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**IDENTIFICATION AND CHARACTERIZATION OF BACTERIA AND FUNGI ASSOCIATED WITH NOSOCOMIAL INFECTIONS FOUND ON MEDICAL EQUIPMENT IN THE KNUST HOSPITAL**

**BY**

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**CHAPTER THREE**

1. **MATERIALS AND METHOD**

**3.1 STUDY SITE**

The study was conducted at the University Hospital, which is located on the KNUST campus in the Oforikorm Municipal Assembly. It has latitude of 6.686008640278667 and a longitude of 1.5741280892984622. The hospital has a 120-bed space capacity and is well equipped to provide excellent medical care for the inhabitants of the Oforikrom Assembly.

**3.2 STUDY DESIGN**

The study was a cross-sectional type that sought to uncover the diversity of bacteria and fungi associated with nosocomial infections and are found on medical equipment at the KNUST hospital. Medical equipment like the suction machines, sphygmomanometers, pulse oximeters, drip stands, weighing scales, trays and flowmeters were swabbed with sterile cotton swab sticks as a sample collection method for the study.

**3.3 SAMPLE COLLECTION**

A total of 17 samples were collected from the KNUST university hospital, in two separate occasions within three weeks. On each occasion, cotton-tipped sterile swabs sticks moistened with previously sterilized peptone water were used to collect swab samples from the various medical equipment and kept in zip lock bags, with each swab kit labeled as according to which medical equipment and part of the hospital the swab was taken from. Samples were transported to the microbiology laboratory where further laboratory tests were conducted on the collected samples.

**3.4 Serial Dilution**

1ml of each sample were introduced into 9ml of distilled water using sterile pipettes. The resultant solution was then mixed thoroughly together and was further diluted. For each serial dilution, a marking tape and a marker was used to label each test tube holding 9ml of sterile distilled water. Dilutions of known concentrations (10-1,10-2, 10-3, 10-4 ) were made serially by the addition of 1ml of the solution to 9ml of the diluent.

**3.5 Bacteriological analysis**

All the samples were subjected to bacterial analysis using the total viable bacteria and fecal coliforms. Using culture media, potential isolates were identified and their numbers were expressed in terms of colony forming units.

**3.6 Total viable/bacterial and fungal count**

The pour plate method was used to conduct the bacterial and fungal count. Test tube dilutions of 10-1, 10-2, 10-3 and 10-4 were labelled to correspond with respective labels on sterile petri dishes. Using their aliquot, 1ml of the corresponding dilutions were poured into the sterile petri dishes. The agar which has been added then settles after being fully and evenly stirred. The settled agar was then turned upside down and incubated for 24 hours at 37°C for bacteria and 48 hours at 30℃ for fungi.

**3.7 Culture media preparation**

The culture media used were Plate count Agar (PCA), Potato Dextrose Agar (PDA), nutrient agar (NA), peptone water, tryptophan broth and Eosin Methylene Blue (EMB). All the agars and characterization media used in this investigation were prepared as per the manufacturer’s instructions.

**3.7.1 Peptone water**

In the laboratory, 4.5 grams of peptone powder was measured. The powder was dissolved in 300ml of distilled water. Portions of the solution were transferred into Bijou bottles and sterilized in an autoclave at a temperature of 121°C for fifteen minutes.

**3.7.2 Potato dextrose agar**

Potato dextrose agar was prepared using 19.5 grams of potato dextrose powder dissolved in 500ml of distilled water. The solution was sterilized by autoclaving at a temperature of 121℃ for fifteen minutes.

**3.7.3 Plate count agar**

A total of 7 grams of the powdered plate count agar was weighed and dissolved in 400 millimeters of distilled water and heated for the solute to dissolve thoroughly. The agar solution was then autoclaved for 15 minutes at a temperature of 121℃.

**3.7.4 Tryptophan broth**

Tryptophan powder was used in the preparation of the broth. A total of 8 grams of the powder was measured and transferred into a conical flask. The weighed quantity of the powder was dissolved in 500ml of distilled water by heating the mixture to homogenize it. A total of 5ml was taken from the solution and was pipetted into test tubes which were arranged on test tube racks. The test tubes containing the broth were tightly capped by plugging in cotton wool. The test tubes were later sterilized by autoclaving at a temperature of 121℃ for 15 minutes.

**3.7.5 MacConkey Agar**

MacConkey agar is a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative and enteric bacteria and to differentiate them based on their lactose fermentation abilities. The broth was prepared by adding 40 grams of MacConkey powder to 1 liter of distilled water. The solution was later sterilized by autoclaving.

**3.8 Test for *Escherichia coli***

Tryptophan Broth and Eosin Methylene Blue Agar (EMBA) were used in the test for *E. coli*. Test tubes were labelled based on those that had fecal coliforms present and approved positive. Using a sterile pipette, 1ml of the samples from test tubes that had fecal coliforms were transferred into the relevant test tubes containing tryptophan broth and incubated at 44°C for 24 hours. The resulting mixture was pipetted into test tubes along with a few drops of Kovac’s reagent. In some of the test tubes, the color changed from yellow to a cherry ring layer, indicating the presence of *E. coli*.

