Antibiotic Susceptibility Patterns of Bacterial Strains Isolated from Skin Samples of healthy Individuals

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ABSTRACT The aims of this study were to isolate and identify bacterial strains from facial samples collect from individuals in Nilai, Negeri Sembilan, and to determine the antibiotic susceptibility profiles of these bacteria. The samples were incubated in nutrient broth which were further cultured on nutrient agar. Single colonies were picked and were subjected to biochemical tests such as Gram staining, catalase test and, Mannitol Salt Agar, to confirm the identity of the isolates. Disk-diffusion assay using a range of antibiotics including cefoxitin for the detection of MRSA was carried out. There was a higher percentage of *Staphylococcus aureus* (51%) while *Staphylococcus epidermidis* (15%), *Enterococcus* species (12%), *Propionibacterium acnes* (10%) and *Streptococcus pneumoniae* (2%) were also isolated. A single *S. pneumoniae* was identified as PRSP since it was resistant to penicillin. 18% and 6%, of the isolates were MRSA and MRSE respectively. All the MRSA strains were resistant to cefoxitin, ampicillin and penicillin G while all of the MRSE strains were resistant to cefoxitin and ampicillin. There were five different antibiograms for the MRSA isolates and only two antibiograms for MRSE strains. 40% of the *P. acnes* were resistant to erythromycin. From the study, 5% of the isolates were not sensitive to the antibiotics while 95% were resistant to at least one antibiotic. The results obtained from this study allows the determination if the antibiotic resistant bacteria are increasing in Malaysia and it also enables the determination of which antibiotic can still be used to treat infections caused by the bacteria.

KEYWORDS: Antibiotic susceptibility pattern, Skin samples, Healthy individuals, MRSA, Cefoxitin

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INTRODUCTION

Antibiotic resistance among bacterial strains is becoming more prevalent and alarming in today's world and this has resulted in an increase in the mortality rate among patients in hospitals (Sarkar *et al.*, 2017). This is mainly due to the fact that bacteria that previously were easily killed with antibiotics are now becoming more difficult to treat (Mazel & Davies, 1997). Some bacteria develop resistance to multiple drugs from acquisition of genes harboured in plasmids or integrons (Jones *et* al., 1997; James & Wong, 2015). Resistance of bacteria to drugs is due to gene mutations, acquisition of genes and by passing genetic material through horizontal gene transfer. The resistant genes can be transmitted to the population of bacteria using different mechanisms such as conjugation, transduction and transformation (Oliveira *et al.*, 2017).

Some people who are physically well, harbour resistant strains of bacteria making them carriers of these pathogens (Perron *et al.*, 2008) which is a danger since the bacteria can be transferred to other people thereby increasing the spread of bacterial resistance. Furthermore, the ability of certain pathogens to develop biofilms in indwelling devices such as catheters, is made more difficult to treat due to antibiotic resistance (Nillian, Rukayadi and Radu, 2016). This might result in the ineffectiveness of many drugs to cure the infections caused by these pathogens and will result in different types of diseases (Jaiswal *et al.*, 2016). Thus, antibiotic susceptibility patterns should be known to be able to know which antibiotic are still effective against the pathogens which will prevent the spread of such pathogens which cause diseases and might result in death. The aim of this study was to determine different species of bacteria that could be isolated from the facial skin of

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healthy individuals, and to determine the antibiotic susceptibility profiles of the identified bacterial isolates. The overall aim was to identify if the individuals were carriers of pathogens that are resistant to antibiotics and what antibiotics are still effective in the treatment of these skin pathogens.

METHODOLOGY

Sample Collection

A survey form and a consent form were given to each individual participating in this study. Samples were obtained from skin by the swabbing technique using sterile cotton swabs on each of the participants. These swabs were inoculated into 2 mL of nutrient broth, and incubated at 37℃ for 24 hours, with aeration.

