground to powder. Methanol extracts were obtained by soaking 200 g of grounded material in 1 L of methanol and left overnight on a rotating platform. The crude extracts were then filtered through Whatman No. 1 filter paper and filtrate was evaporated to a small volume at 40°C using a rotary evaporator. The concentrated extracts were subsequently dried under a fan at room temperature.

Antibacterial activity

Minimum inhibitory concentration (MIC) of extracts for antibacterial activity was determined using the micro-dilution bioassay (Eloff, 1998) as previously described (Amoo et al., 2009). Overnight cultures (incubated at 37°C in a water bath with a rotary shaker) of the bacterial isolates were diluted with sterile Mueller-Hinton (MH) broth (1 ml bacterial suspension/50 ml MH). The crude plant extracts were dissolved in sterile water to make 50 mg/ml. 100 µl of each extract were two fold serially diluted with sterile distilled water in a 96-well micro-plate for all the isolates. A similar two fold serial dilution of neomycin (Sigma) (0.1 mg/ml) was used as a positive control against each isolate. 100 µl of each isolate culture was added to each well. The methanol solvent and bacteria-free broth were included as negative controls. The plates were covered and incubated overnight at 37°C. Bacterial growth was indicated by adding 50 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37°C for at least 30 min. Bacterial growth in the wells was indicated by a reddish-pink colour, whereas clear wells indicated inhibition by the tested extracts. MIC values were recorded as the lowest concentrations of extracts showing clear wells.

RESULTS

Out of the 185 samples that were analyzed, 88 (47.5%) isolates were identified as CNS both on coagulase test and species identification by the MicroScan. Five species isolated were: *Staphylococcus auricularis* (n = 5), *Staphylococcus capitis* (n = 6), *S. epidemidis* (n = 36), *Staphylococcus hominis* (n = 23) and *Staphylococcus saprophyticus* (18). Prevalence of antibiotic resistance to the following antimicrobial agents: oxacillin, amoxicillin, ampicillin, amp/sulb, cefazolin, ceftrixor, imipenem, meropenem, linezolid, clindamycin, daptomycin and vancomycin was 100%. About 85, 82, 83 and 86% of CNS isolates were resistant to rifampin, tetracycline, erythromycin and synercid, respectively. 42% were resistant to levofloxacin and moxifloxacin, respectively, while prevalence of resistance to gentamicin was 33 and 72% for trimethoprim and chloramphenicol. The frequencies of resistance of the isolates to different antibiotics are shown in Figure 1.

Results obtained from the plant extracts showed that all the medicinal plant extracts exhibited potential antibacterial activity against representatives of the test isolates with a MIC value of 0.39 mg/ml as shown in Table 1. The back extracts of the medicinal plants demonstrated significant antimicrobial activity (>14 mm)

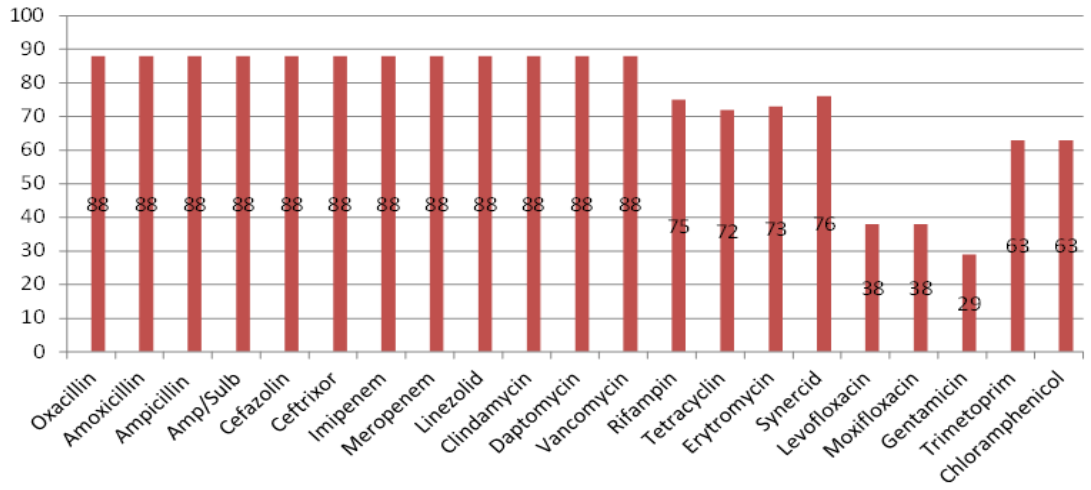

Figure 1. Frequency of antibiotic resistance among the CNS isolates from HIV positive patients.

