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Isolation and Identification of Gram-Positive Bacteria from the Epidermis of Students of Nnamdi Azikiwe University, Awka.

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ABSTRACT

Serving as the defense mechanism of the human body with the environment, the human skin acts as a contact barrier to prevent the interference of foreign invaders, while providing a accommodation to the microflora. In circumstances where the defense is altered, the skin disease or even systemic disease can result. The composition of microbial communities on the human skin, is primarily dependent on the physiology of the skin position. Sebaceous sites are dominated by lipophilic *Propionibacterium* species, whereas bacteria that thrive in humid environments, such as *Staphylococcus* and *Corynebacterium* species, are abundant in moist areas of the skin. Colonization by microorganisms, on the skin surface is also driven by the ecology of the skin surface, which is highly variable depending on topographical location, endogenous host factors and exogenous environmental factors. The skin is composed of two distinct layers; the epidermis and dermis. Most microorganisms dwell in the topmost part of the skin (epidermis), and others reside in the innermost areas of the hair follicles (dermis). In this study, however, culture-based techniques were used to analyze the gram-positive microbes, colonizing the human epidermis. Ten different body sites of ten different students in Nnamdi Azikiwe University were sampled. All ten specimens, gave positive results to the gram stain test. From the results derived, after several biochemical tests were carried out, suspected organisms include: *Propionibacterium acne*, *Corynebacterium* species, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *other Staphylococcus* species, *Micrococcus* species, *Streptococcus* species and *Clostridium* species. All suspected organisms are a major part of the human skin microflora, although organisms such as *Staphylococcus aureus* could exhibit pathogenicity in some cases.

Corynebacterium specie and *Staphylococcus aureus* were shown to have very high susceptibility to all tested antibiotic, followed by *Propionibacterium acne* and *Staphylococcus epidermidis*. *Clostridium* specie had the highest resistance to Amoxil, streptomycin and nofloxacin. Furthermore, this study is added proof that gram-positive bacterial species are more abundant on the human skin than the gram-negative, as all ten different sites, sampled from ten different students of Nnamdi Azikiwe University, using culture-based analysis, yielded only gram-positive bacterial species.

INTRODUCTION

The skin is home to billions of active microbes that compose the skin microflora (Timm *et al.*, 2020). The skin microflora are microorganisms that are resident on our skin. Such microorganisms are the skin microbiota or the skin microbiome (Grice and Segre, 2011). After the gut, there are more microorganisms on the skin than anywhere else in the body (Chen and Tsao, 2013). Bacterial species are by far the most numerous, however fungi, viruses and mites are also found on the skin of normal healthy humans (Alexeyev, 2013).

Resident microbiotas are found in the upper parts of the epidermis and congregated in and around the hair follicles (Findley *et al.*, 2013). They include bacteria such as *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Brevibacterium* and *Dermabacter* (Grice and Segre, 2011). Some microbiotas are considered transient as they can be only isolated and cultured from skin samples from time to time (Alexeyev, 2013). These are mainly Gram-positive bacteria including *Clostridia* in the perineal area (Alexeyev, 2013). Occasionally, moist areas of the skin allow the growth of Gram-negative *Acetinobacter (*Jo *et al.*, 2016.) Other gram-negative bacteria such as *Pseudomonas* or even *Escherichia colil* are not considered part of the normal skin microbiota, as the low humidity and high osmotic pressure of the skin are unfavorable for their growth (Alexeyev, 2013).

Human skin sites can be categorized by their physiological characteristics, i.e, whether they are sebaceous (oily), moist or dry (SanMiguel and Grice, 2015). These regions of the human body can be thought of as different ecosystems (SanMiguel and Grice, 2015).

Moist body sites which is made up of the skin folds, such as elbow creases, beneath the breast in between the toes and the ground are predominated by *Corynebacterium*, which is also Gram-positive (Timm *et al.*, 2020). Sebaceous (oily) skin sites includes the forehead, neck and trunk, where the sebaceous glands secrete an oily substance, sebum, allowing Gram-positive *Cutibacteria* to thrive (Szabo *et al.*, 2016). The quantitative differences found at these sites may relate to the amount of moisture, body temperature and varying concentrations of skin surface lipids (Oh *et al.*, 2014).

