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The Relationship of Opportunistic Gram-Positive Bacterial Biofilms and Nosocomial Infections

Hamido M. Hefni[1], Tarek R. Mohamed[1], Hassan A.M. Samaha[1] and Sameh R. Ahmed[2]

[1]Microbiology and Immunology Department, Faculty of Pharmacy (Boys Al-Azhar University, Cairo, Egypt.
[2] Faculty of Pharmacy, Al-Maarefaa Colleges, Riyadh, Saudi Arabia.

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Two hundred and twenty one Gram-positive bacterial isolates obtained from Intravascular catheters (IVC), blood culture, and environmental samples of three Egyptian hospitals were identified as Corynebacteria species (48%), Coagulase negative staphylococci (CNS) (25.8%), *Bacillus cereus* isolates (25.8%), and *Staphylococcus aureus* isolate (0.45%). Most of isolates were multiple drug resistant (MDR) confirming their nosocomial source. Matching the antimicrobial resistance patterns of microorganisms isolated from skin around catheter, IVC and blood specimens of the same patient showed that 29% CNS IVC isolates were exogenously implanted versus 6.5% isolates were endogenously implanted from blood. Alternatively, 7.7% *B. cereus* IVC isolates were of exogenous origin, whereas 25.3% isolates were endogenously implanted. Finally, 11.3% Corynebacteria species isolates were exogenously implanted and 33% were endogenously implanted. Statistical analyses showed the lack of any specific type of reaction as synergism, mutualism and antagonism between any couple of the investigated microorganisms. Semi-quantitative biofilm assay results were directly related to viable count of IVC which is directly related to blood stream symptoms, e.g. fever. Both viable count and bioadherence assay results were directly related. Also, data may suggest that the more the number of multidrug resistance markers per isolate, the higher the level of microbial bioadherence degree.

Key words: Gram positive, Bioadherence, Nosocomial infection.

Intravascular catheters (IVC) and urinary catheters are the two most commonly inserted medical devices with a dramatic increase in nosocomial bloodstream infection as a result of biofilms formation on their surfaces (Lynch, and Robertson, 2008). The most frequently involved microorganisms were *S. aureus*, coagulase negative staphylococci (CNS) (Fredheim *et al.*, 2009), *Enterococci*, aerobic Gram-negative bacilli, *Klebsiella pneumoniae, Pseudomonas aeruginosa* (Lynch, and Robertson, 2008). The incidence of MDR Gram-positive infections is partly increasing because drug development in the last twenty years concentrated largely on agents active against Gram-negative pathogens (Baquero, 1997). Therefore, this study aimed to investigate opportunistic Gram-positive biofilm role in nosocomial infection.

* To whom all correspondence should be addressed.
E-mail: samaha1964@yahoo.co.uk

MATERIALS AND METHODS

Microorganisms

Two hundred and twenty one microbial isolates were obtained from IVC, blood, skin specimens of both patients, medical staff, besides environmental samples from three Egyptian university hospitals.

Media

Prepared media were prepared according to published method (Kumar, 2007), and ready-made media was prepared according to instruction of the manufacture.

Biomaterials: PTFE intravenous catheters

Collection and Processing of the Intravenous Catheters

IVC internal part inserted for up to 72 hours was transferred to 3ml of modified Amie's transport medium in 5 ml sterile screw capped tubes. These tubes were sonicated using Becton Dickison, USA, sonicating system for 1 minute, and vortexed for twenty seconds. To assure the removal of all adherent microorganisms, sonicated IVCs were longitudinally cut, Gram stained and observed under microscope. Five µls loop was surface streaked on blood agar plates, for viable count morphological, and biochemical identification. The remaining of the transport media were incubated at 37 ˚C in a sterile double strength tryptone soy broth (TSB) tube for seven days to detect any microbial growth. Moistened swabs obtained from patients, medical staff skin and environmental samples were streaked on a nutrient agar. Blood samples of patients from with IVC_s were collected in sterile vacuum tubes with lithium heparin (Eastern Chemicals Limited Company, Italy). Five µls was surface streaked on Brucella base agar plates and the remainder was inoculated in tryptone soy broth (TSB) tubes and incubated for 2-7 days at 37 ˚C (Sherertz, *et al.*, 1990).

