CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

3.1.1. Type of study

This is a cross-sectional study conducted to assess the load and types of Gram-negative bacteria on mobile phones in Khartoum State.

3.1.2. Study area

The study was conducted in Sudan University of Science and Technology, University of Khartoum, Al-Nilain University, University of Bahri, Al-Mughtaribeen University, National Ribat University, International University of Africa, Al-Zaiem Al-Azhari University, University of Science and Technology, Omdurman Islamic University and University of Holy Quran and Islamic Sciences. The practical part was carried out in the Research laboratory of Sudan University of Science and Technology.

3.1.3. Study duration

The study was conducted during the period from April to June 2014.

3.2. Collection of specimens

Simple random technique was adopted to collect specimens from mobile phones using sterile cotton swabs. The swabs were soaked in sterile normal saline before swabbing the target phones. Then the swabs were put quickly into their containers and immediately transported to the laboratory for processing (Kawo and Musa, 2013).

3.3. Ethical considerations

Ethical clearance and approval for conducting this study was received from Sudan University of Science and Technology. All owners of mobile phones were informed of the purpose of the study before swabbing their mobile phones.

3.4. Materials

All chemical reagents, culture media compositions and preparation protocols as well as the equipment and instruments used throughout the study were presented in appendices (1, 2 and 3).

3.5. Methods

3.5.1. Cultivation of the specimens

The cotton end of each swab was aseptically broken in a tube containing 2 ml of normal saline labelled with the specimen number, then mixed using vortex machine in aseptic environment. The bacterial load on each mobile phone was calculated as viable bacterial cells. The method done as follows;

Serial 10 fold dilutions of the bacterial suspension were prepared by pipetting 9ml of sterile normal saline into each of 6 sterile test tubes ($10^{-1}, 10^{-2}, 10^{-3}, \ldots., 10^{-6}$). The suspension of bacteria was mixed uniformly without vigorous shaking, then 1.0 ml of the suspension was aseptically pipetted into the first tube with diluent with sterile 1.0 ml delivering pipette. The content was mixed thoroughly, and then 1.0 ml was transfared into the next tube of diluent was done. All the remaining dilutions were made in the same way by using a fresh sterile pipette for each. A 1.0 ml quantity from

each of the test tubes containing 10^{-4}, 10^{-5}, and 10^{-6} dilution was aseptically pipetted and transferred into correspondingly labeled Petri dishes, in triplications. This was followed by pouring 15 ml of prepared, molten nutrient agar medium of 45˚C onto the plates. The contents were gently swirled and allowed to solidify at room temperature. Finally, the plates were inverted and aerobically incubated at 37˚C for 24 hours. The colonies developed after incubation period in the three plates that were seeded with the dilution giving between 30-300 colonies/plate were counted, which are statistically valid and then the average was taken.

Calculation

The standard formula =

CFU/ml =

$$\frac{\text{colony count on an agar plate}}{\text{amount plated in ml} \times \text{dilution of tube (used to make plate for colony count)}}$$

Plates with colonies greater than 300 and less than 30 have high degree of error and are statistically invalid. Plates with more than 300 colonies could not be counted and were designated too many to count (TMTC). Plates with fewer than 30 colonies were designated too few to count (TFTC) (Reynolds, 2011).

3.5.2. Storage

Isolates were preserved in sloped nutrient agar medium at 4˚C for further investigation (Collee *et al.*, 1996).

3.5.3. Colonial morphology

The colonies on MacConkey's agar were examined for color, lactose fermentation, consistency, size and appearance (Cheesbrough, 2000).

3.5.4. Gram's stain

A smear was prepared and fixed by gentle heating, then covered with crystal violate for 30-50 seconds, washed with water. Lugol's iodine was added, washed with water and decolorized few seconds with alcohol. The smear was covered with suffranin for 2 minute, washed off with clean water and dried with air then examined under the microscope (Cheesbrough, 2000).

3.5.5. Biochemical Tests

3.5.5.1. Oxidase test

The colorless redox reagent, tetramethyl-p phenylenediamine dihydrochloride (or dimethyl can be used) used in the test will detect the presence of the enzyme oxidase and reaction with oxygen turn the color. The oxidase reagent contains a chromogenic reducing agent, a compound that changes color when it becomes oxidized. A positive reaction will usually occur within 10-15 seconds, and will be a bluish-purple color that progressively becomes more purple. Late positive reaction read within 30 seconds (Reynolds, 2012).