Most microorganisms live in the superficial layers of the skin (the statin cornerman) and in the upper parts of the hair follicles (Grice and Segre, 2011). Some bacteria however, reside in the deeper areas of the hair follicles where they may be beyond the reach of ordinary disinfection procedures such as

washing with soap and water or an antibacterial product (Findley *et al.*, 2013). These out of reach bacteria serves as reservoirs for recolonization of the skin environment after the surface bacteria are removed (Grice and Segre, 2011). This study aimed at isolation and identification of gram-positive bacteria, from the epidermis of students, of Nnamdi Azikiwe University, Awka.

MATERIALS AND METHOD

Study area and collection of specimens

A total of 10 sterile swab sticks were moistened in sterile saline solution and were subsequently used to collect specimen from 10 different sites of the skin surface (volar forearm, palm, chest, toe web, navel, auxiliary vault, antecubital fossa, forehead, back and interdigital web space), of students of Applied Microbiology and Brewing, of Nnamdi Azikiwe University, Awka, by rubbing the swab sticks vigorously against the sites. The swab sticks were labeled accordingly and taken to Nnamdi Azikiwe University's microbiology laboratory, for studies/culturing

Sterilization of glasswares

All glasswares were washed with detergent and water and then wrapped up in an aluminum foil and put in an auto clave for sterilization at 121°C for 20 minutes.

Preparation of culture media

The bench was cleaned thoroughly with disinfectant before every procedure, to ensure it was contaminant free, after which all media were prepared by following manufacturer's instructions.

Nutrient agar preparation

An electronic laboratory scale was used to weigh out 5.6 g nutrient agar powder and poured in a conical flask. Nystatin was added to serve as an antifungal agent, after which 200 ml distilled water was measured using a measuring cylinder and poured into the conical flask. The conical flask was then closed up with cotton wool and sealed with aluminum foil. The solution was mixed by shaking the conical flask before it was taken to an auto clave for sterilization at 121°C for 20 minutes. After that, the solution was brought out and allowed for some time to cool before been poured into appropriately labeled petri dishes, bijou bottles and test tubes close to a lit bunsen burner and allowed to gel.

Simmon citrate agar preparation

Simmon citrate agar powder (4.9 g) was weighed out and dissolved in 200 ml of water. The solution was autoclaved at 121°C for 20 minutes and allowed to cool before it was poured into appropriately labeled Petri dishes (close to a lit bunsen burner), and allowed for 20 minutes to gel.

Blood agar preparation

Nutrient agar was prepared following manufacturers instruction and 10 ml of human blood was added. Then it was poured into appropriate petri dishes close to a lit bunsen burner and labeled appropriately.

Mueller Hinton agar preparation

Mueller hinton agar (7.6 g) was measured and dissolved in 20 ml sterile water in a conical flask. The solution was mixed by shaking, closed up with cotton wool and sealed with aluminum foil. After that, it was sterilized at 121°C for 20 minutes in an auto clave, allowed to cool and poured into appropriately labeled Petri dishes under sterile condition.

Sterility check

All media prepared and poured into respective petri dishes as well as test tubes and Bijou bottles, were allowed for 24 hours prior to inoculation time, to ensure they were free of contaminants before use.

Isolation of bacteria

The specimen that were obtained previously with sterile swabs sticks, were inoculated onto the sterile petri dishes containing solidified nutrient agar, close to a lit Bunsen burner. The plates were incubated at room temperature for 24 hours. After that, developed colonies were observed, counted and recorded before being subcultured onto new nutrient agar plates, using sterile inoculation loops, to obtain pure cultures. Isolates from selected colonies were taken and subcultured on new nutrient agar medium (using streak technique). The inoculation loop was first sterilized by putting the loop into the flame from a bunsen burner, until it was red hot. It was allowed to cool and dipped into the inoculum to obtain small amount. The culture containing loop is then streaked onto the surface of a solidified nutrient agar medium, to make series of parallel, non-overlapping streaks. After that, the plates were closed and the inoculation loop was flamed and put away. The isolates were incubated for 24 hours, after which gram staining and other biochemical analysis was carried out. The isolates were also smeared on nutrient agar bijou bottle slants, labeled appropriately and incubated at room temperature.