Identification of Microbial Isolates

Microbial isolates were identified manually according to published method (Kumar, 2007).

Antibiotic Susceptibility Test

Susceptibility test was done by disk diffusion method according to published method (Sherertz, *et al.*, 1990).

Semi-quantitative bioadherence Assay

Semi-quantitative bioadherence Assay was performed according to the previously published method (Christensen, *et al.*, 1985). Briefly, Five ml of TSB in sterile polystyrene tubes were inoculated with 5 ml loop of each isolate grown on nutrient agar plate. *S. aureus,* CNS and *Corynebacterium spp*. isolates tubes were incubated at 37°C for 18 hours, whereas *B. cereus* isolates were kept for 24 hours. All tubes were emptied without washing stained with crystal violet and left for 1 minute, emptied, tap water washed and air dried. A sterile TSB tube was used as control. All tubes were visually examined for intensity of biofilm stain lining the tube wall. The intensity of stain was visually scaled from negative as in control to ++++ as judged by the help of three observers.

Statistical Analyses

Statistical analyses were performed using the Microsoft Excel for windows and Statistical Package for Social Science (SPSS) 10.0 for Windows on an IBM-PC. Comparisons between groups were made using the Pearson's correlation coefficient (*r*) as appropriate. A level of significance value of <0.01 (two-tailed) was considered statistically significant.

RESULTS AND DISCUSSION

As a result of drug development during the last two decades, the incidence of multidrug-resistant Gram-positive infections is increasing, since drug industry concentrated largely on agents active against Gram-negative pathogens (Baquero, 1997) with concomitant increase in the prevalence of gram- positive bacteria involved in chronic nosocomial infections associated with the more frequent use of in-dwelling medical devices(Cramton, 2000). The incidences of IVC implantation with Gram-positive opportunistic pathogens were arranged in the following descending order, *Corynebacterium spp.* 106(48%), CNS 57 (25.8%), *B. cereus* 57(25.8%) and one isolate *S. aureus* (0.4%), respectively. Besides serious multiple drug resistant nosocomial infection with *C. jeikeium* associated with plastic devices inserted into patients (Riebel, *et al.*, 1986), *Corynebacterium* spp. had been implicated as causative organisms in endocarditis associated with indwelling IV device and prosthetic heart valves (Knox, and Holmes, 2002). CNS, which is considered as the third most common causative agent of nosocomial infections are frequently associated with nosocomial bloodstream infections (Spencer, 1996). Also, *B. cereus* isolates were implicated in opportunistic infections following trauma, placement of artificial devices and catheters (Strohl, *et al.*, 2001).

Clinical CNS isolates were most frequently isolated from IVC (54.8%), Skin (35.5%) and blood (9.7%), whereas environmental CNS isolates were (26.32%), respectively. All CNS clinical isolates were 100% sensitive to methicillin, oxacillin, and gentamicin with prevalences for antimicrobial sensitivities were in the following descending order as follows: cefoperazone and ofloxacin (96.77%), clindamycin (93.55%), erythromycin and doxycycline (90.32%), erythromycin(80.65%), vancomycin (70.97%), rifampicin (45.2%) and amocxicillin + clavulanic acid (38.7%), respectively (Figure 1). Literature reported variable incidences for methicillin sensitive CNS isolates from 71.1% in coronary cardiovascular surgery to as low as 25.6% (Khadri, and Alzohairy, 2010), where these methicillin sensitive CNS isolates, as in this study, were, also, sensitive with different degrees to other antibiotics.

There were both qualitatively and quantitatively similiraties between the antimicrobial sensitivity markers of clinical and environmental frequencies percentages. Thus, all environmental and CNS staff isolates were 100% methicillin, gentamicin and oxacillin sensitive, whereas 100% resistant to both penicillin and ampicillin. The absence of methicillin resistance both in clinical and environmental isolates refers to common origin for both isolates and probable short hospitalization (Altoparlak, *et al.*, 2004). Also, the similarities in the incidences of multidrug resistant between clinical and environmental CNS isolates attested for their nosocomial origin and that clinical isolates could not originate from community environment.

