Evaluation of MicrocinE492 gene presence in *Klebsiella pneumoniae* isolated from clinical samples.

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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iDECLARATION

I, hereby, solemnly declare that the research work titled "**Evaluation of MicrocinE492 gene presence in *Klebsiella pneumoniae* isolated from clinical samples**" submitted by the undersigned has been carried out under the supervision of **Mr. S M Rakib-Uz-Zaman**, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. I also confirm that this work is completed by me and has never been submitted to this institution or in any other university.

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Abstract

Bacteriocins are small peptides with anti-bacterial properties. Among which, micrrocin E492 (MccE492) is a low-molecular-weight, channel-forming bacteriocin produced by *Klebsiella pneumoniae*. However, micrrocin E492 is active on strains of *Enterobacteriaceae* family. Moreover, it also has a cytotoxic effect on several malignant human cell lines. Recently, there is a growing rate of development of antibiotics-resistant bacterial strains. Secondly, Drug Related Problems (DRPs) in cancer is also a severe issue in the world. Thus, these two emerging concerns require related advanced researches for better therapeutic outcome. The present descriptive analytical study is carried out to determine the presence of *Klebsiella pneumoniae* with mceA gene, encoding for microcin E492 in clinical specimens isolated from patients with burn infection and respiratory tracts diseases. However, in this study, 65 clinical samples from burn wound and respiratory tract infection were collected. Then using different phenotypic characterization and biochemical testing, *Klebsiella pneumoniae* were isolated and identified from the specimens. Finally, polymerase chain reaction (PCR), using gene specific primers, was carried out to confirm the presence of mceA gene in the selected samples. A total of 7 *Klebsiella pneumoniae* samples were found to be positive with mceA gene, which means 14% of the total sample contained the gene. This thesis work helped to establish a data for the very first time in Bangladesh based on the frequency of *Klebsiella pneumoniae* containing mceA gene in hospitalized patients with burn wound and respiratory tracts infection.

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LIST OF ABBREVIATIONS

MccE492	Microcin E492
bp	Base Pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
KPN	*Klebsiella pneumoniae*
kb	Kilobytes

MIC	Minimum Inhibitory Concentration
nm	Nanometer
PCR	Polymerase chain reaction
rpm	Revolutions per minute
UV light	Ultraviolet light
TSI	Triple sugar iron
MIU	Motility indole urease
R.B.C	Red blood cell
µl	Microliter
SDS- PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Eletrophoresis
LAB	Lactic acid bacteria

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Chapter 1

Introduction

1.1 Background

Bacteriocins are bacterially synthesized ribosomal antibacterial proteins that either kill or inhibit the growth of closely related bacteria and against which the producer itself has a specific immunity mechanism. In 1925, André Gratia discovered bacteriocins [1]. Bacteriocins may have a narrow spectrum or sometimes even a broad spectrum of antibiotic activity and can inhibit the growth of taxonomically close or even a wide variety of bacteria at very low concentrations. (Cotter et al., 2005; Mills et al., 2011). However, bacteriocins are produced by gram positive bacteria, gram negative bacteria and archaea. It is estimated that 99% of bacterial strains produce at least one bacteriocin [25]. Bacteriocins comprise a diverse group of peptides with respect to size, structure, mode of action, antimicrobial potency, immunity mechanisms and target cell receptors and etc. Thus, are classified in numerous ways, like, producing strain, common resistance mechanisms, and mechanism of killing, genetics, molecular weight and chemistry and method of production [2]. However, the categorization of the bacteriocins is described briefly below:

A. Gram positive bacteriocins are typically classified into different classes [3] [4].

1. ***Class I bacteriocins***

The class I bacteriocins are small polycyclic peptide antibiotics containing characteristic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine, and 2-aminoisobutyric acid. Examples are nisin and other lantibiotics.

2. ***Class II bacteriocins***

The class II bacteriocins are small (<10 kDa) heat-stable proteins. This class is subdivided into five classes.

- **Class IIa** contains strong anti-Listeria activity and broad range of activity, due to which they have numerous applications food preservation and medical applications.
- **Class IIb** bacteriocins: It contains two different peptides for its activity. However, almost all of these bacteriocins have a GxxxG, which interacts with the motifs in the membranes of the target bacterial cells.
- **Class IIc** contains cyclic peptides, in which the N-terminal and C-terminal regions are covalently linked.
- **Class IId** comprises the single-peptide bacteriocins, which are not post-translationally modified and do not show the pediocin-like signature.

3. Class III bacteriocins

Class III bacteriocins are large, heat-labile (>10 kDa) protein bacteriocins. This class is subdivided in two classes.

- **Subclass IIIa** includes peptides that kill bacterial cells by cell wall degradation, thus causing cell lysis.
- **Subclass IIIb** comprises those peptides that kill the target cells by disrupting plasma membrane potential.

4. Class IV bacteriocins

Class IV bacteriocins are complex bacteriocins containing lipid or carbohydrate moieties.

B. Gram negative bacteriocins are characteristically classified by size but this size classification also coincides with genetic, structural and functional similarities [3] [4].

1. Microcin

Microcins are very small bacteriocins, composed of relatively few amino acids. Molecular weight of microcins are less, they are less than 10 kDa in size. These proteins can be found in two different

classes. Microcins which are post- translationally modified fall into one class and the unmodified microcins belongs to a different class. Examples of few microcines are, microcinE492, microcinB17, microinC7, ColicinV.

2. Colicin-like bacteriocins

Colicin-like bacteriocins are 20 to 90 kDa in size and tailocins or so called high molecular weight bacteriocins which are multi subunit bacteriocins that resemble the tails of bacteriophages.

The classification of bacteriocins is shown in figure 1.1.

	Bacteriocins	Type/ Class	Size (kDa)
Gram-negative bacteria	Colicins	Pore former Nucleases	20-80
	Colicin-like	NA	20-80
	Phage-tail-like	NA	> 80
	Microcins	Post- translationaly modified Unmodified	< 10
Gram-positive bacteria	Class I	Type A- positively charges and linear Type B- uncharged or negatively charged globular Type C- synergistic	< 5
	Class II	Type IIa- antilisterial Type IIb- synergistic	< 10
	Class III	TypeIIIa- bacteriolytic enzymes Type IIIb- nonlytic peptides	> 10
	Class IV	Cyclic peptde	< 10
Archea	Halocins	Microhalocins Protein halocins	< 10 > 10
	Sulfolobicin	NA	~ 20

Table 1.1: Bacteriocins of Bacteria and Archaea (Bakkal et al., 2012).

1.2 Applications of bacteriocins

The phenomenon properties of bacteriocins have evoked immense interest among scientists to study the detail mechanism of action and the effect of chemical modifications of bacteriocins.

Understanding the strategies used by bacteriocins suggest that bacteriocins have numerous applications in different sectors.

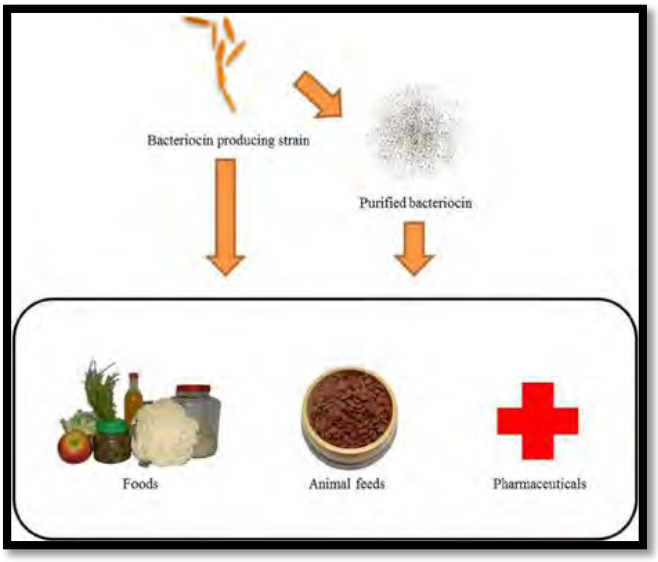


Figure 1.1: Applications of bacteriocins in different sectors (Thanner, 2002).

1.2.1: Application of bacteriocin in pharmaceutical sector:

Bacteriocins are being tested as narrow-spectrum antibiotics and have been proved to become a potential drug candidate for replacing antibiotics in order to treat multiple drugs resistance pathogens in the future. For instance, the hybrid bacteriocin purified from *E. coli* extracts, named Ent35eMccV, showed inhibitory activity against enterohemorrhagic *E.coli*, *L. monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria (Acuña et al. 2012).