Gram staining

A total of 10 glass slides were labeled using the letters of the alphabet, A to J respectively. Under sterile condition, a drop of sterile water was added on each glass slide using a pipette. A smear was made by emulsifying 24-hour old bacterial culture on each slide containing water. The air-dried smear was

heat fixed by passing each slide through the flame of a bunsen burner three times. After that each smear was flooded with crystal violet and allowed for 60 seconds before it was rinsed off with sterile water. Next, each smear was flooded with Lugol's iodine, allowed for 60 seconds and rinsed off with water. The slides were then rinsed with 95% ethanol followed by sterile water. Again, each smear was counter-stained with safranin for 30 seconds, rinsed with sterile water and allowed to air dry. Slides were viewed under a microscope, by the addition of immersion oil on each slide, using ×100 objectives lens and results were recorded accordingly.

Biochemical tests

Motility test

Under sterile condition, an inoculation needle was used to pick a loop from 24-hour old Culture and then stabbed once, deep into nutrient agar slants in test tubes. Each test tube was covered with cotton wool, sealed with aluminum foil, labeled appropriately and incubated for 24 hours. Test tubes were then observed for diffused zone of growth flaring out from the line of inoculation and observations were recorded accordingly.

Catalase test

A drop of hydrogen peroxide solution was added on well labeled sterile glass slides. A sterile inoculation loop was used to introduce a loopful of 24-hour old inoculum on each slide. The slides were observed for the presence or absence of gas bubbles within five seconds. The production of gas bubbles, indicated a positive result whereas non-production indicated in negative results.

Hemolysis test

Sterile blood agar plates we are labeled appropriately. Under sterile condition, young cultures of 24 hours were inoculated on the plates by streak method. Then, plates were incubated at room temperature for 24 hours. The plates were observed for complete clear zones, partial clear zones and no clear zones at all, as an indication of beta-hemolysis, alpha-hemolysis and gamma-hemolysis respectively. All observations were accurately recorded.

Citrate test

Under sterile conditions, 24-hour old cultures were inoculated on well prepared citrate agar plates. Each plate was labeled appropriately, sealed with masking tape and incubated at room temperature. After 24 hours, observations were made for color change in plates, from green to blue, to indicate a positive or negative result. Observations were recorded accordingly.

Coagulase test

Human blood (5 ml) was collected using a syringe, transferred into an EDTA container and centrifuged to obtain Plasma. A drop of physiological saline was added on labeled sterile glass slides, after which a smear was made with 24-hour old cultures. A drop of human plasma was added on each of the slides, and observations of presence or absence of clumps after 10 seconds was noted, to indicate a positive or negative result. Observations were recorded accordingly.

Sugar fermentation test

Sucrose, fructose, glucose, galactose, lactose, maltose, mannitol, and dextrose (1 g each), were dissolved in 100 ml of water in separate conical flasks. Peptone water (1.5 g) was equally added to each conical flask containing sugars, followed by the addition of a few drops of bromothymol blue indicator, to achieve color homogeneity in each conical flask. Each solution (6 ml), was dispensed in well labeled test tubes after which Durham tubes were placed in inverted position inside each test tube. All tubes were closed with cotton wool and sealed with aluminum foil and then autoclaved at 121°C for 20 minutes to obtain sterility. On cooling, cultures were carefully inoculated in each test tube, and afterwards incubated at room temperature. After 24 hours, each test tube was examined for acid and gas production, which was indicated by color change from green to yellow as well as production of bubbles in the upper part of the inverted Durham tubes respectively. Results were recorded.

Maintenance of Test Organisms

The isolated test organisms were used for the antibacterial sensitivity testing. Prior to the test, the organisms were subcultured on nutrient agar plate at 37^0C for 24 hours. Then the 24-hour cultures were transferred into nutrient broth and incubated aerobically at 37^0C for 24 hours.