Bacterial isolation and confirmation

A loopful of the mixed culture was used to perform dilution streaking onto nutrient agar which was incubated overnight at 37^{o}C. A single colony with different morphologies was picked and a dilution streaking was performed on a nutrient agar to obtain pure and homogenous colonies. Confirmatory tests were then performed to confirm the identity of the pure cultures. The confirmation tests were carried out on pure, overnight cultures to confirm the identity of the bacteria isolated. Gram staining was performed using a smear of culture on a slide that was heat fixed and stained with Crystal violet, washed with iodine, and counter-stained with Gram's safranine (Claus, 1992). The catalase test was performed by reacting a loopful of pure culture with 3% H_2O_2 and observing the bubble formation (Reiner, 2010). Gram positive cocci isolates were plated out on mannitol salt agar and incubated overnight at 37^{0}C. The indole test was carried out on Gram negative isolates by adding Kovac's reagent (5 drops) into an overnight bacterial culture grown in tryptophan medium (MacWilliams, 2009a). Gram positive cocci-shaped isolates that were catalase negative and did not grow on MSA agar, were cultured onto blood agar to determine hemolysis patterns, if any (Buxton, 2005). MacConkey agar was used to culture the Gram negative isolates by streaking them on the agar and incubating the agar overnight at 37^{0}C (Mossel et al., 1962). Methyl red (MR) and Voges-Proskauer (VP) tests were carried out on Gram negative isolates where the pure cultures were grown in MRVP broth overnight (McDevitt, 2009). For Methyl red test, five drops of Methyl red reagent was added to the overnight culture while for the Voges Proskauer test, Barrits reagents A (0.6 ml) and B (0.2 ml) were added to the overnight culture. The cultures were then incubated at room temperature for 30 minutes for color development to take place. Pure cultures of Gram negative isolates were inoculated into Citrate agar and incubated at 35^{0}C for 18 to 48 hours, before recording any change in the agar color (MacWilliams, 2009b). Triple sugar iron (TSI) test was carried out on all Gram negative isolates. Briefly, pure cultures were inoculated into TSI agar slants by streaking and stabbing the agar, before incubation at 37^{0}C overnight. The TSI slants were then observed for sugar fermentation, gas production and hydrogen sulphide production (Lehman, 2005). Oxidase test was carried out by soaking a filter paper in Kovac's reagent and smearing a pure, overnight bacterial culture on the paper (Shields and Cathcart, 2010). All bacterial isolation and confirmatory tests were performed in a biosafety cabinet.

Antibiotic Susceptibility Assay

A suspension of each bacteria tested was compared to the McFarland standard, 0.5. Approximately 50 µL of the inoculum was used to obtain 5 x10^{5} CFU/mL was spread onto Mueller-Hinton agar plates by lawning technique using sterile cotton swabs (Citron *et al*., 2005). The plate was then allowed to dry and after 15 min the appropriate antibiotic discs were placed on each

quadrant of the plate using a sterile forceps. The diameter (mm) of the inhibition zone was measured after incubation at 37℃, overnight and compared with the Clinical and Laboratory Standards Institute (CLSI) values. The disc diffusion assay was done in triplicates to obtain more accurate results. The common antibiotics used in the treatment of these bacterial strains were used in the disc diffusion assay including cefoxitin (30 μg), penicillin (10 μg), ampicillin (10 μg), rifampicin (5 μg), gentamicin (10 μg), oflaxicin (5 μg), tetracyclin (30 μg), clindamycin (2 μg), oxaxillin (1 μg), erythromycin (15 μg), amoxillin (30 μg), amikacin (30 μg), and doxycyclin (30 μg).

Confirmation of MRSA using Brilliance MRSA 2 agar

Pure, overnight cultures of cefoxitin-resistant *S. aureus* were inoculated onto Brilliance MRSA 2 agar (Thermo Fisher Scientific), and the plates were incubated overnight at 37^0C to confirm the identification of MRSA isolates.

Statistical Analysis

The results of the triplicates were analyzed using a one-way analysis of variance (one-way ANOVA) followed by Post-Hoc test. The significance should be at $P \leq 0.05$. The one-way ANOVA for multiple antibiotic resistance of each bacteria found on the skin for all the individuals was analyzed (Pathak *et al*., 1993).

RESULT AND DISCUSSION

Collection of samples

A total of 30 samples were taken from 30 individuals in Nilai who filled the survey and consent forms. There were more female (57%) than male (43%) participants. Out of the 30 individuals, 29 were under antibiotic treatment and 26 individuals visited hospitals and clinics recently. Furthermore, eight individuals were under acne treatment.

Isolation and identification of bacteria

From the 30 samples collected, 49 isolates were obtained. The total number of gram positive bacteria obtained were 48 out of the 49 isolates (98%). One gram negative cocci-shaped bacteria were obtained. From figure 1, more than half of the bacteria isolated were *Staphylococcus aureus* (51%) (Kontiainen & Rinne, 1987). The rest of the bacteria isolated were deduced to be *Staphylococcus epidermidis* (15%), *Propionibacterium acnes* (10%), *Enterococcus* sp. (12%) and *Streptococcus pneumoniae* (2%).

The percentage of both indoles negative and positive was 50% for *P. acnes* showing that there are different variants of *P. acnes* which showed different activity when tested. Therefore, it can be deduced that two variants of *P. acnes* were successfully isolated in this study (Mcginley *et al*., 1978).