IVC implantation could be either exogenous from patient skin as during IVC implantation, with early contamination onset or late onset of endogenous source (Von Eiff, *et al.*, 2002). When the antimicrobial susceptibility patterns of CNS isolates on IVC, blood and patient skin were compared, no CNS isolates match was found on the three compartments, i.e. skin, IVC and blood, which meant no exogenous CNS blood stream infection. However, major CNS match was found on CNS isolates on skin and IVC compartments (29%), i.e. exogenous IVC implantation. Minor CNS match was detected on IVC and in blood compartments (6.5%) referring to endogenous implantation (Figure 3). The lack of match between CNS isolates on skin, IVC and blood is not unprecedented in the literature, several investigators failed to find a match between CNS isolates on the three compartments using biotyping methods (Patrick, *et al.*, 1989). Even, if match

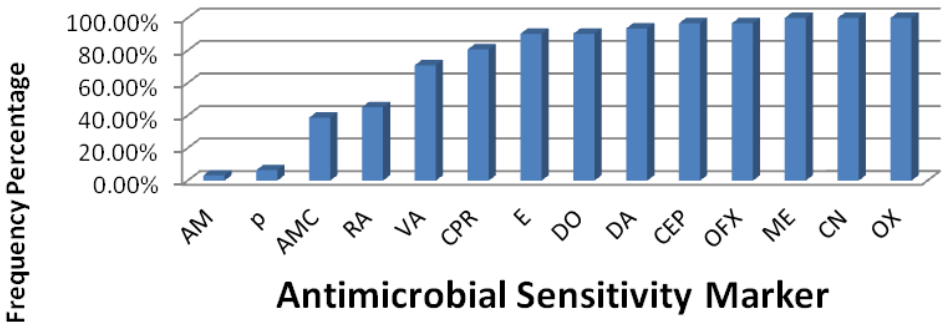

Fig. 1. Frequency percentages of CNS clinical isolates according to antimicrobial sensitivity marker

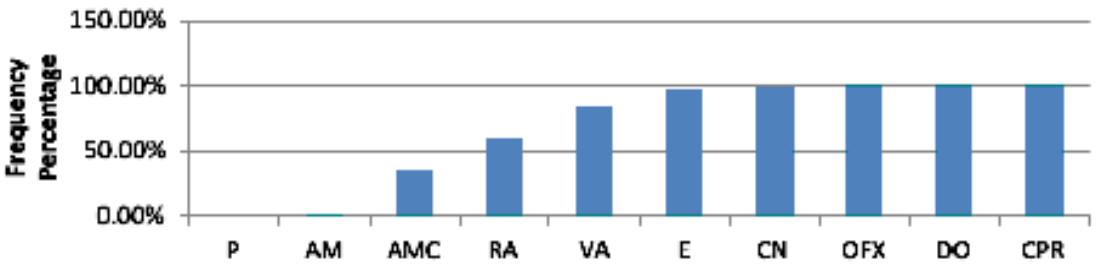

Fig. 2. Frequency percentages of antimicrobial sensitivity markers of Corynebacterial isolates

happens between the three compartments, it was found to be a rare event (Costa, *et al.*, 2006), except for blood stream infection (BSI) detected in neonatal intensive care units probably due to severely impaired immune response and vulnerability of neonates in ICUs (Jain, *et al.*, 2004).

CNS isolates, sometimes, were detected on a single compartment only, i.e. IVC or skin or blood. Arranged in descending order, CNS isolates without antimicrobial susceptibility match with other isolates on other compartments, were detected on IVC (35.5%), skin (19.4%) and in blood (3.2%). CNS existence on IVC without detection on skin could be due to skin disinfection after IVC implantation (Leonidou, and Gogas, 2010). Alternatively, CNS skin detection without existance on IVC could be due to cross transmission from another patients, medical staff, and surrounding medical objects or due to too short period to allow biofilm formation on IVC followed by infection (Agvald-Ohman, *et al.*, 2004).