Standardization of Inoculum

The innocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4^0C and subcultured onto nutrient broth using a sterilized wire loop. The density of suspension innoculated onto the media for suceptibility test was determined by comparison with 0.5 McFarland standard of Barium sulphate solution.

Antibiotic sensitivity test

Under sterile condition, isolates of gram-positive bacteria, that has been standardized using McFarland standard of concentration (0.5 colony forming units per ml), was spread plated on all labeled mueller hinton agar plates accordingly, using a sterile swab glass spreader, and allowed for few minutes to dry. Antibiotic sensitivity discs containing ciproflox (10 mcg), norfloxacin (10 mcg), gentamycin (10 mcg), Amoxil (20 mcg), streptomycin (30 mcg), rifampicin (20 mcg), Erythromycin (30 mcg), chloramphenicol (30 mcg), ampiclox (20 mcg), levofloxacin (20 mcg), were placed on each agar plate and

gently pressed with a sterile wire loop to ensure it was firmly attached to the agar medium. Plates were incubated for 24 hours, at room temperature. After that, plates were observed for zones of inhibition around the discs; zones were measured using a transparent ruler and values were recorded.

RESULTS

A total of 10 specimen were inoculated on nutrient agar medium and their colony morphology were observed after 24 hour of incubation (Table 1).

Plate 1: showed the isolates of pure cultures, obtained after 24 hours, by streak plate method. Table 2: showed the Biochemical examinations, such as Gram stain, motility, catalase, hemolysis, citrate and coagulase tests respectively. The various biochemical tests aided the inferential identification of suspected organisms for each isolate, also listed in table 2. The results of sugar fermentation tests for the isolates, against 8 different sugars are shown in table 3, and table 4 showed the sensitivity testing of the isolates against 10 different antibiotics to determine their susceptibility or resistance to each antibiotic with their zones of inhibition measured in millimeters.