**3.9 Confirmatory test for *E. coli* with EMBA**

The EMBA was heated, and 5ml of it was put in a petri dish marked for the test tubes that indicated the presence of *E. coli* during the Indole test. This was done to confirm the presence of *E. coli*. After a quick shake, all tubes that retained the crimson red color ring were chosen. EMBA was used to stain them at 37°C, the plates were kept for 24 hours. The presence of *E. coli* was confirmed by the plate’s shiny metallic appearance.

**3.10 Isolation and characterization of the isolates**

To ensure pure staining of cultures, different colonies on the plates were selected and sub-cultured using the streak plate method on sterile nutrient agar. Following isolation, characterization and identification based on the morphological, biochemical, and gram staining techniques, various pure cultures were examined. The characteristics considered included color, size, shape, surface elevation, and margin of colony morphology.

**3.10.1 Colony Counting**

For every petri dish, a colony counter was employed to tally the aggregate number of bacteria colonies. Subsequently, these counts were documented.

**3.10.2 Gram staining**

Sterile swab sticks were used to take colonies from the sub-culture plates. The colonies were mixed with saline to liquify the agar which has been solidified. The saline also aided in visible identification. A smear was made on a sterile slide and was allowed to dry before it was heat fixed. After the slide was heat fixed, crystal violet solution was poured onto the microscope slide for two minutes. The crystal violet solution was then washed off with water. Iodine solution was also poured on each slide to cover its entire surface for two minutes before being washed off with water. Safranin was added thereafter, timed for two minutes and washed off with water. The slides were allowed to dry, and with the aid of an immersion oil applied on each slide, the various slides were viewed under the oil immersion lens which had a magnification of times hundred (x100) to determine the various shapes of the bacteria due to the Gram staining. Gram positive organisms retain a purple stain on viewing, whereas Gram negative bacteria shows a pink to reddish coloration.

**3.10.4 Microscopy**

Immersion oil was applied to the slide and viewed under a microscope’s oil immersion lens with a magnification of times one hundred (x100).

**3.10.5 Biochemical Tests**

Due to distinct biochemical reactions of various bacteria with different chemicals, numerous biochemical tests are employed to identify bacterial species. The biochemical tests conducted included the catalase, indole, citrate test and methyl red test.

**3.10.6 Catalase test**

The catalase test usually edifies facultative anaerobes as well as strict aerobes. The enzyme catalase, which breaks down the toxic hydrogen peroxide (H2O2) into water (H2O) and free oxygen (O2), producing noticeable bubbles is detected using the catalase test. In this test, isolates from sub-cultured media are placed and spread onto a clean slide with a sterile loop. A drop of 3% hydrogen peroxide prepared was then added to the smear. Immediate bubbling indicates a positive result, while no bubbling indicates a negative result.

**3.10.7 Indole test**

Sterilized tryptophan broth were prepared, 5ml of which was pipetted into test tubes and inoculated with samples from sub-cultured media. The test tubes were then incubated for 24 hours at 37°C. A few drops (about 2 to 3) of indole reagent also known as Kovac’s reagent was added to the solution after the incubation period; a positive test is indicated by the development of a cherry red ring at the surface, whereas a negative test is indicated by the surface being yellow or slightly hazy.

**3.10.8 Citrate test**

The Simmons citrate test evaluates whether microbes can utilize citrate as their sole carbon source or not. Microbes capable of this usually incorporate citrate into their cells with the aid of their citrate-metabolizing enzymes. To conduct the test, 5 ml of prepared citrate broth was pipetted into sterile test tubes, which were then inoculated with the sub-cultured colonies. These test tubes were incubated at 37°C for 24 hours, and observed for cloudiness. A cloudy solution indicated a negative result, while a turbid solution indicated a positive result.

**3.10.9 Methyl red test**

This test is conducted to determine the fermentation pathway used in glucose metabolism by microbes. First, 5 ml of sterilized peptone water was pipetted into test tubes. A small colony from the sub-cultured sample was then collected with a sterilize loop and placed into the test tubes containing the peptone water. The tubes were incubated at 37°C for 24 hours. After incubation, a few drops of methyl red reagent were added to the peptone water. The appearance of a red ring at the surface indicated a positive methyl red test, while the absence of a ring indicated a negative methyl red test.

**3.10.10 Sugar fermentation test**

All the isolates from the sub-cultured media were examined for their ability to induce fermentation of various sugars. The samples were inoculated in test tubes containing various solutions of the sugars and bromothymol blue indicator for 24 hours. Bubbles trapped within the test tubes indicate the production of gas. Test tubes with no gas production were recorded as negative.

**3.11 Data management and analysis**

The data were entered into Microsoft Excel 2016 and the outcomes were shown in tables and charts. The means of the viable coliform counts from the samples were compared using an analysis of variance (ANOVA) at 5% level of significance. With a p-value of less than or equal to 0.05 being considered significant, all analysis was completed.