3.5.5.2. Fermentation of sugars, production of gas and H_2S

The organism under test was inoculated in KIA medium with sterile straight wire loop by stabbing the butt and streaking back and forth (zigzag) along the surface of the slant. After overnight incubation (18-24 hours) the medium was examined for change

of colour due to pH change, production of gas and H_2S. When KIA is inoculated with a glucose fermenter, the acid producted lower the pH and turn the entire medium yellow within a few hours. Then the organism begins to break down available amino acids, producing NH_3 and raising the pH. This process is called a reversion and only occurs in the slant because of the anaerobic conditions in the butt. Organisms that are able to ferment glucose and lactose turn the medium yellow throughout. However, because the lactose concentration is ten times higher than that of glucose, greater acid production results and both slant and butt will remain yellow after 24 hours. Gas produced by carbohydrate fermentation will appear as fissures in the medium or will lift the agar off the bottom of the tube. Hydrogen sulfide (H_2S) may be produced by the reduction of thiosulfate in the medium or by the breakdown of cysteine in the peptone. Ferrous sulfate in the medium reacts with the H_2S to form a black precipitate. An organism that does not ferment either carbohydrate but utilizes peptone and amino acids will alkalinize the medium and turn it red. If the organism can use the peptone aerobically and anaerobically, both the slant and butt will appear red. An obligate aerobe will turn only the slant red (Leboffe and Pierce, 2011).

3.5.5.3. Urease test

The organism under test was inoculated in urea agar medium which contained urea and phenol red as indicator. After overnight incubation the medium was examined for change of color to pink color due to production of ammonia after the breakdown of urea by urease enzyme in which the medium becomes alkaline, which meant a +ve result and the organism under test is urease producer (Cheesbrough, 2000).

3.5.5.4. Indole test

Testing for indole production is important in differentiating enterobacteria. The test was done by inoculation of organism under test in peptone water or trepton water medium which contained tryptophan. After overnight incubation presence of red ring when Kovac's reagent was added (which react with the indole) meant a +ve result and organism under test broke down the amino acid tryptophan with the release of indole (Cheesbrough, 2000).

3.5.5.5. Citrate utilization test

The test is based on the ability of an organism to use citrate as its only source of carbon and ammonia as its only source of nitrogen. Organism under test was inoculated in Simmon's Citrate Agar medium which contained sodium citrate, an ammonium salt and the indicator bromo-thymol blue. After overnight incubation change of color of the indicator from light green to blue color due to the alkaline reaction following citrate utilization meant a +ve result while presence of the light green colour mean –ve result (Cheesbrough, 2000).

3.5.6. Microbact™ 24E Gram-negative identification system

Identification of some isolates was done by Microbact™ 24E Gram-negative system (Oxoid, UK) for the identification of aerobic and facultatively anaerobic Gram-negative bacteria. The Microbact™ 24E Gram negative system is standardized micro-substrate system designed to stimulate conventional biochemical substrates used for the identification of enterobacteriaceae and common miscellaneous Gram-negative bacilli (MGNB). It consisted of dehydrated substrates for 24 different biochemical tests placed in the wells of a microtiter tray. Four organisms could be tested in each 96

well tray. Organism identification was based on pH change and substrate table of reactions or the substrates contained in each well, the specific reaction principle and colour changes.

3.5.6.1. Preparation of inoculums

Bacterial isolates were subcultured onto nutrient agar to check for purity. Three isolated colonies from 24 hours culture were picked and emulsified in 5.0 ml of sterile saline. Mixed thoroughly to prepare a homogeneous suspension. The plate was placed in the holding tray and by using a sterile Pasteur pipette 4 drops (approximately 100µl) of the bacterial suspension were added. A dropper bottle used to overlay the substrates underlined on the holding tray with sterile mineral oil to provide anaerobic condition in wells No 1, 2, 3, 20 and 24.

3.5.6.2. Incubation

The inoculated rows were resealed with the adhesive seal and the specimen identification number was written on the end tag with a marker pen. Tray was incubated at 37˚C for 24 hours for enterobacteria and for 48 hours for miscellaneous Gram-negative bacilli (MGNB).

3.5.6.3. Reading the plate

The 24E plate was read after 24 hours for enterobacteriaceae and within 48 hours for miscellaneous MGNB. The tray was removed from the incubator, the sealing tape peeled and the plate was observed for colour change with or without the addition of suitable reagents according to the manufacturer instructions. All positive results were recorded. The reactions evaluated as positive or negative by comparing them with the colour chart and the results recorded under the appropriate heading on the report form.

3.5.6.4. Interpretation of results

An octal coding system has been adopted for Microbact™. Each group of three reactions produced a single digit of the code. Using the result obtained, the indices of the positive reactions were circled. The sum of these indices in each group of three reactions formed the code number which was entered into the computer package.

3.5.6.5. Computer aided identification package

The Microbact™ computer aided identification package was consulted for the identification choices. The percentage figure shown against the organism name was the percentage share of the probability for that organism as a part of the total probabilities for all choices (Oxoid, UK).

3.5.7. Quality control

The quality of the study was kept by preparing and using standard operational procedures for laboratory investigation and media preparation. Sample collection and processing were carried out using aseptic techniques. The samples were labelled properly. Culture and bacterial colony count were determined by experienced laboratory personnel. The performance and sterility test of prepared media were checked by incubating at 37˚C and inoculating with control strain organism. The performance and sterility test of the Microbact 24E were checked by using control strain organism.

3.5.8. Data analysis

Figure was performed by using statistical package for social sciences (SPSS) version (15.0).