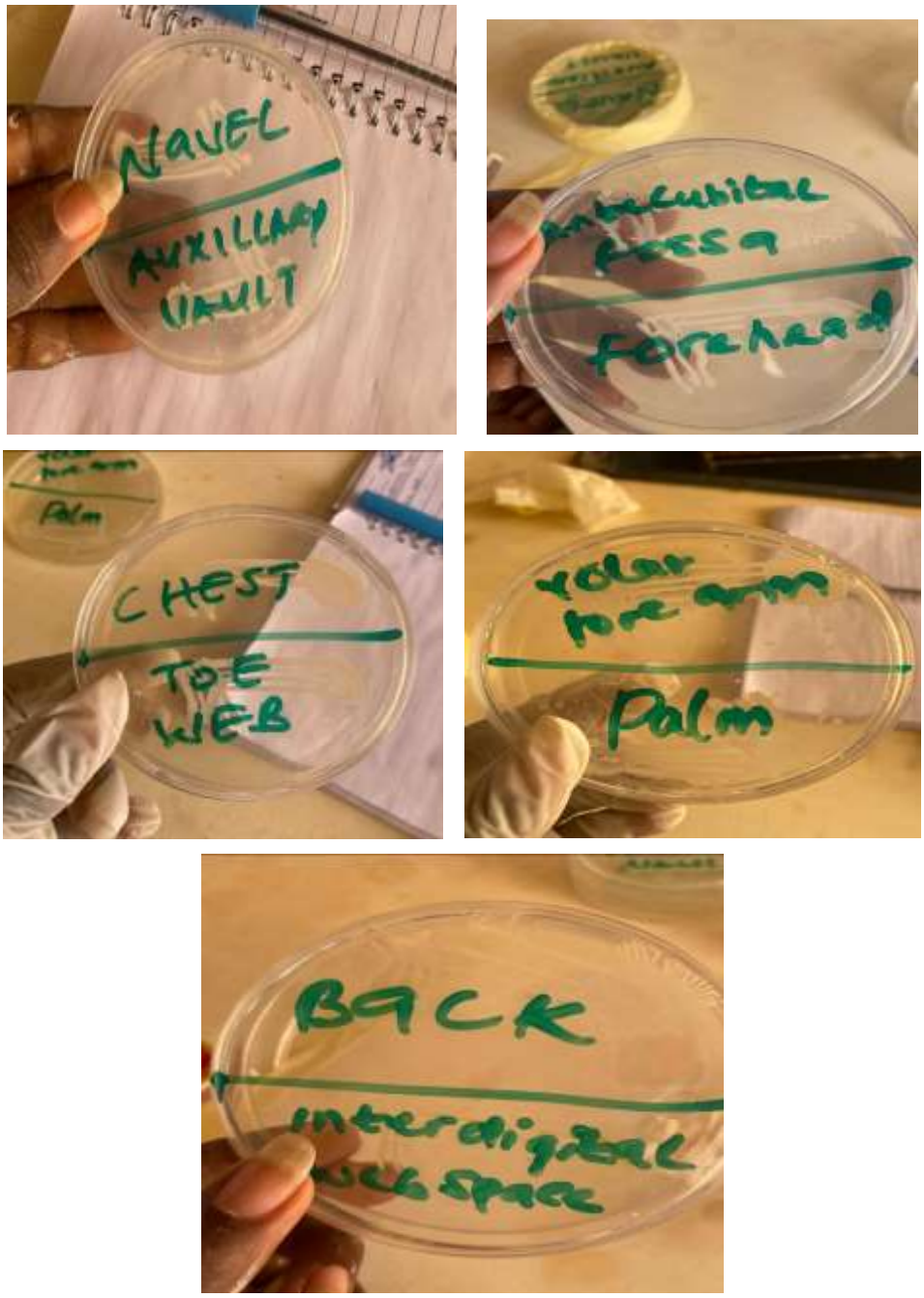

Plate 1: Isolates of pure culture by streak technique.

Table 1: The morphology of colonies on nutrient agar medium

Specimen	Colony Morphology on Nutrient Agar Medium
A	opaque, milky, orange, round, punctiform, irregular and filamentous colonies
B	opaque, round and irregular colonies
C	milky, round and punctiform colonies
D	opaque, milky, orange round and punctiform colonies
E	opaque and punctiform colonies
F	opaque, and punctiform colonies
G	opaque, milky and round colonies
H	opaque, milky, orange, round and punctiform colonies
I	opaque, round and irregular colonies
J	opaque, milky, orange, round, punctiform and irregular colonies

Key: A = volar forearm, B = palm, C = chest, D = Toeweb, E = navel, F = auxiliary vault, G = antecubital fossa, H = forehead, I = back, J = interdigital web space.

Table 2: Biochemical test results for isolates and suspected bacterial organisms.

Iso	GS	Shape	Mot	Cat	Hem	Cit	Coag	Suspected Organisms
A	+	rod	-	+	α	-	-	*Propionibacterium acne*
B	+	rod	-	+	β	-	-	*Corynebacterium specie*
C	+	cocci	-	+	γ	-	-	*Staphylococcus epidermidis*
D	+	rod	+	-	β	-	-	*Clostridium specie*
E	+	cocci	-	+	γ	-	-	*Staphylococcus epidermidis*
F	+	cocci	-	+	γ	-	-	*Micrococcus specie*
G	+	cocci	-	+	β	+	+	*Staphylococcus aureus*
H	+	cocci	-	+	γ	-	-	*Staphylococcus specie*
I	+	cocci	-	-	β	+	-	*Streptococcus specie*
J	+	cocci	-	-	β	+	-	*Streptococcus specie*

Keys: ISO = isolates, GS = Gram staining, Mot = motility, Cat = catalase, Hem = hemolysis, cit = citrate, Coag = coagulase + = positive, - = negative, α = alpha, β = beta, γ = gamma.