1.2.2: Applications of bacteriocins in food:

Among bacteriocins produced by many Gram-positive and Gram-negative species, those produced by lactic acid bacteria (LAB) are of particular interest to the food industry, since these bacteria

have generally been regarded as safe. Nevertheless, purified and identified bacteriocins are being used in food technology, which aims to extend food preservation time [5]. As of 2016, nisin, a bacteriocin is recognized as safe by the FDA and is used as a food preservative in several countries [11].

1.2.3: Applications of bacteriocins in livestock health and aquaculture:

Bacteriocin also has applications in animal farm and aquaculture. Bacteriocin producing microorganisms can be used as a source of probiotics and can be feed to livestock and fishes, to avoid the use of different vaccine and antibiotics in their diet [11]. Many works reported that the administration of bacteriocin producing bacteria as probiotic exclude competitively pathogenic bacteria through the production of inhibitory compounds, improve water quality, enhance the immune response of host species, and enhance the nutrition of host species through the production of supplemental digestive enzymes (Taoka et al., 2006; Wang 2007). Moreover, several bacteriocins including nisin have been tested against the causative bacteria of bovine mastitis. The positive results have been reported for *in vivo* studies (Sears et al., 1992; Wu et al., 2007).

1.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a gram-negative, facultative anaerobic and nonmotile bacteria. The reason for its pathogenicity is the thick capsule layer surrounding the bacterium. It is 160 nm thick of fine fibers that protrude out from the outer membrane at right angles [16], [17]. It belongs to Enterobacteriaceae family. *Klebsiella pneumoniae* is ubiquitous and opportunistic pathogen which is commonly found in the environment and it can colonize and infect both plants and animals [13]. Widely, it can sustain asymptomatically in the intestinal tract, skin, nose, and throat of healthy individuals, but it can also cause a range of infections in hospitalized patients, most commonly

pneumonia, wound, soft tissue, or urinary tract infections. As opportunistic pathogens, *Klebsiella pneumoniae* primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction [8]. *Klebsiella* pneumoniae is getting resistant to many antibiotics including one of the newest antibiotics—carbapenem (Clock et al., 2013; Alp et al., 2013). Carbapenemase-resistant *Klebsiella pneumoniae* (CRKP) is very challenging to treat, especially in Intensive Care Units (Borer et al., 2009; Ben-David et al., 2010; Marchaim et al., 2011; Llaca-Díaz et al., 2012).

K. pneumoniae acquire resistance against existing antimicrobials by multiple mechanism results in increased multidrug-resistant (MDR) of *K. pneumoniae* that leads to serious problem in hospital settings and health concern. Emergence of resistance occurs not only in MDR isolates but also exist in pan-drug resistant (PDR) isolates of *K. pneumoniae*. PDR refers to the resistant strains those are specifically resistant to 7 antimicrobial agents such as cefepime, imipenem, meropenem, ceftazidime, ciprofloxacin, piperacillin-tazobactam, and levofloxacin [14]. Continuous use of antibiotics leads to resistance in microorganisms via different pathways. It can be mediated by plasmids, transposons, and gene cassettes in integrons [15]. As they survive in the environment so need to compete with other microorganisms for resources. As a result, for their survival, bacteria produce antimicrobial compounds or bacteriocin, microcin E492, to inhibit or kill other competing strains [8].

Figure 1.2: *Klebsiella pneumoniae* are gram-negative, encapsulated, non-motile, enteric, rod-shaped bacteria (Trivedi et al., 2015)

1.4 Microcin E492

Microcin E492 (MccE492) is a pore-forming bacteriocin produced by *Klebsiella pneumoniae* RYC492. MccE492 is actively secreted into the extracellular medium when cells approach early log phase (Delorenzo, 1984). MccE492 secretion is favored when nutrients are depleted and the cells become ravenous. Microcin E492 is coded by mceA gene, 277 bp. Moreover, few other nutrient deficiency agents such as the *fur* locus also regulate the production of this protein, (Salomon and Farias, 1994). Analysis of the genetic determinants indicates that, microcin E492 ais chromosomally encoded on DNA fragments [9]. Microcin E492 has a molecular mass of 7,887 Da and is a highly hydrophobic. Furthermore, the deduced amino acids sequence also indicates no similarity between the sequences of any known channel-forming bacteriocin with MccE492. It is neither related to colicin-like channel-forming bacteriocins from gram negative microorganisms nor to channel-forming bacteriocins from gram-positive bacteria. On the other hand, it is also determined that microcin E492 shares some characteristic cleavage site features with lactococcins

and related bacteriocins found in gram-positive bacteria [9]. In addition, MccE492 has the capacity to form amyloid-like fibrils, both *in vivo* and *in vitro* [6]. All together, it appears that this microcin defines a novel class of low molecular- weight pore-forming bacteriocin with unique characteristics.

Figure 1.3: 3D model of microcin E492 (Karpiński et al., 2018).

1.4.1 Antimicrobial activity of microcinE492

Microcin E492 (MccE492) acts as an antibacterial agent against strains of *Escherichia*, *Klebsiella*, *Salmonella*, *Citrobacter*, and *Enterobacter* [7]. However, MccE492 is not active on other Gram-negative bacteria, Gram-positive bacteria or fungi. In a study, highly purified MccE492 was investigated against *Escherichia* and Salmonella species and has showed potent antibacterial activity at minimal inhibitory concentrations in the range of 0.02-1.2 μM [7]. This bacteriocin is a modular protein, with a toxic domain at the *N*-terminal and an uptake domain at the *C*-terminal. The mechanism by which the *C*-terminal of microcin E492 is recognized by catecholate siderophore receptors is called the "Trojan horse" strategy, because the *C*-terminal structure mimics essential bacterial elements, which are recognized by the respective receptors and translocated across the outer membrane to exert antibacterial action [8].

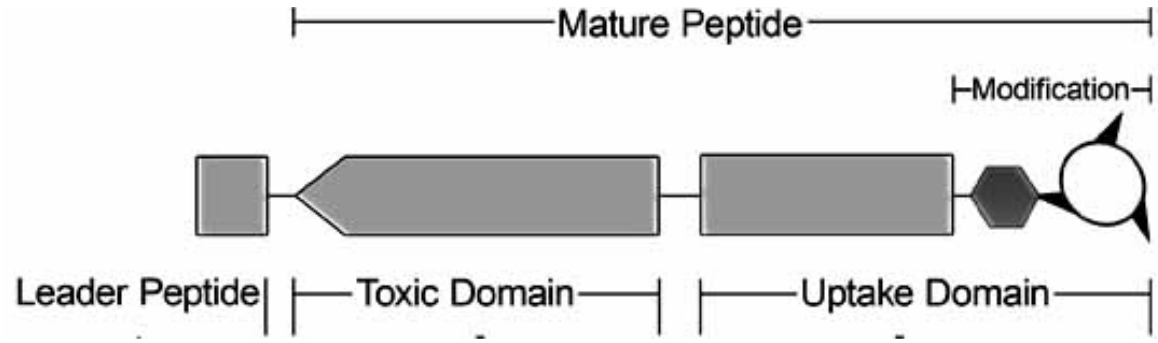


Figure 1.4: Modular structure of MccE492 contains precursor with three domains: leader peptide, toxic, and uptake domain (Trivedi et al., 2015).

1.4.2 Anti-cancer activity of microcin E492

Microcin E492 has cytotoxic effect on malignant human cell lines. The cytotoxicity of M-E492 was detected in various malignant human cell lines, including cervical adenocarcinoma (HeLa), acute T cell leukaemia (Jurkat), B cell line originated from Burkitt's lymphoma (Ramos) and B-lymphoblastoid cell lines transformed by infection with Epstein-Barr virus (RJ2.25, a variant of the Raji B-LCL). At the same time, no effect was determined against human endothelial cells from human tonsils (AMG-3) and a monocyte-macrophage cell line (KG-1) . Among all, Jurkat cell line was the most sensitive to microcin E492, with 96% viability decrease after 24 h of incubation. At a low concentration (5–10 _g/mL), M-E492 induced apoptosis of cancer cells, while at a higher concentration (20 _g/mL) it caused necrosis of them [8]. It was also reported that M-E492 causes morphological and biochemical modifications during apoptosis such as, cell shrinkage, fragmentation of DNA, extracellular exposure of phosphatidylserine, caspase activation, decline of potential of mitochondrial membrane and also release of calcium ions from intracellular stores [8][10].