Table 1. Antibacterial activity (MIC mg/ml) of different extracts from *P. africanum, C. edulis, Securidaca* spp. and *C. molle.*

Plant	Plant part	Average minimum inhibitory concentration (mg/ml)				
		S. auricularis	***S. capitis***	***S. epidemidis***	***S. hominis***	***S. saprophyticus***
P. africanum	Leaf	1.56	1.56	1.56	1.56	1.56
	Bark	**0.78**[a]	**0.78**	**0.39**	**0.78**	**0.78**
C. edulis	Leaf	3.13	1.56	1.56	1.56	1.56
	Bark	**0.39**	**0.78**	**0.39**	**0.39**	**0.39**
Securidaca spp.	Leaf	3.13	1.56	1.56	1.56	3.13
	Bark	**0.39**	**0.78**	**0.78**	**0.78**	**0.78**
C. molle	Leaf	3.13	3.13	1.56	1.56	1.56
	Bark	**0.78**	**0.78**	**0.78**	**0.78**	**0.39**
Neomycin (µg/ml)		9.75×10^{-2}	3.13	3.13	3.13	1.56

[a]Values in bold are considered as quite active (<1 mg/ml).

against the test isolates when compared with those extracts from the leaves (<10 mm).

DISCUSSION

The trend in resistance in CNS over the last 14 years has continued to show escalation in the frequency and expression of resistance determinants. Resistance to β-lactam agents (methicillin resistance; MR) has remained the foremost strain determinant. Strains of MRCNS are often linked to multi-resistance, like their MRSA counterparts, thus presenting an ongoing therapeutic challenge (Mongkolrattanothai et al., 2004; Noto et al., 2006).

Jones et al. (2003) in a study testing the susceptibility of only skin and soft tissue isolates in 283 US hospitals and 301 hospitals in Europe for 9 antimicrobials, including amoxicillin-clavulanate, cefotaxine, cetriaxone, ciprofloxacin, erythromycin, gentamicin, levofloxacin,

trimethoprim/sulfamethaoxazole and vancomycin revealed that almost all MSCNS isolates were susceptible to amoxicillin-clavulanate. In this study, all the isolates were resistant to amoxicillin-clavulanate, 33% of isolates were resistant to gentamicin, and 72% showed resistance to trimethoprime/sulfamathoxazole, while all were resistant to vancomycin.

The prevalence of resistance among the isolates was 100% to cefazole, ceftriaxor, imepenem, meropene, vancomycin, clindamycin and oxacillin, respectively, while majority were resistant to tetracycline, refampicin, erythromycin and synercid, respectively, and 42.9% to levofloxacin and moxifloxacin. Also, the prevalence of resistance to chloramphenicol and trimethoprim was 72%, respectively. The prevalence of resistance to ciprofloxacin among MRCNS across Europe and US has been shown to be between 65 and 67% and about 47%, respectively (Jones et al., 2003). Cuevas et al. (2004) studied resistance in clinical isolates of CNS in Spain for five periods from 1986 to 2000 (Cuevas et al., 2004). For all the years together; oxacillin resistance was 61%, gentamicin resistance was 41.4% while resistance to ciprofloxacin rose from 1.1 to 45%. However, there was no vancomycin resistance even though they had one isolate that was resistant to teichoplanin.

In another study done in Uppsalla, Sweden to determine more specifically how an ICU stay will influence resistance, high rates of oxacillin and ciprofloxacin resistance (92 and 83% respectively) in 20 ICUs were observed. In a study from Greece on intravenous catheter infections associated with bacteremia, over a 2 year period CNS were responsible for nearly 60% of infections (Paragioudaki et al., 2004). For these pathogens in 1999, the resistance rate was 72% for ampicillin, oxacillin, ceflazidine, ceftriaxone, cofactor, amoxicillin/clavulanate and imipenem, with slightly lower rates for ciprofloxacin and amikacin. However, there was no vancomycin resistance among CNS isolates.