Table 3: Sugar Fermentation Test Results For Isolates

Isolates	Sucrose	Glucose	Mannitol	Lactose	Fructose	Dextrose	Maltose	Galactose
A	-	A	-	-	A	A	A	A
B	-	A/G	-	-	A	A	A	A
C	A	A	A/G	A/G	A	A	A	A
D	-	A	A	A	A	A	A	A
E	A	A	A/G	A/G	A	A	A	A
F	-	-	A	A	A	A	-	A/G
G	A	A	A	A	A	A	A	A
H	-	A/G	A	-	A	A/G	-	-
I	A/G	A	A	A	A	A	A	-
J	A	A/G	A	A/G	A	A	A	-

Keys: A = Acid, A/G = Acid and Gas, - = negative.

Table 4: Antimicrobial susceptibility test results for the Gram-positive isolates in millimeters

Iso	AML 20mcg	S 30mcg	NB 10mcg	CH 30mcg	CPX 10mcg	E 30mcg	LEV 20mcg	CN 10mcg	APX 20mcg	RD 20mcg
A	25	25	10	19	25	25	25	25	25	25
B	25	25	25	25	25	25	25	25	25	25
C	25	25	21	16	25	25	25	25	25	25
D	R	R	R	16	25	25	25	25	25	19
E	25	25	24	23	25	25	19	20	19	25
F	15	17	R	18	25	18	16	24	15	25
G	25	25	25	25	25	25	25	25	25	25
H	6	R	5	20	25	25	17	5	4	12
I	18	R	10	20	25	25	25	14	16	10
J	10	R	10	25	25	25	25	25	25	25

Keys: ISO = isolates, AML = Amoxil, S = streptomycin, NB = nofloxacin, CH = chloramphenicol, CPX = ciproflox, E = erythromycin, LEV = levofloxacin, CN = gentamicin, APX = ampiclox, RD = rifampicin, R = resistant.

Plate 2: Antimicrobial Susceptibility Zones of inhibition for isolates

DISCUSSION

For the various results derived from all biochemical tests, carried out on all 10 isolates, suspected bacterial organisms were *Propionibacterium acne, corynebacterium* specie, *Staphylococcus epidermidis, Clostridium* specie, *Micrococcus* specie, *Staphylococcus aureus, other Staphylococcus* specie, and *Streptococcus* specie. These bacteria form a major part of the normal microflora of the skin, although in some cases, they could be involved in various skin infections.

Gram staining was done as a preliminary form of identification of the bacterial isolates. After the gram staining was concluded, organisms were observed under the microscope and were all identified to be gram positive, as they all stained purple. This gives more proof that gram negative bacterial organisms are only rarely isolated from the skin. Isolates A, B and C were gram positive rods, while every other isolate were gram positive cocci.

Every other isolate tested negative to the Coagulase test, aside Isolate G, hence *Staphylococcus aureus* was the suspected gram-positive bacterial organism for isolate. On the other hand, only one isolate tested positive to motility test, which was isolate D, which showed that the bacterial organism suspected was of the *clostridium* specie.

Corynebacterium specie and *staphylococcus aureus* were shown to have very high susceptibility to all tested antibiotic, followed by *Propionibacterium acne* and *Staphylococcus epidermidis. Clostridium* specie had the highest resistance to Amoxil, streptomycin and nofloxacin.

CONCLUSION AND RECOMMENDATIONS

The human skin plays a major role as a barrier, protecting the human body from mechanical impacts and pressure, variations in temperature, pathogens, radiation and chemicals. As an interface with the outside environment, the human skin is colonized by a diverse collection of microorganisms, including bacteria, fungi, viruses, as well as even mites. This study is added proof that gram-positive bacterial specie are more abundant on the human skin than the gram-negative, as all ten different sites, sampled from ten different students of Nnamdi Azikiwe University, using culture-based analysis, yielded only gram-positive bacterial specie. Six different bacterial specie were isolated and *Staphylococcus* specie was the most prevalent. Further analysis is therefore recommended, to analyze the gram-negative species colonizing the human skin. On the other hand, owing to technological advancements in the world today, various cosmetics and beauty products are produced and applied on the skin; most of which causes damage. Hence, proper analysis should be carried out, to determine the relationship that these microbes share with different cosmetic products, in causing damage on the skin.

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