Nevertheless, MccE492 is toxic in its modified, unmodified and amyloid-like fibrils form [8]. Hence, the ability of microcin E492 to form amyloid-like fibrils can be exploited in the formulation

of this bacteriocin as an antitumoral agent, because these fibrils are very stable and potentially they could be employed as sustained release of a biologically active molecule. Alternatively, live bacteria can be used as a continuous source of microcin E492 production in specific tumors [8] [10].

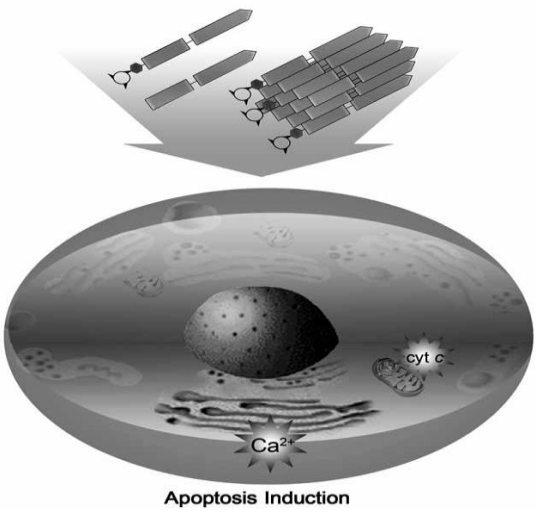


Figure 1.5: The cytotoxic effect of MccE492 on eukaryotic cells involves the liberation of calcium (Ca2+) from intracellular stores, mitochondria dysfunction with cytochrome c(cyt c) release, and activation of caspases and DNAses to induce the cellular death (Trivedi et al., 2015).

1.5 The objective of the thesis

- To observe the frequency of *Klebsiella pneumoniae* in patient with burn wound and respiratory tract infection of Bangladesh.
- To identify the *Klebsiella pneumoniae* with mceA gene, which is encoded for the production of micocinE492 protein.
- Finally, to do a statistical analysis for determining the frequency of *Klebsiella pneumoniae* containing microcinE492 producing gene in clinical samples

Chapter 2

Method

2.1 Clinical sample collection

In this study, 65 clinical samples ,including, 20 burn wounds specimens from Dhaka Medical College Burn and Plastic Surgery Unit and 45 respiratory tract specimens from National Institute of Diseases of the Chest and Hospital (NIDCH), were collected.

2.1.1 Clinical specimen collection from burn infection

The wound swab from the burn infection was collected from clinically deep areas prior to any cleansing. A sterile cotton swab was moistened with sterile normal saline. Then the swab was rubbed onto the burn wound surface. Swab was taken from areas which appear deep and areas with discharge. The swabs were transported to the laboratory in 0.9% normal saline.

2.1.2 Clinical specimen collection from patients with respiratory diseases

Sputum from the patients was collected into clean and sterile plastic cups. The caps of the cups were tightly screwed and were transported to the laboratory.

2.2 Isolation and identification and of *Klebsiella pneumonia* from the clinical samples

After sample collection samples were streak on blood agar, nutrient agar and MacConkey agar plate for microbiological culture of the isolated colony. Samples were identified routinely to distinguish *Klebsiella pneumoniae*. The cultural properties of the organisms were observed and recorded.

For confirming the bacterial isolates, several methods were followed. These methods include-

- Analysis of colony morphology
- Gram staining
- Biochemical tests

2.2.1 Analysis of colony morphology

For analyzing the colony morphology of the bacteria, the collected specimens were streaked on blood agar, nutrient agar, UTI Hi-Chrome agar and MacConkey agar plate within 4 hours of collection. Different media helped to determine morphology and different features of the bacteria.

Blood agar

Blood agar is an enrichment media containing trypticase soya agar with 5% sheep blood. Certain bacterial species produce extracellular enzymes that lyse red blood cells in the blood agar (hemolysis), which diffuses outwards from the colony to cause complete or partial destruction of the red cells (RBC) and hemoglobin to colorless products [29]. Thus, bacteria were cultured on blood agar for 24 hours at 37^{o}C to determine the type of hemolysis produced by the bacteria.

UTI Hi-Chrome agar

UTI HiCrome agar media is a differential chromogenic medium used for presumptive identification of *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus aureus*. This media contains peptic digest of animal tissue for and chromogenic mixture. It facilitates the identification of bacteria on the basis of different contrasted colony colours produced by reactions of genus or species produced specific enzymes. The chromogenic substrates are specifically cleaved by the enzymes. Presence of amino acids like phenylalanine and tryptophan from peptones helps for detection of tryptophan deaminase activity,

indicating the presence of *Proteus* species. The samples were grown for 24 hours at 37°C for identification of mentioned bacteria.

MacConkey agar

The bacteria were also grown for 24 hours at 37°C in MacConkey agar for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria. This media contains pancreatic digest of gelatin and peptones (meat and casein) which provide the essential nutrients, vitamins and nitrogenous factors required for growth of microorganisms. The selective action of this medium is attributed to crystal violet and bile salts, which are inhibitory to most species of gram-positive bacteria. Sodium chloride maintains the osmotic balance in the medium. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colorless at any pH greater than 6.8. For distinguishing lactose fermenters from non-lactose fermenters.

Isolation of pure colony

Four quadrant streaking method was used for obtaining pure single colonies. From the culture grown in the media above, bacteria that match colony morphology of KPN were isolated in nutrient agar. In this technique, the wire loop was sterilized and cooled by touching it on the edge of the sterile agar plate. A single colony was touched with the loop and the lid was lifted just enough to insert the loop. The loop was dragged over the surface of the top one-third of the plate back and forth in a "zig-zag" formation. Then the loop was sterilized in the flame. The plate was turned 90 degrees and the loop was dragged for 2-3 times through the area of previously streaked area and continued to drag the loop in a "zig-zag" formation in the remaining half of the plate without touching that area again. The loop was again sterilized in the flame and the plate was turned 90

degrees. Same procedure was repeated. In this way four quadrants streaking was done. All the plates were incubated at 37^{o} temperature for 24 hours.

2.2.2 Gram Staining

The Gram staining technique is the most important and widely used microbiological differential staining technique. It categorizes bacteria according to their gram character (gram positive or gram negative). In addition, this stain also allows determination of cell morphology, size, and arrangement [18].

Materials Required:

1. Clean glass slides
2. Inoculating loop
3. Bunsen burner
4. Bibulous paper
5. Microscope
6. Immersion oil

Reagents Required:

1. Primary Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Safranin

Procedure

Grease or oil free slides were taken and labeled. Then with a sterile cooled loop, a loopful of 24 hours fresh colony was taken and a thin whitish layer smear was made on the slide using normal saline. The smear was then heat fixed. Smear was flooded with crystal violet and let stand for 1 minute. The slide was tilted slightly and gently rinse with tap water using a wash bottle. The smear was flooded with Gram's iodine and let stand for 1 minute. Then the slide was tilted slightly and gently rinse with tap water using a wash bottle. Using 95% ethyl alcohol the smear was decolorized for 10 seconds and washed with water. The smear was flooded with safranin to counter-stain and let stand for 45 seconds and then washed with water. The slide was blot dried with bibulous paper. The smear was viewed using a light-microscope under oil-immersion.

2.2.3 Biochemical tests

Biochemical tests were used for the identification of *Klebsiella pneumoniae* species based on different biochemical activities.

Cultures were grown for 24 hours at 37°C on nutrient agar media prior to each biochemical tests.

TSI (Triple Sugar- Iron agar) test:

Triple Sugar Iron Agar (TSI Agar) is utilized for the separation of gram-negative enteric bacilli in view of carbohydrate fermentation furthermore, the generation of hydrogen sulfide. TSI Agar contains three sugars (dextrose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production (indicated by blackening in the butt of the tube). Carbohydrate fermentation is demonstrated by the creation of gas and an adjustment in the shade of the pH pointer from red to yellow. 1.0 % lactose/1.0% sucrose causes a large amount of acid turns both butt and slant yellow, thus indicating the ability of the culture to ferment either lactose or sucrose. Ferrous sulfate is present by indicating H2S formation. Phenol red act as an indicator of acidification (It is yellow in acidic condition and red under alkaline conditions). It also contains Peptone which acts as source of nitrogen. For the test of TSI, a single bacterial colony of the bacterium to be tested was picked up by a sterile straight needle and stabbed through the center of the medium to the bottom of the tube. Then streaking on the surface of the agar slant into the TSI containing dextrose, lactose and sucrose. Cap of the tube was loosened and incubated at 37°C for overnight and were examined after 24 hours for carbohydrate fermentation, CO_2 and H_2S production.

