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Isolation of the biofilm producing bacteria from osteomyelitis patients at Hospital Tengku Ampuan Afzan (HTAA), Kuantan

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ABSTRACT

Aims: Accurate diagnosis and proper treatments of osteomyelitis are often difficult and ineffective due to several reasons such as less sensitive sample collected and the formation of biofilm following prosthetic use. Thus, our goal of this study is to identify suitable sample for laboratory diagnosis and also microbial species that cause osteomyelitic infection and discriminate between biofilm and non-biofilm producing strains in patients at Hospital Tengku Ampuan Afzan, Kuantan.

Methodology and results: Samples of bone, prosthetic material, tissue and swab were collected from patient with suspected osteomyelitis at the hospital. Bacteria were isolated from sample using methods such as homogenization, direct transfer, and sonication. Then, species identification was done by colony characterization, biochemical test and the API identification system. Once species identified, tissue culture plate method was performed to discriminate the biofilm-producing strain from the non-biofilm-producing strain. The total number of 57 samples were collected from 17 cases of suspected osteomyelitis with 34 samples were found positive bacterial growth. Prosthetic samples produced highest positive growth with 81.3%, following by bone samples with 66.7% while swab and tissue samples with 46.2% and 43.8% respectively. We found that 14 from total 16 pathogens identified were biofilm producing-strains.

Conclusion, significance, and impact of study: Prosthetic and bone samples produced higher bacterial growth, in contrast to other type of samples. Sonication method improves bacterial detection. Biofilm producing-bacteria were also the most common isolated strains from osteomyelitic infection. These have underscored the need to revise current clinical and laboratory practice as proper identification biofilm bacteria may influences management an outcome.

Keywords: Osteomyelitis; Biofilm; Prosthetic; Sonication.

INTRODUCTION

Osteomyelitis results from inflammation of the bone caused by infection of pathogenic organisms. It may progress into a chronic and persistent state, leading into necrosis, destruction of bone and interruption in the formation of new bone, which will increase the risk of pathological fracture (Carek *et al.*, 2001). Osteomyelitis can be classified into acute and chronic state of osteomyelitis. Acute osteomyelitis developed within 2 weeks after onset of infection and predominantly occurs in children (Lew and Waldvogel, 2004). On the other hand, chronic osteomyelitis is a persistent, prolonged infection from several months up to years. Unlike acute osteomyelitis, chronic osteomyelitis is more common in adults (Carek *et al.*, 2001).

The most common pathogen in osteomyelitis is *Staphylococcus aureus*, followed by other species such as *Pseudomonas aeruginosa,* and *Enterobacteriaceae* spp. and 90% of osteomyelitis associated with implant are caused by *Staphylococcus epidermidis* (Ciampolini and Harding, 2000)*.* It was thought that monoculture of bacteria is commonly associated with non-prosthetic samples such as tissue (Gristina and Costerton, 1985; Thomaidis *et al.*, 2013). Polymicrobial infection of mixed gram positive and gram negative bacteria occurs most

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commonly in patients with certain conditions, notably diabetic foot infection (Ciampolini and Harding, 2000). Different profile of infection is also seen with a different age-group. In the newborn and infant, most bacteria isolated are *S.aureus*, *Streptoccoccus agalacticae*, *S. pyogenes*, *Escherichia coli*, *and Haemophilus influenza* predominate in children (Brady *et al.*, 2008). Among adult, the most common microorganism causing osteomyelitis is *S. aureus.* Other bacteria that are less frequently found include *Enterococcus* sp., *Streptococcus* sp., *Enterobacter* sp., *Mycobaterium* sp. and *Candida* sp. (Calhoun *et al.*, 2009).

Biofilm is a community or an aggregation of interacting bacteria attached to a solid surface or to each other and encased in an exopolysaccharide matrix. This formation plays an important role in the osteomyelitis giving rise to persistent infection that lead to the chronic state. Bacterial film can comprise of single species or multiple microbial species congregating to form biofilm on a range of biotic and abiotic surfaces (O'Toole *et al.*, 2000) such as the surface of prosthetic material and necrotic bone tissue (Brady *et al.*, 2008). The biofilm that formed have special features and characteristics such as slow penetration of antibiotic through the biofilm matrix, changes in the growth rate of biofilm bacteria, and physiological alteration due to the biofilm mode of growth that cause ineffective treatment with antimicrobial agents (Donlan and Costerton, 2002), raising the concern of treating non-discriminated osteomyelitis with common or wide-spectrum antimicrobial agents. Different species have different virulence factors and required specific antimicrobial treatments. Inability to identify the accurate species and biofilm-producing strain lead to false positive and false negative diagnosis that will cause wrong treatment management. By identifying biofilm formation ability; we can create the proper treatment management plan that can effectively eradicate the biofilm.