Finally, CNS BSI exclusive detection in blood compartment could be ascribed to recent or transient bacteremia without enough time to develop IVC implantation. It should be noted that diagnosis of catheter related BSI could be on the premises of one or more positive blood cultures growing a single morphotype (strain) as a sole isolate (Sidebottom, *et al.*, 1988) using their colonial morphology, species identity and antibiogram susceptibility similar to those detected on IVC (Freeman, *et al.*, 1990). The use of antibiogram alone gives 66% of discriminatory power between CNS species and achieves 95% of the discriminatory power when used along with biotyping and/or phage typing (Ludlam, *et al.*, 1989).

Congruent with morphological and biochemical characters of genus *Corynebacterium* spp was the most frequently isolated bacteria, with incidence (47.9%). Unlike *C. diphtheriae*, isolates were all ²-hemolytic, 100% resistant to penicillin, 98.1% resistant to ampicillin, 65% amoxicillin + clavulanic acid resistant and 40.6% rifampicin resistant (Maple, *et al.*, 1994). Also, unlike MDR resistant *C. jeikeium*, all corynebacterial isolates were fermentative and ²-hemolytic (Funke, *et al.*, 1997).

As shown in (Figure 2), corynebacteria isolates were 100% resistant to penicillin and 100% sensitive to ofloxacin, ciprofloxacin and doxycycline. This underscores the clinical importance of ciprofloxacin and doxycyclin as empiric antibiotic treatment for corynebacteria nosocomial infection unlike cefotaxime used in this study. In descending order, antimicrobial sensitivity of corynebacterial isolates were 99.1% gentamicin, 98.1% erythromycin, 83% vancomycin,

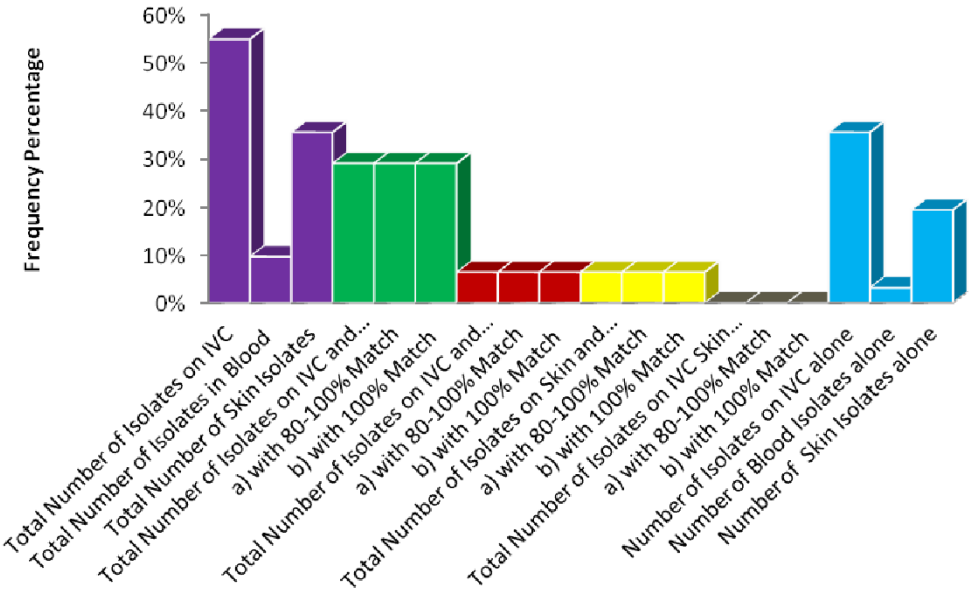

Fig. 3. Distribution of clinical CNS isolates on IVC, Skin and in blood specimens of patients

59.4% rifampicin, 34.9 amoxycillin + clavulanic acid and 1.9% ampicillin. Unlike previous studies which reported 100% corynebacterial isolates sensitivity to vancomycin (Renom, *et al.*, 2007), this study detected the emergence of vancomycin resistant corynebacteria, reaching 17% in a background showing 65.1% of amoxycillin + calvulanic acid resistance (Figure 2).