Citrate utilization test:

The Citrate Utilization test was used to identify bacteria which utilize citrate as one of their starting products of metabolism. Simmon's citrate media contain mineral salts, sodium citrate for carbon, and ammonium phosphate for its nitrogen source. The pH indicator bromo thymol blue is also present, which is green at neutral pH, yellow at acidic pH <6.0 and turns blue at alkaline (basic) p*H* >7.6. For citrate test, firstly Simmon's citrate agar slant was prepared, a single pure bacterial colony of the bacterium to be tested was picked up by a sterile straight needle. Then transferred aseptically to a sterile tube of Simmons citrate agar. The inoculated tube was incubated at 37^{o}C for 24 hours and the results were determined.

Motility indole urease test:

MIU medium is used for checking motility, urease production and indole production. MIU contains casein enzymic hydrolysate provide amino acids and other nitrogenous substances. Sodium chloride maintains osmotic equilibrium. Dextrose is fermentable carbohydrate. Phenol red is the pH indicator which turns pink- red in alkaline condition. For MIU test, single pure bacterial cultures was stab-inoculated with the help of a straight sterile needle throughout the center of the media. Then it was incubated overnight at 37°C.

Indole test:

Indole test was used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce is indole. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth. The bacteria were inoculated on tryptone broth and incubate at 37°C for 24 hours in ambient air. After incubation, 0.5 ml of Kovac's reagent was added to the broth culture.

Methyl Red (MR) test and Voges-Proskauer (VP) test:

Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. Pyruvic acid, the pivotal compound in the fermentative degradation of glucose, is further metabolized through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathways result in the production of acetion (acetyl methyl carbinol), a neutral-reacting end product.

MR-VP broth were used for both MR test and VP test. Only the addition of reagent differs, and both tests were carried out consecutively.

Reagents:
For VP test

- Barrit's reagent A
    1. Alpha Naphthol-5g
    2. Absolute ethyl alcohol- 100 mL
- Barrit's reagent B
    1. Potassium hydroxide 40g
    2. Distilled water to: 100 mL

For MR test

1. Methyl red indicator

Procedure

Two tubes containing MR-VP broth with a pure culture of the microorganisms were inoculated and incubated at 37 °C for up to 4 days. About 5 drops of the methyl red indicator solution in one tube was added for MR test. For VP test, 3-5 drops of Barrit's reagent A, followed by 0.2 mL of Barrit's reagent B were added. The tubes were shaken gently and allowed to remain undisturbed for 10 to 15 minutes.

Catalase Test:

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). 3% H_2O_2 was used for the test. Using a sterile wooden stick a small amount of colony was transferred in the surface of a clean, dry glass slide. A drop of 3% H_2O_2 was placed in the glass slide. Evolution of oxygen bubbles was observed for positive result.

Oxidase test:

The oxidase test is used to identify bacteria that produce Cytochrome C Oxidase. When present, the Cytochrome C Oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. A filter paper was soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. A colony was picked with a wooden stick and a smear was made in the filter paper. Inoculated area of paper was observed for a color change to deep blue or purple within 10-30 seconds.

2.3 Identification of *Klebsiella pneumoniae* using Microrao software

After conducting all physiological and biochemical tests, online bacterial identification Microrao software was used**.** Microrao software application enable the users to identify the organisms based on the results of their biochemical tests. The software uses probability matrix for identification and the results are expressed in percentage probabilities. Accuracy of the results are dependent on the accuracy of the test results.

Steps to identify the bacteria using Microrao software are shown below:

Step 1: Visit http://www.microrao.com/identify.htm link from the internet browser.

Step 2: The desired option for the identification of the bacteria has been selected, which in this case, is the 'basic identification', shown in the figure, marked by red box (Figure 2.1).

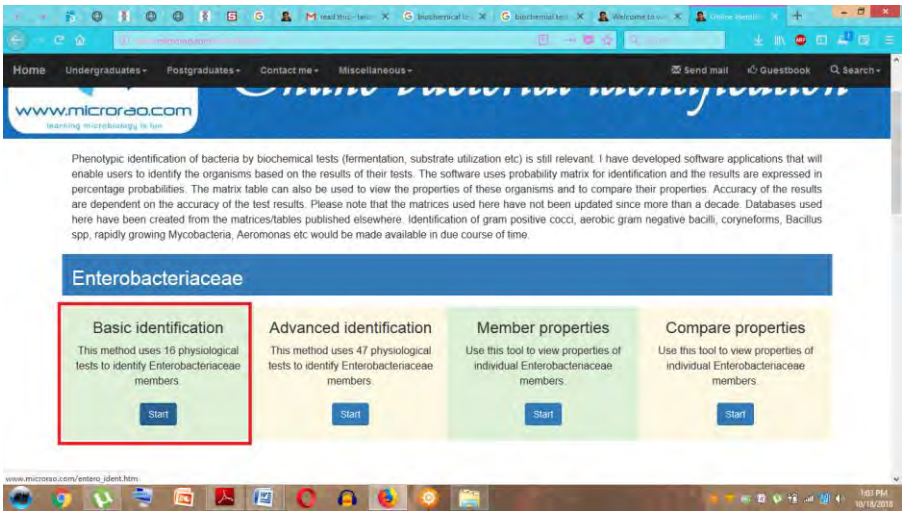


Figure 2.1: 'Basic Identification' option is being chose, which has been marked by red box.

Step 3: Then, the results of the biochemical tests are selected from the options provided by the software (Figure: 2.2).

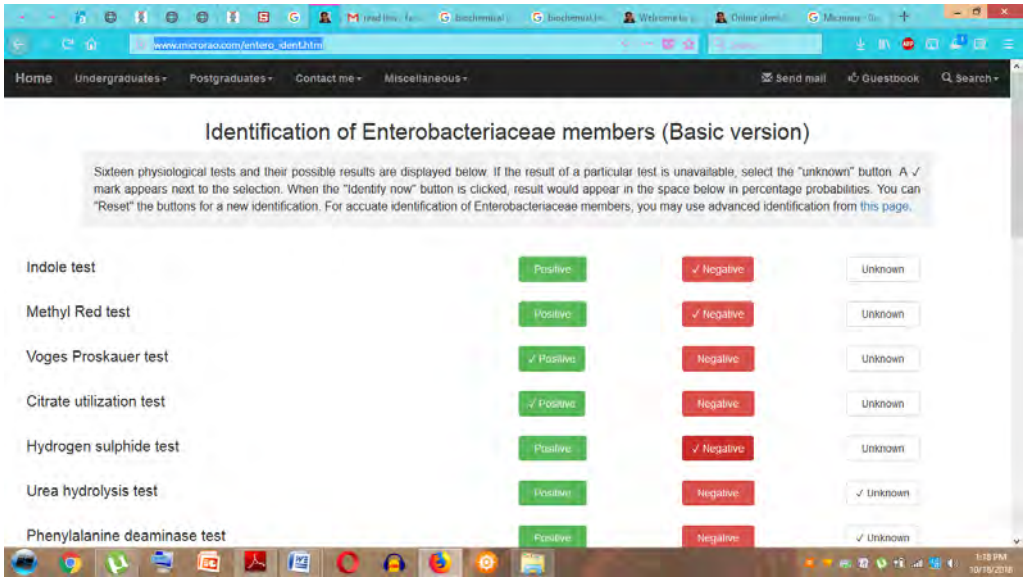


Figure 2.2: Results of the biochemical tests are being selected.

Step 4: The software predict the best possible result for identification of the bacteria and expressed the result in percentage probabilities. (Figure: 3.10)

2.4 DNA extraction of *Klebsiella pneumoniae*

DNA extraction by boiling method were done of the *Klebsiella pneumoniae* obtained from the clinical samples. Sample organisms were grown overnight on Luria bertani broth. Overnight culture were centrifuged at 13,000 rpm for 10 minutes. Supernatant was removed. 0.5 ml autoclaved DH_2O was added and centrifuged at 12,000 rpm for 10 minutes. Supernatant was removed again. Cells were disrupted at 98^{o}C in water bath for 5 minutes. Then the sample was immediately kept in ice for cold shock for 10 minutes. The sample was then centrifuged at 13,000rpm for 5 minutes. Finally the supernatant was collected, containing DNA sample, and stored at -20^{o} C. Extracted DNA was qualified by using 1.5 % agarose gel electrophoresis.