The prevalence of CNS has increased over the past 20 years. They are commonly encountered in hospitals patients, particularly in infections related to heart valve disease intravascular catheters, and neurosurgical and arterovascular shunts. Resistant strains of CNS are becoming an alarming problem. Clinical failure of teicoplainin and vancomycin treatment in CNS and in MRSA in Japan (Hiramatsu et al., 1997) and in the US (Center et al., 2003) has already been reported. Resistance to vancomycin among coagulase-negative staphylococci was first reported more than 20 years ago (Siebert et al., 1979). However, the first report of a clinically significant isolate was in 1987 (Schwalbe et al., 1987). Since that time, there have been several other case reports of clinically relevant coagulase-negative staphylococci that had diminished susceptibility to vancomycin (Aubert et al., 1990; Dunne et al., 2001; Krcmery et al., 1996; Sanyal et al., 1991).

Since the initial report of reduced susceptibility to vancomycin in coagulase-negative staphylococci, there have been at least 20 studies that have screened large numbers of isolates in an attempt to define the prevalence of this problem. 11 of these studies did not find any isolates of coagulase-negative staphylococci with reduced vancomycin susceptibility (Barelli et al., 1999; Ena et al., 1993; Udo et al., 1995; Hanberger et al., 1997; Biavasco et al., 2000; von Eliff et al., 2000), and the 9 that are not described in more details in Table 1. From the studies, it appears that the incidence of these organisms is very low. In a study by Froggatt et al. (1989), 42% of *S. haemolyticus* isolates were intermediately resistant (MIC 6.25 g/ml) to vancomycin. Kimberly et al. (2003) in a study involving 321 coagulase-negative staphylococci, reported that a 3.9% of the CNS isolates had decreased vancomycin susceptibility while Natoli et al. (2009) reported a prevalence of 5.4% of CNS with reduced susceptibility to glycopeptides. Del et al. (1999) evaluated the antimicrobial susceptibility of 239 CNS isolated from blood samples and reported that all isolates were susceptible to vancomycin. Maugein et al. (1990) studied the *in vitro* activities of vancomycin and teicoplanin against 185 coagulase-negative staphylococcal strains isolated from 80 neutropenic patients who received different antibiotic treatments and reported that all strains were susceptible to vancomycin.

All the isolates in this study showed resistance to vancomycin, the commonly used antimicrobial agents that were tested against them; a finding that is quite alarming and different from other studies reported in other literatures. CNS infections preferentially affect immnunocompromised, long-term hospitalized and critically ill patients (Ziebuhr, 2001). Increasing antibiotic resistance of nosocomial isolates of CNS aggravate the problem and pose a great challenge for the management of hospital acquired infections in general (Goossens, 2005). Also, the implications of this finding is that little choice is available to the patients and this obviously will have impact on the general health care delivery in the region on the long run. As a consequence, the urgent need for an alternative therapy cannot be overemphasized and search therefore, should be extended to medicinal plants that are known to have antibacterial properties.

Plants are important sources of potentially useful structures for the development of new chemotherapeutic agents and the first step towards this goal is the *in vitro* antibacterial activity assay (Tona et al., 1998). Many

reports are available on antiviral, antibacterial, antifungal, antihelmintic and anti-inflamatory properties of plants (Samy et al., 2000; Palombo et al., 2001; Kamaraswamy et al., 2002; Stepanovic et al., 2003; Obi et al., 2002). In this study, the methanol bark extracts of *P. africanum, C. edulis* and *Securidaca spp* and *C. molle* showed potent antibacterial activity against CNS isolates that were isolated from HIV-1 positive drug naïve patients in Limpopo Province, South Africa. The results of this study clearly indicated that the medicinal plants investigated inhibited the growth of the test isolates of CNS.

There are no previous reports, known to us, on antibiotic resistant patterns of CNS from HIV patients in Limpopo Province in South Africa. HIV patients due to their immune compromised status are prone to various bacterial infections and antibiotics are usually indicated for such infections. Isolation of organisms with antimicrobial resistance patterns, as reported above, may impact on the choice of medication for these patients. Consequently, antibiotic sensitivity tests are warranted prior to prescription as most CNS bacteria are likely to show some degree of resistance as a result of transfer of resistant plasmids among different genera. Glycopeptide susceptibility of CNS and Gram positive pathogens can no longer be assumed and hence consistent routine susceptibility testing and elaborate monitoring are necessary imperatives for effective therapeutic strategies and interventions.

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