Osteomyelitis diagnosis is often based on clinical findings, including information from medical history, physical examination and laboratory tests. However the benchmark and important measures of osteomyelitis are based on the laboratory tests. The microbiology examination that is included in the laboratory tests is the gold standard for this diagnosis (Carek *et al.*, 2001). An accurate organism identification result depends on the type of specimen such as bone, tissue, and prosthetic implants (Larsen *et al.*, 2012). Other specimen such as sinus tract is not reliable. Sometimes previous antibiotic administration may interrupt the diagnostic accuracy (Legout and Senneville, 2013).

Thus, in this present study, we identify suitable sample for laboratory diagnosis and also microbial species that cause osteomyelitic infection and discriminate between biofilm and non-biofilm producing strains in patients at Hospital Tengku Ampuan Afzan, Kuantan.

The modified criteria that are used in this study are; (1) positive organism cultured from bone, (2) evidence of osteomyelitis on direct examination during surgery or histopathologic examination and (3) at least 2 signs of symptom such as fever, localized swelling, tenderness,

heat, or drainage at suspected site of bone infection. These criteria are based on Centre of Disease Control (CDC)'s criteria for osteomyelitis infection (ECDC, 2012).

The present study would like to highlight the importance of discriminating type of samples, sonication method and biofilms strains. The accurate identification and accurate diagnosis will result in the proper treatment for osteomyelitis.

MATERIALS AND METHODS

Sample collection

All samples were collected from patients with suspected osteomyelitis infection during surgery at the Orthopaedic Operation Theatre Hospital Tengku Ampuan Afzan from September 2013 until January 2014. Diagnosis was made based on the Centre of Control Disease (CDC)'s (as mentioned earlier in the introduction) (ECDC, 2012). The sample collected once the patient read and signed a consent form. Samples were stored in suitable containers at temperature 2-8 °C and transported to the Microbiology Research Laboratory, Kuliyyah of Allied Health Science, IIUM Kuantan. Samples were processed within 48 h of collection.

Bacterial Isolation

Swab sample

Swab samples were obtained using Amies Transport Medium with Charcoal (Healy and Freedman 2006). In the laboratory, each swab sample was spread directly on the agar media and incubated at 37 °C for 24 h (Figure 1).

Tissue sample

Tissue samples were homogenized by shaking them with 5 mL phosphate buffer solution (PBS) (pH 7.2) and 10 ballotini beads in accordance to tissue size at 250 rpm for 10 min as described by Patridge and Towsend (2012). After homogenization, 100 µL of solution was transferred and spread onto the agar media. Plates were then incubated for 24 h at 37 °C (Figure 1).

Bone, prosthetic and beads

Bone and prosthetic samples were processed with the sonication method as described by Trampuz *et al.* (2007). PBS (pH 7.2) was added into each container until the sample was fully submerged in the solution. The sample were later shaken at 250 rpm for 10 min and subjected to sonication in the ultrasonic water bath (Dentsply Neytech, USA) at a frequency of 44 ± 2 kHz for 5 min. The process was repeated for two times and 100 µL of sonicated fluid was spread on the agar media and incubated for 24 h at 37 °C (Figure 1).

Bacterial identification

After 24 h of incubation, the colonies from each agar media (blood agar, tryptic soy agar, MacConkey agar) were subjected to identification assays to determine species by colony characteristics, biochemical test, growth on selective agar media (mannitol salt agar, cetrimide agar) and API Identification System (Biomerieux, France). Plates with low numbers of colony were incubated for another 24 h at the same temperature. Plates that were completely lacked in bacterial growth after 48 h of incubation were classified as no growth plates. As biofilm-producing strain is the only one that is able to adhere to solid surface, positive growth from either prosthetic or bone sample from each patient was selected. They are subjected for species identification and biofilm detection. Bone and prosthetic samples are solid type of material that possesses high chances of biofilm growth compared to tissue and swab samples (Brady *et al.*, 2006; Larsen *et al.*, 2012)