Most of Corynebacterial isolates were multidrug resistant. In descending order, 36.7% were resistant to three antimicrobial agents, 34% to four resistance markers, 17% resistant to two markers, 11.3% to five resistance markers and 0.9% resistant to six antimicrobial markers, i.e. nosocomial pathogens. Unlike CNS isolates, corynebacterial isolates were neither detected on staff skin nor on hospital equipment. However, staff skin, hospital equipment, bed sheet linen and other environmental components were not exhaustively searched. Thus, 106 corynebacterial spp. isolates were exclusively detected in clinical samples obtained from 52 patients from burn and general surgery wards. Corynebacterial isolates were most frequently detected on IVC 48.1%, followed by

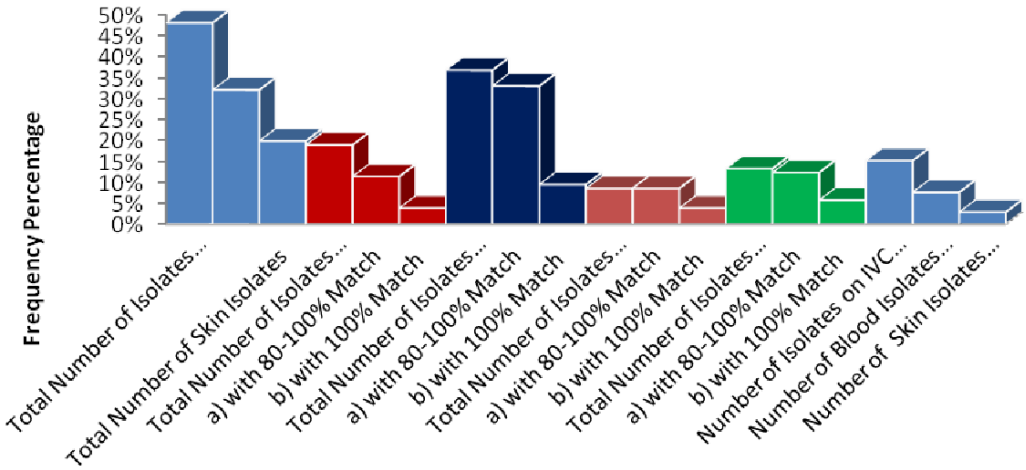

Fig. 4. Distribution of Corynebacteria in blood, on IVCs and on patient skins

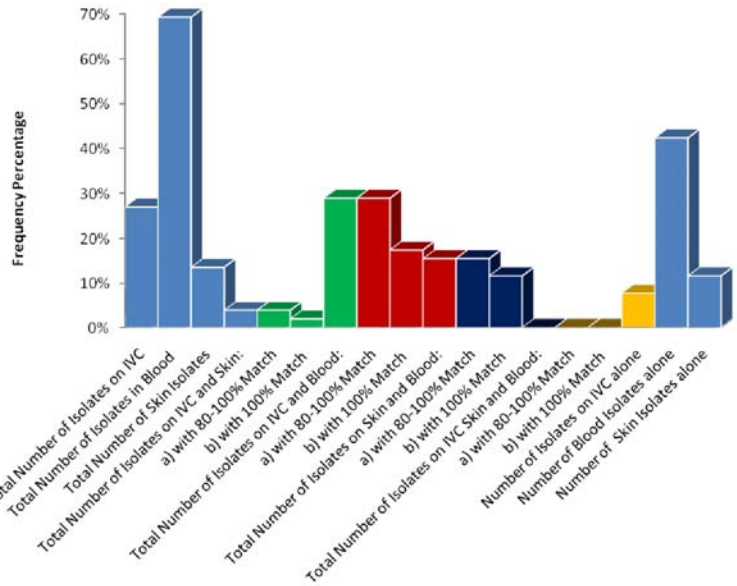

Fig. 5. Distribution of *B. cereus* isolates between IVCs, Skin and blood compartments

blood 32.1% and least frequently detected on skin 19.8%. Presence of 34 corynebacterial blood isolates out of 52 patients refers to presence of corynebacterial outbreak between patients. Several reports showed corynebacterial spp. invasiveness between hospital in-patients (Renom, *et al.*, 2007).