2.5 Polymerase chain reaction (PCR)

After DNA isolation, through PCR, *Klebsiella pneumoniae* containing microcinE492 producing, mceA gene, was determined. The main purpose of this technique is to amplify a particular sequence of the DNA extracted from the sample which may contain mceA gene, using gene specific primers.

2.5.1Gene specific primer

Gene specific primers for gene encoding microcin E492 with the access number “AF063590.3” in the National Center for Biotechnology Information (NCBI) genome data bank, were used in the PCR reaction [20]. The primer sequence is shown in the table 2.1.

Primers Name	Primer Sequence	Length
FMN (forward)	5'-ATCGACGGATCCGGGCGAGACCGATCCAAATAC-3'	33
RMN (reverse)	5'-CAACATCTCGAGACTACCACTACCGGAACTGGATG-3'	35

Table 2.1: Gene specific primers sequences of microcin E492.

2.5.2: PCR reaction mixture

The composition of reaction mixture of PCR are given in the table 2.2.

Reagent	Amount
10X *Taq* buffer($MgCl_2$)	2.5µl
2 µM dNTP mix	2.0µl
10µM Forward Primer	2.0µl
10µM Reverse Primer	2.0µl
Taq DNA Polymerase	0.2µl
DNA template	2.0µl
Nuclease free water	14.3µl
	Total 25µl

Table 2.2: PCR reaction mixture for each sample.

2.5.3 PCR thermal cycler condition:

The target DNA sample was initially heat denatured at 94°C for 45 sec. In this temperature main DNA is separated in its complementary strands, this process is called melting of target DNA. Annealing of primers was done at temperature 64°C for 45 sec. As a result the primers form the hydrogen bonds and anneal to the DNA on both sides of the DNA sequence. Finally, different nucleoside triphosphate (dATP, dGTP, dCTP, dTTP) and a thermo-stable DNA polymerase are added. It helps in polymerization process of primers and, therefore, extends the primers (at 72°C) resulting in synthesis of multiple copies of target DNA sequence. Moreover, initial denaturing was carried out at 94°C for 5 minutes and final extension was done at 72°C for 5 minutes. After

completion of all these steps in one cycle, again the second cycle was repeated following the same process. PCR was carried out for total 35 cycles (table 2.3) [20].

Stage	No. of Cycle	Temperature °C	Incubation Time
Initial denaturation	1	94	5 min
Denaturation	35	94	45 sec
Annealing	35	64	45 sec
Extension	35	72	30sec
Final extension	1	72	5 min

Table 2.3: PCR thermal cycler condition.

2.6 Electrophoresis of PCR products

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here, DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. A 0.7% gel will show good separation for large DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of 0.2-1kb. There is an important factor that works behind the gel electrophoresis that is voltage applied. The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases. But voltage should be limited because it heats and finally causes the gel to melt.

2.6.1 Agarose gel preparation

50ml TBE buffer and 0.5 gm agarose were added in a beaker and heat was applied to dissolve the agar. After that, adding 2.5 µl of $EtBr_2$ into the buffer. After that, an appropriate comb for sample slots in gel was chosen and gel was poured in the mold. When the gel was settle down, the comb was removed carefully.

2.6.2 Detection of mceA gene band using PCR product by following gel electrophoresis

At first 2µl of loading dye was taken for each samples. Then took 3µl sample (PCR product) was added into the specific dye and properly mixed it up and input the well. In one well 6 µl ladder was added. Extra buffer added in the electric field if it is necessary. Electrophoresis was done for 50 minutes at 80 volts. Then the gel was visualized under UV light.

Chapter 3

Result

3.1 Isolation and identification of *Klebsiella pneumonia* from clinical sample

After growing the organisms on different media, gram staining and biochemical tests, *Klebsiella pneumonia* were isolated and identified for further molecular studies. The results obtained are discussed below.

3.1.1 Morphology of Klebsiella pneumonia on different media

Blood agar media

After 24 hours incubation period at 37º C, *Klebsiella pneumoniae* produced opaque mucoid colonies and did not cause hemolysis on blood agar. This means it caused gamma hemolysison blood agar media.

Figure 3.1*:* *Klebsiella pneumoniae* in blood agar media after 24 hours of incubation, showing gamma hemolysis.

UTI Hi-Chrome Agar

After the mentioned incubation period in UTI Hi-Chrome agar media, colonies of different morphology and colors were observed. *Klebsiella pneumoniae* produced purple large mucoid colonies. Those colonies were recognized and streaked again to get single colonies.

Figure 3.2: UTI Hi-Chrome media after 24 hours incubation with bacterial inoculation.

MacConkey agar

In MacConkey agar media *Klebsiella pneumonia* produced pink, circular and smooth colonies with entire margin. The pink color was shown because of the production of acid from the fermentation of lactose.

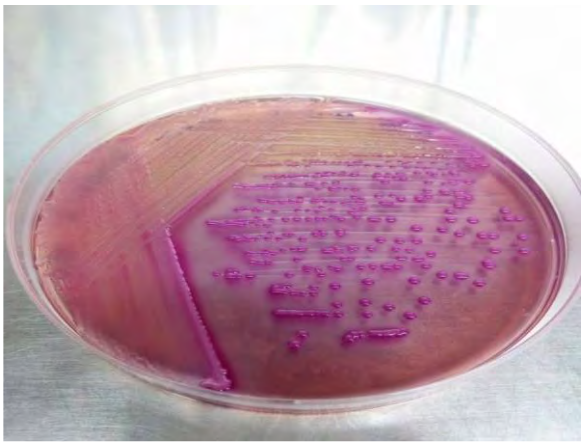


Figure 3.3: The growth of lactose fermenter *Klebsiella pneumoniae* on MacConkey agar.

3.1.2Gram staining:

After gram staining, the slides were observed under microscope and *Klebsiella pneumoniae* appeared pink, which means it is a gram negative bacteria. The shape was also observed as rod shaped.

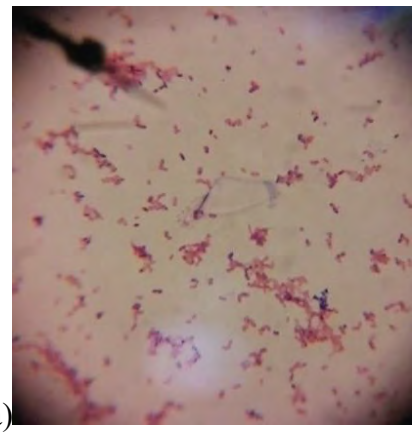
a) 

Figure 3.4: *Klebsiella pneumonia* under light microscope after gram staining.

3.1.3 Biochemical tests result

Isolates showed pattern of biochemical reactions typical for K. pneumoniae.

Results of Triple Sugar Iron Agar Test

After mentioned incubation period, *Klebsiella pneumonia* produces yellow butt and yellow slant on TSI media. Production of gas was observed as positive but no production of H_2S was seen. The acidic slant/acidic butt indicates that *Klebsiella pneumonia* is glucose, lactose and/or sucrose fermenter.

Figure 3.5: TSI media after incubation of 24 hours with *Klebsiella pneumonia*.

Citrate utilization test result

After 48 hours of incubation in Simmon's citrate agar, the color change of the media were observed. Abundant growth on the slant and a change from green to blue in the medium was indicated, which means *Klebsiella pneumonia* shows positive result.

Figure 3.6: Citrate utilization test result.

MIU test result

After 24 hours incubation period in MIU media, *Klebsiella* showed growth along the stabline. The color of the media changed to pink-red and no indole production was observed. The change of pink-red color indicated that *Klebsiella pneumonia* produces urease and growth along the stabline indicates that it is non motile.

MR & VP test result

In case of *Klebsiella pneumonia* a negative methyl red test was indicated by lack of development of red color ring after the addition of methyl red reagent, and a positive Voges-Proskauer result was indicated by the development of cherry- red color after the addition of Barritt's A and Barritt's B reagents.

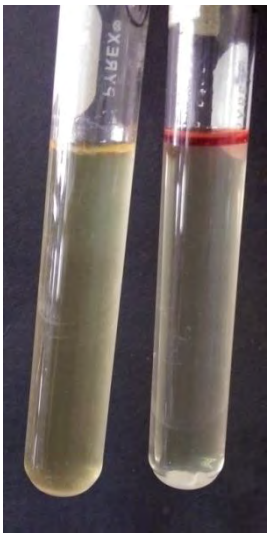
a)  b) 

Figure 3.7: a) MR posive sample produces red ring on the top and negative does not change color. b) VP positive test is indicated by cherry-red color change, while for negative result no change of color is seen.