Biofilm detection

The bacterial biofilm assessment was performed using the Tissue Culture Plate method for biofilm detection as described by Stepanović *et al.* (2007) with some modifications. The test strains from stock cultures were prepared as working cultures from overnight incubation on the tryptic soy agar (TSA). After verifying the strain purity, 3 to 4 identical colonies were suspended in 5 mL tryptic soy broth (TSB) and incubated for 18 h at 37 °C. After incubation, the cultures in TSB were vortexed and diluted in 1:100 Tryptic soy broth supplemented with 1% glucose (TSBg). The diluted bacteria were vortexed and transferred into a tissue culture plate with flat bottom (Corning, USA) and incubated for 24 h at 37 °C. The contents of incubated tissue culture plates were decanted. Each well was washed three times with a 300 µL PBS (pH 7.2). After washing, the plates were dried at room temperature before fixation. Then, the biofilm in the well were fixed with 150 µL methanol for 20 min and left to air dried overnight in an inverted position at room temperature. The dried plates later were stained with 150 µL of 1% crystal violet for 15 min. After staining, the plates were washed with distilled water and dried. Then the crystal violets were resolubilized with 150 µL of 95% ethanol. Optical density (OD) reading was taken for assessment. Wells with OD reading more than ODc (average OD of negative control + 3 (SD of negative control) were assumed as indicative of biofilm production (Figure 2).
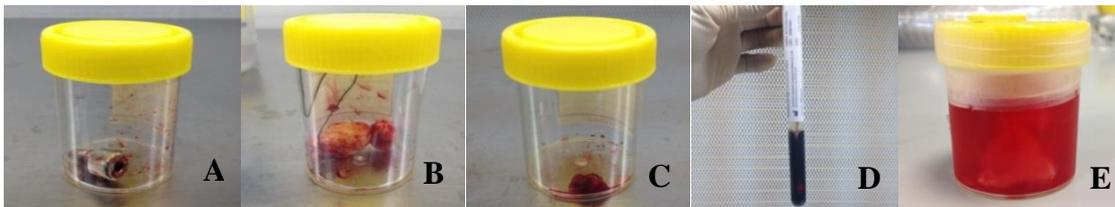

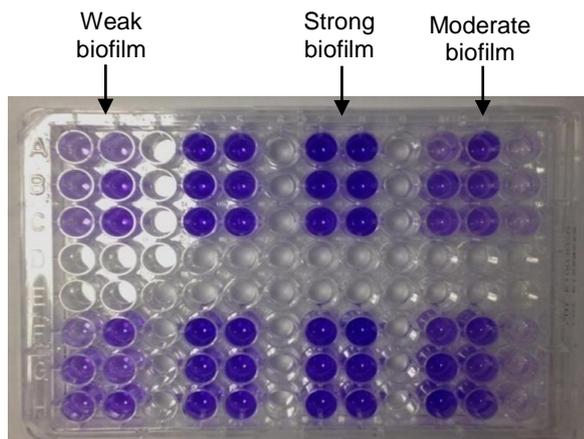**Figure 1:** Type of samples A, prosthetic implant; B, antibiotic beads; C, tissue; D, swab; and E, bone

Figure 2: Tissue Culture Plate method for biofilm detection showing strength of biofilm production indicated by colour intensity with crystal violet. Strong purple indicates high biofilm production and weak purple indicates low biofilm production.

RESULTS

Since September 2013 until July 2014, total number of samples taken was 57 from 17 cases (Patient ID OS001-OS017) of orthopaedic surgery with suspected osteomyelitis at Hopital Tengku Ampuan Afzan, Kuantan. Total sample that found positive growth was 34 which were 86.7% prosthetic samples, 66.7% Bone samples, 46.2% Swab samples and 43.8% Tissue samples (Table 1). These samples with bacterial growth were then identified as coming from only 14 patients, two of which were also polymicrobial in character.

It was also found that the most common pathogen identified was *S. aureus* (n=10), followed by *S. epidermidis* (n=3) and *P. aeruginosa* (n=3) (Table 2). From total ten, nine *S. aureus* isolated were biofilm producing strains with five being strong producers, two moderate and two weak producers. For *S. epidermidis*, two were biofilm producer with one being a strong and one being a moderate biofilm producer. All three *P. aeruginosa* isolated were moderate biofilm producer (Table 2).

Table 1: Number of samples with bacterial growth.

Sample	Collected	Bacterial growth	Mono growth	Poly growth
Bone	12	8 (66.7%)	6	2
Prosthesis	15	13 (86.7%)	9	4
Tissue	16	7 (43.8%)	5	2
Swab	13	6 (46.2%)	4	2
TOTAL	57	34 (59.6%)	24	10

Table 2: Species of bacteria isolated from each patients.