In this study, 23.5% of blood isolates exclusively existed in blood, i.e. without IVC and/or skin implantation (Figure 4). Some investigators (Funke, *et al.*, 1997), reported six *C. mucifaciens* sp. nov. isolates in blood of different patients without detecting the same isolates on skin and IVC. Others (Granok, *et al.*, 2002) reported *C. minutissimum* cellulites in immunocompetent patient without detection on IVC devices. Also, 163 corynebacteria strains were detected in different clinical specimens of Brazilian university hospital patients, where 46.62% of Corynebacteria were from blood and GIT sites (Camello, *et al.*, 2003). Renom *et al.*, 2007) reported nosocomial outbreak of *C. striatum* infections in 21 patients with chronic obstructive pulmonary disease. The authors concluded that *C. striatum* can be transmitted between patients, from person to person, and via caretakers. Thus, Corynebacteria could be colonized internally Renom *et al.*, (2007) in GIT.

The increasing reports of Corynebacterial species, other than diphtheria, diagnosis as a etiological agent for disease (Funke, *et al.*, 1997) could be due to recent better taxonomy of these organisms, better identification schemes and/or growing population of immunocompromised patients (Camello, *et al.*, 2003). Clinical sheet data showed that 46.2% of patients involved in this study were immunocompromised. Eight out of 24 had general surgery, 25% were suffering from burns, 20.8% had appendicitis, 8.4% had abdominal wound, 4.2% was diabetic and 4.2% had HIV.

Fourteen Corynebacterial isolates (13.2%) were detected on IVC, skin and in blood samples of the same patients. Thirteen of them (92.9%) showed 80-100% match in antimicrobial spectrum which alluded to probable blood stream infection through IVC. A previous study (Lagrou, *et al.*, 1998) showed that out of 150 identified Corynebacterial spp., 30 isolates (20%) obtained from blood and IVC use were related. Granok *et al.*, (2002) detected two *C. minutissimum* bacteremia related to catheter use in immunocompromised patients. Finally, It was found that most of 34 *C. striatum* nosocomial infections were associated with IVC use.

In addition, 18.9 % of isolates were found on both IVC and skin without concomitant existence in the blood specimens of the same patients. Twelve of them (60%) showed 80-100% match in antimicrobial sensitivity patterns,

Table 1. linear Regression equations, R^2, and SD of CNS, Corynebacterium, and CNS

Item	Linear regression equation	R^2	SD
CNS	Y=0.6449x+3.358	0.8337494	0.630344
Corynebacterium	Y=0.6325x+2.9708	0.819151	0.634513
B. cereus	Y= 06513x+3.1677	0.656818	0.636872

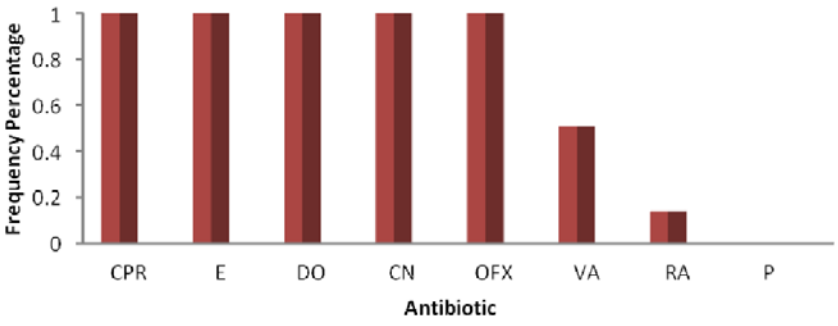

Fig. 6. Frequency percentages *B. cereus* isolates arranged in descending order according to antimicrobial sensitivity

suggesting exogenous source of IVCs implantation with Corynebacteria, i.e. skin. Absence of Corynebacteria in blood may be due to recent IVC implantation. On the other hand, 36.8% isolates were found simultaneously on IVC and blood samples of the same patients without concomitant existence on the skin of the same patients. Thirty-five of them (89.7%) had 80-100% match in antimicrobial sensitivity pattern which may suggest an endogenous IVC implantation. Detection of 33.3% of Corynebacterial isolates on IVC only, without coexistence in blood and/or skin of the same patient suggested plugging of contaminated IVC, IVC implantation after transient bacteremia or recent skin disinfection after IVC implantation. Finally, 14.29% of Corynebacterial isolates were found on skin samples alone. This could be associated to person-to-person transmission, which occurs frequently in hospital environment (Otsuka, *et al.*, 2006).