The gram negative isolate could be cocci bacilli which could not be conclusively identified. In fact, in a previous study pink colonies on MacConkey agar that were cocci gram negative could not be identified but it was suggested that the isolates were opportunistic pathogens. It could also be because the individual from which the sample was taken was under clinical treatment for diseases such as diabetes or leukemia (Wallace *et al*., 1990). It could be from the *Klebsiella* species as all the biochemical tests were nearly identical except that gas should have been produced when it was inoculated in TSI (Acharya, 2013) and MR should have been negative while VP should have been positive (Aryal, 2015). Previous studies have reported that some bacteria were not identifiable since they could have been subjected to different conditions due to stress such as nutrients which makes

them adapt to the environment (Poole, 2012). There is also a possibility where mutation occurred which gave rise to difference in phenotype (Coli *et al*., 1955)

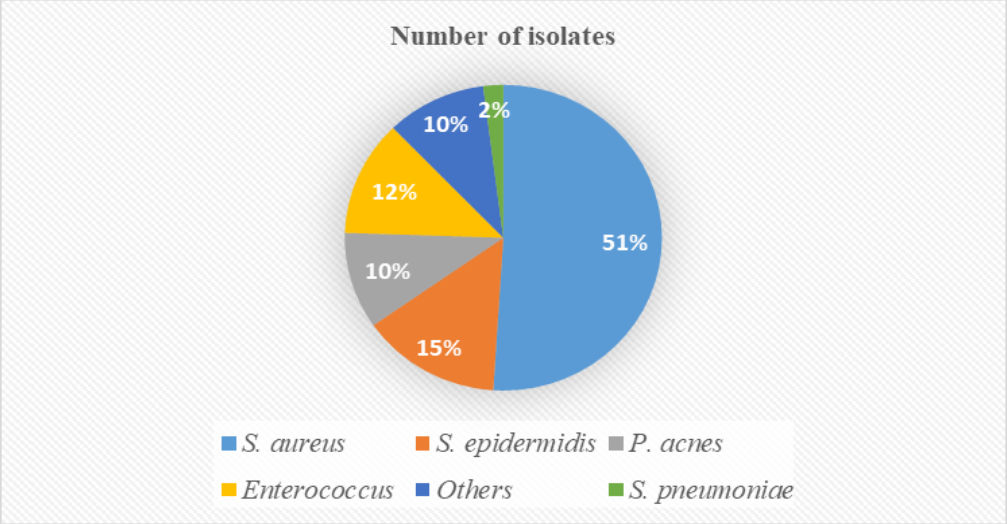

Figure 1. Percentage of known bacteria isolated from the samples collected

Disc diffusion assay

The *mecA* gene is responsible for the expression of a penicillin-binding protein (PBP) known as PBP2a which accounts for the resistance of the bacteria against β-lactam (Bonjean *et al*., 2016). Out of the 25 isolates of *S. aureus*, 36% were identified as Methicillin-resistant *Staphylococcus aureus* (MRSA). These isolates were resistant to ampicillin while three (12%) were resistant to tetracycline. All *S. epidermidis* were sensitive to tetracycline and 43% were identified as Methicillin-resistant *Staphylococcus epidermidis* (MRSE) which were resistant to cefoxitin and ampicillin. Penicillin has been shown to be completely ineffective against all the isolates tested in this study. There were three individuals that were not associated with medical institutions and were not under antibiotic treatment but were carriers of resistant strains including MRSA and PRSP. In a study in Iran, it was seen that antibiotics such as gentamicin, tetracycline and erythromycin could still be used to treat MRSA. This was concurrent with the findings in this study since the antibiotics that were observed to be still effective against the MRSA strains were oflaxicin and gentamicin (Japoni *et al*., 2010). Only three out of the seven *S. epidermidis* isolates were MRSE which were resistant to cefoxitin, ampicillin and oxacillin. All three MRSE isolates had different antibiogram patterns. However, these isolates could still be treated with a range of antibiotics from tetracycline, gentamicin to clindamycin which are still commonly used to treat infections cause by MRSE.

40% of the *P. acnes* isolated were resistant to erythromycin only. In a study carried out in Egypt, 80% of *P. acnes* were resistant to erythromycin and this is not concurrent with the findings in this study which showed only 40% of the *P. acnes* isolated were resistant to erythromycin. Furthermore, all the *P. acnes* isolated were sensitive to doxycycline and amikacin (Hassan *et al*., 2015). These conflicting results could be due to the small number of *P. acnes* isolated. Further studies need to be carried out to validate these findings.