Patient ID	Species isolated	Biofilm formation
OS001	*S. aureus*	+++
	S. epidermidis	+++
OS002	*S. epidermidis*	-
OS003	*S. aureus*	+++
	S. aureus (Small Colony Variant)	-
OS004	*P. aeruginosa*	++
OS005	*S. aureus*	+
OS006	No growth	-
OS007	*S.epidermidis*	++
OS008	No growth	-
OS009	*P. aeruginosa*	++
OS010	*S. aureus*	+
OS011	*P. aeruginosa*	++
OS012	No growth	-
OS013	*S. aureus*	+++
OS014	*S. aureus*	+++
OS015	*S. aureus*	+++
OS016	*S. aureus*	++
OS017	*S. aureus*	++

+++, High biofilm; ++, Moderate biofilm; +, Low biofilm; - , None biofilm producer

DISCUSSION

The purpose of this study is to describe the profile of main bacteria which cause osteomyelitis in Kuantan, Pahang and look into the frequency of the biofilm microorganism. The differences arising from variation in samples, isolated using sonication method, are also characterized. These are done in the background of some limitations namely the short duration of the study and the limited number of source of samples (i.e. from only one hospital). Besides, the study does not look into age, racial, gender and financial/social status differences among the patients. The results are thus non-representative for Pahang or Malaysia but would still be useful for expanding the view of current issues involving osteomyelitis and prosthetic joint infection (PJI).

The role of *S. epidermidis*

The highest frequency of isolated bacteria is *S.aureus.* This is not surprising as this microorganism is already established as the leading pathogen in osteomyelitic infection (Lew and Waldvogel 2004). Other species found were *P. aeruginosa* and *S. epidermidis.* Most osteomyelitis are related to secondary infection and nosocomial infection; both *S. aureus* and *P. aeruginosa* are already known worldwide to be common in these types of infections (Hughes *et al.*, 2005). However, *S. epidermidis* (coagulase negative staphylococci) is not well-established as an osteomyelitis-causing pathogen. This may be the major reason why it is normally not discriminated in microbiology laboratory as coagulase-negative are considered as microorganism with little virulence and usually as contaminant (Aragón-Sánchez *et al.*, 2010)

In the study, 3 out of 16 identified species (19%) were *S.epidermidis* infection; 2 out of these 3 are biofilm producer and both are gentamicin-resistant. Bacteria other than *S. aureus* and *P. aeruginosa* have been suspected of having a more central role in inflammatory conditions and osteomyelitis. Here, we also call for an increase in the attention given to *S. epidermidis* and a longer, and more sampling to affect changes at management and clinical lab level – such as change in the practice where polymixed growth should be further discriminated.

Staphylococcus epidermidis has been described as a significant emerging pathogen in osteomyelitic infection especially in implant-related infection. O'Gara and

Humphreys (2001), Ziebuhr *et al*. (2006), and Valour *et al*. (2013), described *S. epidermidis* infection as an infection which is not as invasive as *S. aureus.* In contrast to *S. aureus* and *P. aeruginosa* that can be virulence in both planktonic or biofilm forms, all infections associated with *S. epidermidis* are only ascribed to when the pathogen achieve the capability to form biofilm. According to Aragón-Sánchez *et al*. (2010) the outcomes of surgical treatments are similar in both *S. epidermidis* and *S. aureus* infection. This observation however may not be similar if the treatment is based on antibiotic administration only, especially if the infected pathogens have developed resistant against antimicrobial agents. It is thus imperative to develop a method where *S. epidermidis* infection and its clinical repercussion could be analysed to provide an accurate diagnosis and deliver better treatment plan especially in terms of antibiotic and debridement surgery.

Biofilm-producing bacteria

More than 3/4 of the bacteria isolated were biofilm producers. Biofilm are the attachment of microorganism communities and formation of exopolysaccharide matrix on the solid surface. Most treatments for osteomyelitis have failed or become ineffective due to the development of biofilm at the site of infection (Brady *et al.*, 2006). There are several biofilm-related mechanisms that lead to antibiotic resistance such as the role of the biofilm matrix forming a barrier for antibiotic penetration , slow growth of the biofilm due to nutrient limitation, activation of the general stress response and activation of specific biofilm phenotype (Mah and O'Toole, 2001). Just as it is important to detect the specific type of bacteria involved, it is also imperative to discriminate the difference in strains' ability to produce biofilm as this may be reflected in the difference in clinical outcome and management.

In this regard, a change in current hospital lab policy may be needed. The results of the study contrasts with the current prevailing assumption that non prosthetic sample only possess monogrowth compared to polygrowth on prosthetic samples. This assumption may have been based on previous findings by the experts of the time such as the report by Gristina *et al*. (1985). Here, we found that 6 out of 10 (60%) polygrowths came from non-prosthetic samples (tissue, bone and swab). The type of bacterial species isolated from prosthetic samples is always the same species found in non-prosthetic samples. Also, the absence of culture in prosthetic samples will also produce no positive in non-prosthetic samples.