Catalase test result

In case of *Klebsiella pneumonia* after the addition of H_2O_2 on the colony on the slide, the production of gas were observed, which indicated a positive catalase result.

Figure 3.8: Positive catalase test.

Oxidase test result

After the addition of the substrate, tetramethyl-p-phenylenediamine dihydrochloride, and no color change of color was indicated, thus negative result was observed for *Klebsiella pneumonia.*

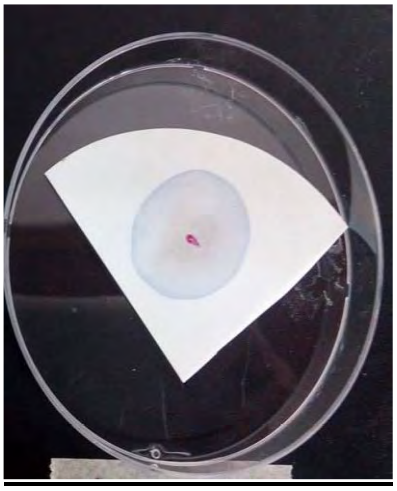


Figure 3.9: Positive oxidase test result.

All the morphological characterization, gram staining and biochemical tests results of *Klebsiella pneumonia* are summarized on the Table: 3.1.

Characteristic	***Klebsiella pneumoniae***
Gram Staining	-
Shape	Rod
Motility	-
Citrate	+
Catalase	+
Gas	+
H2S	-
Hemolysis	Gamma Hemolysis
Urease	+
Methyl Red	-
Voges Proskauer	+
Indole	-
Nitrate reduction	+
Oxidase	-
Fermentation of Glucose	+
Fermentation of Lactose	+

Table 3.1: Phenotypic characteristics and biochemical tests results of the isolated bacteria. '+' indicated positive result, '-' indicates negative result.

3.2 Identification of bacteria using Microrao software:

After all tests and processes mentioned and describes, bacteria present in the clinical samples were identified. The results are expressed in percentage probability, shown in figure marked by red box. The final results were confirmed with the help of Microrao software, with likelihood probability of more than 90%. All the isolates were identified and among them Klebsiella *pneumoniae* were selected and stored for further studies.

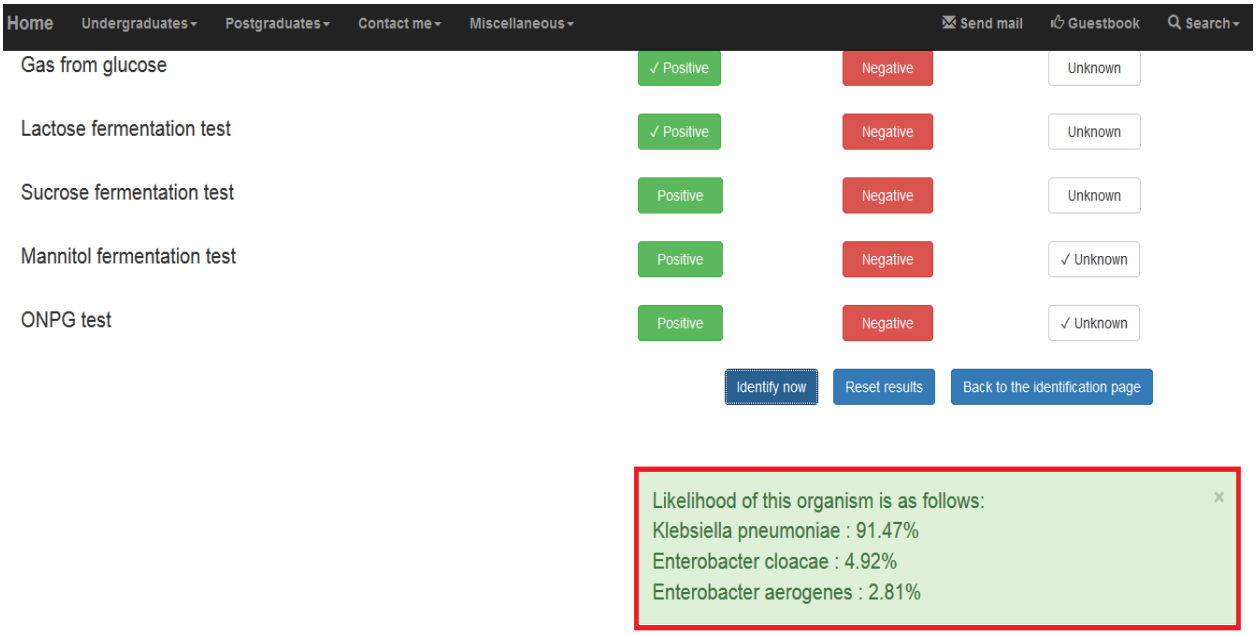


Figure3.10: Identification of bacteria using Microrao software.

3.3 Molecular analysis of mceA gene

The PCR products were run on 1 % agarose gel for gel electrophoresis for 50 minutes at 80V. The gene specific primers used in the PCR helped to detect the samples which are positive with microcinE492 producing gene. After the gel electrophoresis, the gel was seen under UV light to visualize the bands. Out of 50 *Klebsiella pneumoniae* samples isolated from the clinical samples, 7 contained mceA gene. The band were shown at position 277bp. Sample no. 6 and 12 were from burn infection and the rest (25, 31, 35, 45 and 48) were from respiratory tract infection.

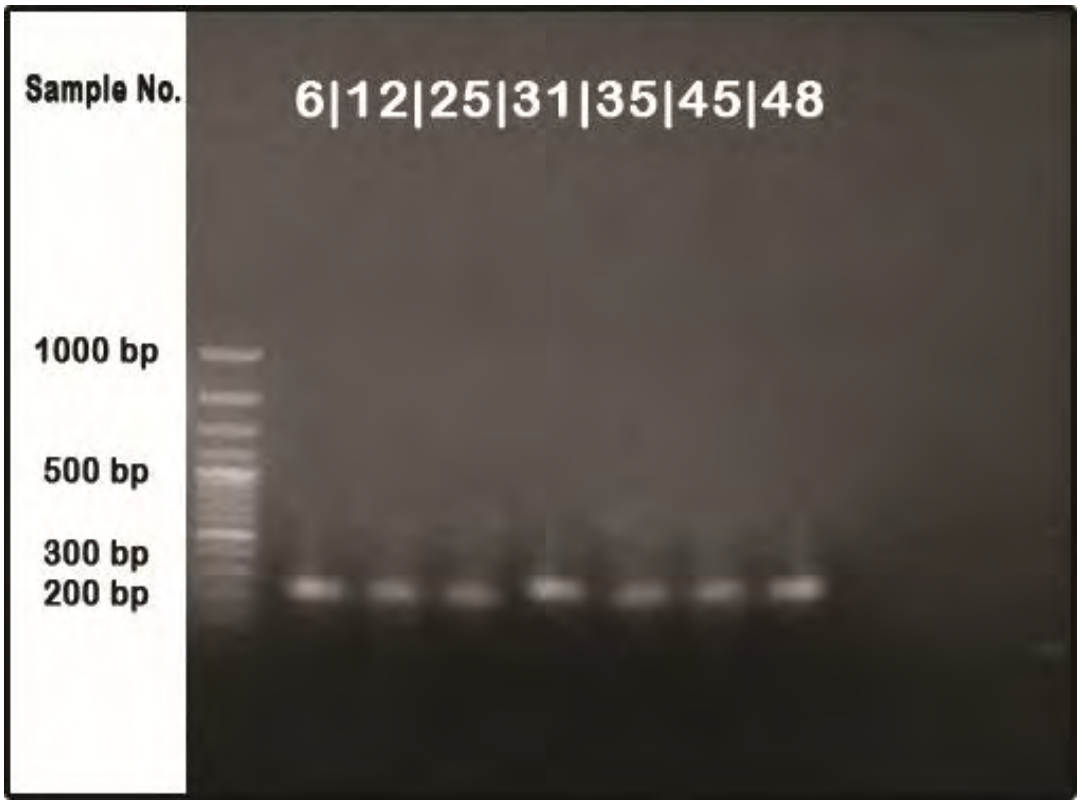


Figure 3.11: Gel electrophoresis of the PCR product on 1% agarose gel.