Fifty-seven *B. cereus* isolates were detected in the clinical samples of fifty-two patients, although environmental as well as staff samples were devoid of *B. cereus*. *B. cereus* isolates were most frequently isolated from blood 63%, followed by 24.6% from IVC and least frequently 12.3% from patient skin. This pattern suggested a *B. cereus* outbreak between patients participated in this study. Absence of *B. cereus* isolates concomitantly detected on the three compartments, i.e. IVC, blood and skin, of the same patient, probably excluded IVC BSI. Thus, all *B. cereus* bacteremia were endogenous (Figure 5).

Detection of *Bacillus spp* in clinical samples was usually regarded as contaminants to clinical samples. That is why, *Bacillus* strains were not identified to species level (Drobniewski, 1993). *B. cereus* BSI was recently reported frequently in hospital infection (Ribeiro, *et al.*, 2010)especially between immunocompromised patients as IV drug abusers, patients receiving hemodialysis or continuous IV infusion, neonates, patients with underlying malignancy, AIDS patients and those with artificial prostheses including orthopedic implants and cerebrospinal shunts(Drobniewski, 1993) as well as patients in burn units as in this study (Jeurissen, *et al.*, 2010).

In general, *B. cereus* clinical infection could be due to: 1) in vitro local implantation of either burns, traumatic postsurgical wounds and eye, or implantation on intact skin followed by introduction to cardiovascular system (CVS) by biomaterial implantation and/or 2) in vivo colonization either on GIT mucosa, as food poisoning, or respiratory tract mucosa followed by introduction to CVS through trauma, surgery causing systemic infections as endocarditis, pericarditis, meningitis, internal abscesses, CNS shunt associated infections(Drobniewski, 1993) and crepitant cellulitis, necrotizing fasciitis as well as gangrene(Ribeiro, *et al.*, 2010). The incidence of non-food poisoning related infections is on the rise due to greater recognition of *B. cereus* as an opportunistic pathogen in immunocompromised patients(Drobniewski, 1993). It should be mentioned that 53.8% patients participating in this study had *B. cereus* bacteremia. Twelve (12/28) 42.9% of them were immunocompromised.

Interesting enough, 63.2% of *B. cereus* isolates were found in the blood stream, where 61.1% of them were found in blood alone without concomitant existence on IVC or skin. The remainder of blood isolates was either 26.3% *B. cereus* isolates having antibiogram matching those on IVC alone or matching *B. cereus* isolates on skin alone (14%), without concomitant existence on IVC. This means *B. cereus* bacteremia is exclusively endogenous (Figure 5).

Although endogenous *B. cereus* infections were reported in literature after postsurgical or traumatic wound, burn, or ocular infection(Ribeiro, *et al.*, 2010)., endogenous *B. cereus* infections were considered minor source of infection.

Most of reports implicated environmental existence of *B. cereus* on hospital linens, carts, tourniquets for blood collection, washstands, washing machines, contaminated with spores, tops of blood culture bottles, non-sporecidal disinfectant as 95% alcohol, telephones, computer keyboards, infusion pumps, blood gas devices, boxes of nitrile gloves, i.e. exogenous sources is the major source of nosocomial infection(Jeurissen, *et al.*, 2010).

Others implicated contaminated water used in extinguishing flame in burn patients, mechanical ventilation tubing system and contaminated air-flow sources, nurse hands and patients forearms (Ribeiro, *et al.*, 2010). Exogenous *B. cereus* existence led to internal bacteremia in

immunocompetent patients via IVC placement and ventricular shunts (Ribeiro, *et al.,* 2010) or through respiratory tract (Kalpoe, *et al.,* 2008).