Importance of prosthetic sample

Malaysian medical microbiology laboratories (bacteriology) currently do not accept prosthetic implant as a sample. This may lead to cases where a negative culture from non-prosthetic sample will lead to false negative situation, in which an existing pathogen may not be recognized as a causing agent. The implication of this is that a generalized treatment may be given, when a

more specific treatment tailored to individual cases could have been offered. The prosthetic samples may be the only place where infective agent, in general, and biofilm-producing agent in specific, may be found.

In this study, all prosthetic samples have been shown to have positive bacteria growth. This indicates the importance to include prosthetic material as a sample for laboratory in the diagnosis of osteomyelitis especially when implant related infections occur and when prosthetic sample is available. Previous studies by Trampuz *et al*. (2007), Esteban *et al*. (2008) and Piper *et al*. (2009) have shownthat prosthetic materials increased sensitivity in isolation of biofilm-producing bacteria.

The biofilm-producing bacteria have been known to be capable to transform from the planktonic to the complex biofilm form, and of recruiting non-adherence bacteria to the biofilm matrix. The mechanism involve may be through gene transfer or quorum sensing mechanism (Sakuragi and Kolter, 2007). As biofilm can only occur on solid surface material, no bacteria can be obtained from tissue sample if entire planktonic bacteria cease to occur. Thus, in theory, if tissue sample is the only sample processed and the prosthetic sample is discarded or ignored, this could cause false negative results and lead to less specific treatment.

The role of sonication method

Isolation of biofilm bacteria especially from prosthetic samples was deemed difficult. A good method with optimization is therefore crucial. We have done sample processing with and without sonication (Figures 3 and 4) and find that without sonication method, the plate showed less or no bacteria colony growth. However, after sonication method, there were significant numbers of colonies growth. Therefore we concluded that the use of sonication method as a processing method for bone and prosthetic sample improved bacterial isolation. The sonication method employed ultrasound at a frequency between 20-40 kHz that is able to remove adherent microorganisms from prosthetic and bone samples (Trampuz *et al*., 2007; Piper *et al*., 2009; Larsen *et al*., 2012). Other non-culture diagnostic methods such as immunofluorescene microscope (IFM), and polymerase chain reaction (PCR), exist and has been shown to have high sensitivity, but determination of antimicrobial sensitivity is not possible due to the non-culture technique in use (Achermann *et al*., 2010). As sensitivity testing is important for proper treatment plans with antibiotic, these methods are not the method of choice for diagnosis or for practical consideration.

CONCLUSION

From this short study, we have found that prosthetic samples and bone samples were more sensitive than other type of sample and also biofilm producing bacteria were the most common causes in osteomyelitic infection. Successful treatment of osteomyelitis depends on a proper identification of the species and strain of pathogen
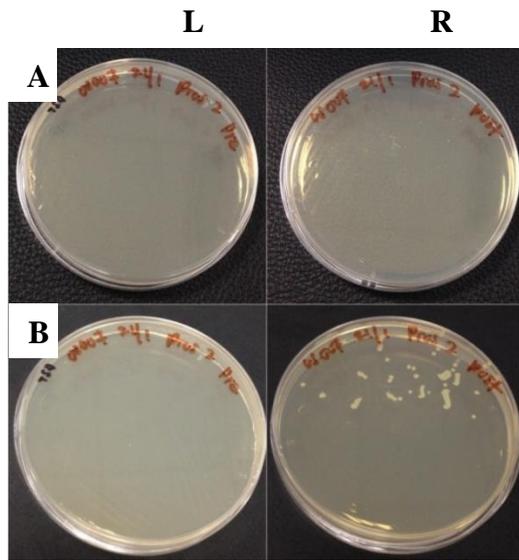
Figure 3: Bacterial culture showing colony isolated from prosthetic sample with (A) denotes culture at 24 h incubation and (B) denotes culture at 48 h incubation. The left-hand sides (L) were cultured in the absence of sonication and showed no bacterial growth. The right-hand side (R) were cultured after sonication and showed colony growth.

Figure 4: Colony isolated from antibiotic beads at 24 h incubation period. The left-hand side (L) was grown in the absence of sonication and showed few colony growths (small, white colonies). The right-hand side (R) was grown after sonication and showed more bacterial growth.

that cause the infection. To get the accurate diagnosis and the best treatment towards patient care, proper sampling with a good diagnostic method such as sonication methods and identification needs to be considered for inclusion in current hospital lab practice.

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