Out of 50 samples, 7 contains microcin E492 gene, which means 14% of the total sample contains the gene. 2 samples out of 15 (13.3%) were from burn wound infections. 5 samples out of 35 (14.3%) are from respiratory tracts diseases.

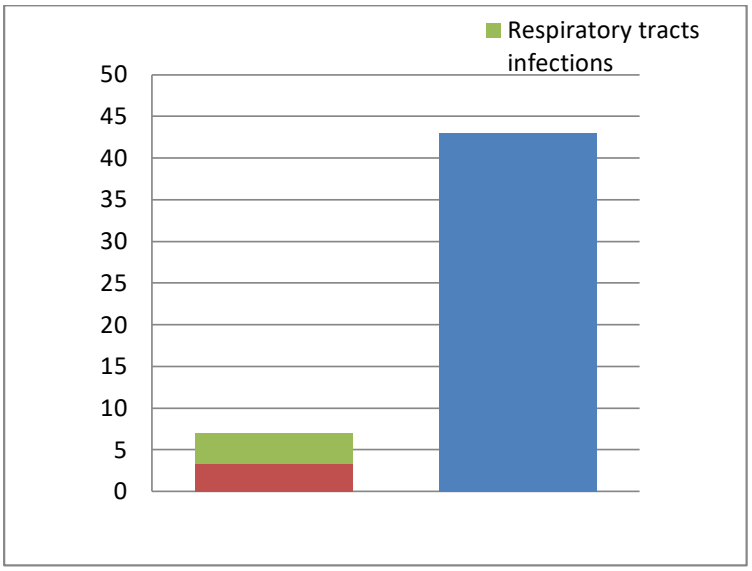


Figure 3.12: Microcin E492 gene from isolated *Klebsiella pneumoniae*

Chapter 4

Discussion and Conclusion

4.1 Discussion

One of the major problems plaguing the world today is that there is an increasing rate of development of antibiotics-resistant bacterial strains. Secondly, Drug Related Problems (DRPs) in cancer is also a severe issue in the world. Thus, these two emerging concerns require related advanced researches for better therapeutic outcome [8]. Considering the properties of microcinE492, it can be said that, microcinE492 has great potential to be used as an alternative drug. However, in Bangladesh no studies have been conducted concerning microcin E492, thus this thesis can be useful to establish a primary data to do further studies relating microcinE492. The objective of this thesis was to establish a data on the frequency of *Klebsiella pneumoniae* containing microcinE492 gene, isolated from hospitalized patients with burn wound and respiratory tract infection.

In this study, a total of 65 clinical samples were collected from patients with burn wound and respiratory tracts infections. *Klebsiella pneumoniae* accounts for a significant proportion of hospital-acquired infection [13]. Nosocomial infections (NI) are very much common in patients with burn wound and respiratory tract infections [13]. Due to this reason, sample were collected from burn infection and respiratory tract infection. The burn samples were collected using cotton swab and for respiratory tract infection sputum samples were collected. From the collected samples, bacteria were isolated and identified.

Basic biochemical tests and phenotypic characterizations were carried out for the identification process of *Klebsiella pneumoniae*. Confirmation of bacterial isolates was done on selective media and gram staining was also done. The test results was interpreted and desired results confirmed the identification of *Klebsiella pneumoniae* (Table 3.1). Moreover, an online software, Microrao was also used for the identification of *Klebsiella pneumoniae.* The software express the result in

percentage probability. Depending on the tests carried out, the software showed 91.47% of chance of the isolated organism to be *Klebsiella pneumonia* (Figure 3.10). All the tests showed satisfactory results and the desired bacteria, *Klebsiella pneumoniae,* was obtained. After the identification and characterization of the bacteria, *Klebsiella pneumoniae* strains were stored for additional studies.

Afterward, to determine the proportion of *Klebsiella pneumoniae* which can synthesize microcinE492 bacteriocin, molecular analysis of the genetic material was done. MceA gene are present in the chromosomal DNA of *Klebsiella pneumoniae* [10]. Hence total DNA was extracted using boiling method protocol. The boiling method protocol is proven to be the best DNA extraction protocol for PCR [18]. Then, gel electrophoresis of the extracted DNA ensued successful DNA extraction and DNA quality was also checked. Later on, using the extracted DNA samples PCR was carried out.

For the molecular analysis of the presence of the desired gene PCR was carried out. For PCR, mceA gene specific primers were used, this gene is responsible for the production of microcinE492 peptide [6]. The specific primers in the PCR mixture ensured only the amplification of the DNA sample containing mceA gene. However, amplification did not occur for the samples with no mceA gene. For mceA gene, the gel electrophoresis analysis result is supposed to give band at the position of 277 bp [20]. In this thesis, the gel electrophoresis result of the PCR products also showed band at the position of 277 bp, confirming the presence of mceA gene.

However, if the results of this thesis is discussed in briefly, it can be divided into 2 sections. Firstly, a basic finding showing that 82% burn wounds specimens and 78% of the respiratory tract specimens contained the *Klebsiella pneumoniae*. Finally, microcin E492 gene from *Klebsiella pneumoniae* was screened, the result of the showed that out of 50 samples, 14% of the total sample contained the gene. 2 samples out of 15 (13.3%) from burn wound infections and 5 samples out

of 35 (14.3%) from respiratory tract infection are found to be containing mceA gene (Figure: 3.11, 3.12). Even though, the sample number for this study was large, yet, if the study can be carried out with more number of samples, the validity of the actual frequency of *Klebsiella pneumoniae* with mceA can be validated.

Through this thesis work, the prevalence of mceA gene in *Klebsiella pneumoniae* has been studied for the first time from different hospitals of Dhaka city. Regardless, the results of this research can support high prevalence of *Klebsiella pneumoniae* in hospital infections. In explanation, it can be justified that, K. *pneumoniae* actually can use microcin E492 as a weapon against other microbial flora and can sustain to be a dominant microbial flora in human body. As mentioned before, Micrrocin E492 is a low-molecular-weight channel-forming bacteriocin produced by *Klebsiella pneumoniae*. Many studies have been conducted concerning this protein and it has been proven to be a potential candidate for pharmaceutical use.

Nevertheless, as *Klebsiella pneumoniae* is a common bacteria found in hospital infection that means it sustains to be a dominant microbial flora by competing with other highly resistant bacteria. In these bacteria, resistance emerged from antibiotic resistant genes, which they develop through natural phenomenon [21]. Some bacteria are naturally resistant to certain types of antibiotics. However, bacteria may also become resistant in two ways, by a genetic mutation or by acquiring resistance from another bacterium [22]. Selection pressure, due to the environment in which they survive and other induced factors, like excessive use of unnecessary antibiotics is, in fact, responsible for development of resentence to antibiotics. As *Klebsiella pneumoniae* itself is exposed to such environment, it is very likely that *Klebsiella pneumoniae* also goes through necessary genetic mutation with times and produces effective bacteriocin with different and effective activities against those newly developed resistant bacteria.

In order to fully understand the MccE492 system it is essential to study both the system of production and the target requirements for the antibacterial or cytotoxic activities. Finally it can be said that information and data of this work can be used to do additional studies to understand the specific protein and its mechanisms in detail. Moreover, it will also assist further researches for efficient drug designing.

4.2. Conclusion

In conclusion, it can be said that the objectives set into view before starting this research project have been successful. The objective of this analytical study was to determine the presence of *Klebsiella pneumoniae* with mceA gene, encoding for microcin E492 in clinical specimens isolated from patients with burn infection and respiratory tracts diseases. Because of the high importance of microcin E492 in medicine, its prevalence in bacteria was focused. The results clearly showed that clinical specimens contained *Klebsiella pneumonia* with microcinE492 producing gene. This study can be used as a guideline to do more researches and understand the mechanism of microcinE492 in details. For further research, SDS-PAGE can be done, to revel and understand its protein expression better. Furthermore, with the help of bioinformatics, homology structure of this protein can be studied. Henceforth, the structural studies can lead this research to a new direction with the chance to improve this proteins activities and stability. To conclude, it is suggested that understanding the bacteriocins processes involved in the synthesis, immunity, and regulation of antimicrobial peptides which later should play a role with emphasis on practical applications of microcinE492 in pharmaceutical sectors.