In this study, it was found that 23% of the individuals harbored resistant strains showing that there is no correlation between the survey and bacterial strains. From the bacteria isolated, 5% were sensitive to all antibiotics tested, while 95% were resistant to at least one antibiotic from which 53%, 13%, 3 %, 8% and 3% were resistant to one, two, three four and five antibiotics respectively (Figure 2) which showed that the individuals tested are not relatively healthy with presumably bad

practices in relation to antibiotic therapy. It may also be due to improper practice of hygiene such as improper hand washing and improper use of soap and sanitizer which would otherwise kill the bacteria present on the hands (Bird *et al.*, 2010). The individuals tested might not be aware about the misuse of antibiotics and used the antibiotics prescribed by doctors in the incorrect dosage and duration (Rather *et al.*, 2017). The individuals concerned could have been ignorant about the dangers of using antibiotics inappropriately (WHO, 2017).

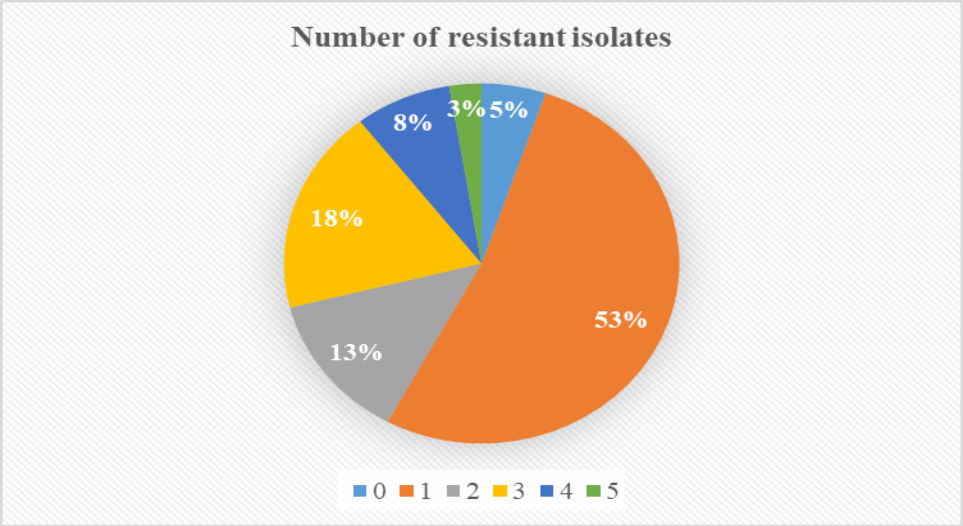

Figure 2. Percentage of isolates resistant to zero, one, two, three and four antibiotics

Table 1 shows the antibiogram of all the MRSA and MRSE isolated from the samples. 56% of the MRSA strains were resistant to two antibiotics while 11% were resistant to three and four antibiotics, each having different antibiotic profiles. 67% MRSE strains were resistant to 3 antibiotics (CFX^R, OX^R, AMP^R) while 33% were resistant to cefoxitin only. All the MRSA isolates were resistant to cefoxitin, ampicillin and penicillin. There was no significance between the antibiotics used and the bacterial isolates showing that different bacteria reacted differently with different antibiotics.

Table 1. Summary of antibiograms in MRSA and MRSE

Isolate	Number of isolates	Antibiogram
MRSA	5	CFX^R, P^R, AMP^R
	1	CFX^R, P^R, RD^R, AMP^R
	1	CFX^R, TET^R, P^R, AMP^R
	1	CFX^R, P^R, DA^R, AMP^R
	1	CFX^R, P^R, RD^R, DA^R, AMP^R
MRSE	2	CFX^R, OX^R, AMP^R
	1	CFX^R, AMP^R

Brilliance MRSA 2 agar

The *S. aureus* strains that were resistant to cefoxitin were streaked on brilliance MRSA 2 agar where growth of blue colonies indicated that they were MRSA strains. Brilliance 2 MRSA agar inhibits all other microbial growth such as MSSA are inhibited except for MRSA strains due to presence of antibacterial agents in the media (Verkade *et al.*, 2009).

CONCLUSION

From this study, 90% of the bacterial strains from healthy individuals isolated could be identified and their antibiotic susceptibility patterns were determined. 95% of the bacteria isolated from healthy individuals in Nilai were resistant to at least one antibiotic. The antibiotic susceptibility pattern of *S. pneumoniae* (PRSP) has to be verified using a larger pool of PRSP to determine if amoxillin and erythromycin can still be used to treat these bacterial infections. Out of the 25 *S. aureus* isolated, 36% were resistant to cefoxitin and ampicillin while 12% were resistant to tetracycline. All the *S. epidermidis* were sensitive to tetracycline and 43% were identified as MRSE since they were resistant to cefoxitin. The MRSA and MRSE strains that were isolated had different antibiogram showing that each bacterium reacted differently to the antibiotics tested by having different diameter of zone of inhibition. MRSA were sensitive to oflaxicin and MRSE were sensitive to tetracycline and clindamycin.

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