Surprisingly, *B. cereus* was neither detected in environmental samples nor on skin of investigated medical staff, since environment and staff skin were not exhaustively investigated. However, it is unlikely that major environmental source is the real cause for *B. cereus* bacteremia due to the facts that skin isolates antibiogram neither matched blood isolates (14%) nor matched isolates on both IVC and blood (26.3%) (Figure 5).

Presence of antimicrobial sensitivity match between IVC and skin isolates on the same four patients (7.69%) could be ascribed to a minor source for probable *B. cereus* implantation on IVC. On the other hand, presence of (14%) *B. cereus* isolates match in antimicrobial sensitivity pattern between skin and blood isolates without IVC *B. cereus* implantation could refer to probable cross contamination between patients' skins with *B. cereus* bacteremia (Figure 4). Also, existence of *B. cereus* isolates on either IVC alone or in blood without IVC implantation could probably due to transient bacteremia (Banerjee, *et al.,* 1988).

Screening of *B. cereus* isolates for antimicrobial sensitivity showed that all isolates were 100% sensitive to ciprofloxacin, erythromycin, doxycylin, gentamicin and ofloxacin, whereas resistant to ampicillin and penicillin (Figure 6). Thus, ciprofloxacin, erythromycin, doxycyclin, gentamicin and ofloxacin are suitable for empiric antimicrobial chemotherapy treatment in these cases. Resistance to ²-lactam was associated to ²-lactamases produced by *B. cereus* that made them resistant to third generation cephalosporins as cefotaxime, which was universally used in this study (Hsueh, *et al.,* 1999). Even clavulanic acid did not inhibit *B. cereus* ²-lactamases since 94.7% of isolates were resistant to amoxicillin + clavulanic acid. Several investigators reported *B. cereus* isolates susceptibility to aminoglycosides, clindamycin, chloramphenicol, ciprofloxacin and erythromycin as found in this study(Hsueh, *et al.,* 1999). What is really alarming is that *B. cereus* clinical isolates were 49.1% resistant to vancomycin, which should alert clinicians about the probable loss of vancomycin efficacy in the treatment of *B. cereus* clinical infections, especially knowing that vancomycin is the drug of choice for treatment of *B. cereus* ocular infections as well as other *B. cereus* infection(Hsueh, *et al.,* 1999).. Recently, vancomycin resistant *B. cereus* was isolated from six ventilated pediatric intensive care unit patients(Kalpoe, *et al.,* 2008).

When *B. cereus* isolates were surveyed for the number of antimicrobial resistance markers per isolate, the most frequent pattern was the existence of five antimicrobial markers resistant 43.9% followed by four markers resistance 40.35%, with a common 84.21% were resistant to penicillin, ampicillin, amoxicillin + clavulanic acid and vancomycin, which is really alarming for resistance to all cell wall antimicrobial agents tested.

Linear regression analyses between log of viable count of CNS, Corynebacteria and *B. cereus* isolates from clinical IVC specimens and their semiquantitative bioadherence to polystyrene tubes showed direct linear relationship between any of the above studied microorganisms log viable count and bioadherence level without significant difference between genera and species, i.e. slopes of these microorganisms were very close to each other (Table 1).

Investigating the relationship between viable counts of CNS isolates and *S. aureus* relation to bioadherence to any of the three different IV catheters, namely silicone elastomer, thermoplastic polyurethane and polyurethane coated with hydrophilic sheath, linear relationship was observed between the two items, i.e. log viable count and bioadherence degree (Kristinsson, 1989). Others (Kadurugamuwa, *et al.,* 2003) proved the same direct linear relationship between viable count of *S. aureus* or *Ps. aeruginosa* and their bioadherence level.

Also, results suggested a relationship between the level of microbial adherence and the number of antimicrobial resistance markers per isolate, where a direct relationship was detected between both. Finally, using Pearson correlation factor (r) statistical analyses, it was found that both microbial bioadherence and fever developments were directly related to duration of IVC insertion. Previous studies showed relationship between clinical signs, as fever and chills, and medical device related infection (Von Eiff, *et al.,* 2005). It should be mentioned that authors are not aware of any previous direct relationship both between clinical signs of catheter related infection versus

bioadherence degree and between the direct relationship between IVC insertion duration and bioadherence degree as the results of this study may suggest.

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