Chapter 5

Bibliography

References

[1] Hassan, M., Kjos, M., Nes, I. F., Diep, D. B., & Lotfipour, F. (2014). Antimicrobial Peptides from Prokaryotes. *Novel Antimicrobial Agents and Strategies,*71-90. doi:10.1002/9783527676132.ch5

[2] Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2011). Bacteriocin Production: A Probiotic Trait? *Applied and Environmental Microbiology,78*(1), 1-6. doi:10.1128/aem.05576-11

[3] Bacteriocin - Definition, Classification, Structure, Production and Uses. (2011, June 29). Retrieved from https://www.chemistrylearner.com/bacteriocin.html

[4] Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2011). Bacteriocin Production: A Probiotic Trait? *American Society for Microbiology*. doi:10.1107/s0108768104030617/bs50122f1sup9.hkl

[5] Ghanbari, M., & Jami, M. (2013). Lactic Acid Bacteria and Their Bacteriocins: A Promising Approach to Seafood Biopreservation. *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*. doi:10.5772/50705

[6] Arranz, R., Mercado, G., Martín-Benito, J., Giraldo, R., Monasterio, O., Lagos, R., & Valpuesta, J. M. (2012). Structural characterization of microcin E492 amyloid formation: Identification of the precursors. *Journal of Structural Biology,178*(1), 54-60. doi:10.1016/j.jsb.2012.02.015

[7] Destoumieux-Garzón, D., Thomas, X., Santamaria, M., Goulard, C., Barthélémy, M., Boscher, B., . . . Rebuffat, S. (2003). Microcin E492 antibacterial activity: Evidence for a TonB-dependent inner membrane permeabilization on Escherichia coli. *Molecular Microbiology,49*(4), 1031-1041. doi:10.1046/j.1365-2958.2003.03610.x

[8] Lagos, R., Tello, M., Mercado, G., Garcia, V., & Monasterio, O. (2009). Antibacterial and Antitumorigenic Properties of Microcin E492, a Pore- Forming Bacteriocin. *Current Pharmaceutical Biotechnology,10*(1), 74-85. doi:10.2174/138920109787048643

[9] Corsini, G., Baeza, M., Monasterio, O., & Lagos, R. (2002). The expression of genes involved in microcin maturation regulates the production of active microcin E492. *Biochimie,84*(5-6), 539-544. doi:10.1016/s0300-9084(02)01415-3

[10] Chakrabarty, A. M. (2016). Proteins and peptides from pathogenic bacteria with anti-viral, anti-parasitic and anti-cancer activity. *Journal of Infectious Diseases & Therapy,04*(07). doi:10.4172/2332-0877.c1.016

[11] Daeschel, M. A. (1993). Applications and Interactions of Bacteriocins from Lactic Acid Bacteria in Foods and Beverages. *Bacteriocins of Lactic Acid Bacteria,*63-91. doi:10.1016/b978-0-12-355510-6.50012-9

[12] Microcin. (n.d.). Retrieved October 22, 2018, from https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/microcin

[13] What is *Klebsiella Pneumoniae* Infection?. Retrieved from https://www.webmd.com/a-to-z-guides/klebsiella-pneumoniae-infection#1

[14] Characterization of *Klebsiella Pneumoniae* | Biofield Treatment. Retrieved from https://www.trivedieffect.com/the-science/publications/microbiology-publications/antibiogram-typing-and-biochemical-characterization-of-*klebsiella-pneumoniae*-after-biofield-treatment/

[15] Kang HY, Jeong YS, Oh JY, Tae SH, Choi CH, et al. (2005) Characterization of antimicrobial resistance and class 1 integrons found in Escherichia coli isolates from humans and animals in Korea. J Antimicrob Chemother 55: 639-644.\

[16] Lawlor, M., Hsu, J., Rick, P., Miller, V. "Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model". *Molecular Microbiology*. 2005. Volume 58, Issue 4. p. 1054–1073.

[17] Amako, K., Meno, Y., and Takade, A. "Fine Structures of the Capsules of *Klebsiella pneumoniae* and *Escherichia coli* K1". *Journal of Bacteriology*. 1988. Volume 170, No. 10. p. 4960-4962

[18] Gram Staining : Principle, Procedure, Interpretation and Animation. (2018, August 06). Retrieved from https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/

[19] Importance of Biochemical Tests of Bacteria. (2014, February 19). Retrieved from http://www.yourarticlelibrary.com/experiments/importance-of-biochemical-tests-of-bacteria/26624

[20] Evaluation of E492 Microcin Gene Presence in *Klebsiella* . (n.d.). Retrieved October 22, 2018, from https://cdn.neoscriber.org/cdn/serve/4c/85/4c8538786a3c2b31473a3685128423b668c431e3/zjrms-inpress-inpress-5038.pdf

[21] Xia, J., Gao, J., & Tang, W. (2016, February). Nosocomial infection and its molecular mechanisms of antibiotic resistance. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/26877142

[22] General Background: About Antibiotic Resistance. (n.d.). Retrieved from http://emerald.tufts.edu/med/apua/about_issue/about_antibioticres.shtml

[23] Wilkens, M., Villanueva, J. E., Cofré, J., Chnaiderman, J., & Lagos, R. (1997, August). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC179325/

[24] Hetz, C., Bono, M. R., Barros, L. F., & Lagos, R. (2002, March 05). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC122410/

[25] Bacteriocin. (n.d.). Retrieved October 22, 2018, from https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/bacteriocin

[26] Thanner, H. (2002). GaPO4 high temperature crystal microbalance with zero temperature coefficients. *Vacuum, 67*(3-4), 687-691. doi:10.1016/s0042-207x(02)00241-5

[27] Trivedi, M. K., Branton, A., Trivedi, D., Gangwar, M., & Jana, S. (2015). Antimicrobial Susceptibility, Biochemical Characterization and Molecular Typing of Biofield Treated Klebsiella pneumoniae. *Journal of Health & Medical Informatics, 06*(05). doi:10.4172/2157-7420.1000206

[28] Karpiński, T., & Adamczak, A. (2018). Anticancer Activity of Bacterial Proteins and Peptides. Pharmaceutics*, 10*(2), 54. doi:10.3390/pharmaceutics10020054

[29] Blood Agar: Composition, Preparation, Uses and Types of Hemolysis -. (2017, May 10). Retrieved from https://microbeonline.com/blood-agar-composition-preparation-uses-and-types-of-hemolysis/

Appendix–I

Media

1.MacConkey Agar.

Ingredients	Amount (g/L)
Peptone	**17.0**
Proteose Peptone	**3.0**
Lactose	**10.0**
Bile Salts No. 3	**1.5**
Sodium Chloride	**5.0**
Agar	**13.5**
Neutral Red	**0.03**
Crystal Violet	**0.001**

3. Motility Indole Urease Agar:

Ingredients	Amount (g/L)	Prepare up to 900ml for autoclave
NaCl (Sigma)	**5**	
Agar (Himedia, India)	**4**	
KH2PO4 (Fisher Chemical, USA)	**2**	
Peptone (Himedia,	**30**	

India)		
Phenol Red (0.25%) (Sigma, India)	**2 ml/L**	
Urea (Amresco, USA)	**20**	**Prepare up to 100ml for filter sterilization**

4. Simmon's Citrate Agar

Ingredients	**Amount (g/L)**
Magnesium sulphate	**0.2**
Ammonium dihydrogen phosphate	**0.2**
Sodium ammonium phosphate	**0.8**
Sodium citrate, tribasic	**2.0**
Sodium chloride	**5.0**
Bromothymol blue	**0.08**
Agar	**15.0**

5. Triple Sugar Iron Agar (Difco™)

Ingredients	Amount (g/L)
Beef Extract	3.0
Yeast Extract	3.0
Pancreatic Digest of Casein	15.0
Proteose Peptone No.3	5.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous Sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Agar	12.0
Phenol Red	0.024

Appendix-II

Reagents

TBE buffer (1x)	5.4 g Tris-HCl 2.75 g Boric acid 2ml 0.5M EDTA Adjusting volume with distilled water pH: 8.0
TE buffer	10 mMTris-Cl (pH 8.0) 1 mM EDTA (pH 8.0)
LB medium	LB medium
Lysis buffer	(10 ml) 9.34 ml TE buffer 600 µl of 10% SDS 60 µl of proteinase K (20 mg ml-1)

Appendix-III

Instruments

Instrument	Company
Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100 µL)	Eppendorf, Germany
Micropipette (100-1000µL)	Eppendorf, Germany
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Weighing balance	ADAM Weighing balance EQUIPMENT™, United Kingdom

Vortex Mixture	VWR International
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Water Bath WiseBath®	Wisd Laboratory Instruments DAIHAN Scientific Co.,