ISOLATION AND CHARACTERIZATION OF PLASMIDS FROM GRAM NEGATIVE CLINICAL ISOLATES

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Philosophy

In

BIOCHEMISTRY/MOLECULAR BIOLOGY

By

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CERTIFICATE

This thesis, submitted by Mr. Muhammad Aamir Khan to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Dated: December 15, 2007.

Dedicated

to

My Dear

"MOTHER"

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LIST OF ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic acid
ECE	Extra Chromosomal Elements
EDTA	Ethylenediamine tetra-acetic acid
g	Gram
Kb	Kilo base pairs
L	Liter
mA	Milliampere
mg	Milligram
ml	Milliliter
mm	Millimeter
pH	− log of Hydrogen ion concentration
PIMS	Pakistan Institute of Medical Sciences
PO_4	Phosphate
psi	Pounds per square inch
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
TBE	Tris borate EDTA
UV	Ultraviolet
μl	Microlitre
μg	Microgram

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ABSTRACT

The purpose of this study was to construct a restriction map of clinical isolates of gram negative bacteria so that it can be further studied for the development of new antibiotic drugs.

A plasmid is an independent, circular, self-replicating DNA molecule that carries only a few genes. The number of plasmids in a cell generally remains constant from generation to generation. Plasmids are autonomous molecules and exist in cells as extra chromosomal genome. Many prokaryotes contain plasmids in addition to their chromosomes. Normally, plasmids are nonessential to their hosts, conferring only an energy burden that can slow cell growth. Plasmids often contain genes or gene cassettes that confer a selective advantage to the bacterium harboring them, e.g., the ability to make the bacterium antibiotic resistant. Plasmids serve as important tools in genetics and biochemistry labs, where they are commonly used to multiply.

Restriction map of four bacterial strains was constructed by using two restriction endonucleases, EcoR1 and HindIII. As all of these bacterial strains carry multiple numbers of plasmids and on gel they gave different bands. However, each of these plasmids has different restriction site for each of the plasmid it is clearly shown by the banding pattern on gel. This restriction map can help in the comparative analysis of the restriction mapping of the locally found strains and mutation rates with those of international strains.

INTRODUCTION

phenotypic features such as shape, metabolic growth requirements and atmospheric growth requirements.

Pathogenesis

A combination of virulence factors come into play beginning with adherence mechanisms such as pili, colonization, penetration, spread and damage mechanisms can be very complex. Only rarely are specific disease-causing exotoxins produced by Gram negative bacteria (e.g. the verotoxin of some *E. coli* and *Shigella* strains, and the cholera toxin of *Vibrio cholerae*). In major infections the cell wall LPS (endotoxin) is released. But the specific features of many Gram negative infections are caused by a combination of species-specific virulence factors and host response. *Salmonella* flagellar protein can translocate across the intestinal wall. *Legionella* produces an acid phosphatase that assists cell adhesion and subsequent incorporation into macrophages. The enzyme DNA adenyl methylesterase (DAM) may act as an upstream regulator for a variety of bacterial housekeeping functions that result in human tissue damage by Salmonellas. Polysaccharide capsules are important in *N. meningitidis, H. influenzae* and other bacteria as a way to resist phagocytosis.

Escherichia coli

Escherichia coli is a bacterium that is a common but certainly not the most abundant inhabitant of the human intestine. It also lives in the intestine of many other animals, wild as well as domestic. Classification of *E. coli* is described in Table 1 on page 4. Cells of *E. coli* are shown in Figure 1 and culture on agar medium is shown in Figure 2 on page 5.

Normally, *E. coli* does not cause disease although some strains frequently cause diarrhea in travelers, and it is the most common cause of urinary tract infections. One strain designated as O157:H7, is particularly virulent and has been responsible for several dangerous outbreaks in people eating contaminated food (usually undercooked hamburger).

Drinking water is tested for the presence of *E. coli* and related bacteria not because these bacteria are dangerous but because they are an indication of contamination by sewage, and sewage may contain organisms (e.g., *Salmonella*, hepatitis A virus) that are dangerous.

The complete sequence of the pathogenic strain O157:H7 was reported in the 25 January 2001 issue of Nature. It contains 5416 genes in 5.44×10^6 base pairs of DNA. Remarkably, these include 1,387 genes that are not present in its harmless laboratory relative *E. coli* K-12 (and K-12 has 528 genes that are not found in O157:H7). So here are two strains of the same species that differ in some 25% of their genes. Compare this with the difference between the genomes of humans and chimpanzees which probably is no more than 1%).

Escherichia coli are the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit (Drasar and Hill, 1974). *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Moreover, even the most robust members of our species may be susceptible to infection by one of several highly adapted *E. coli* clones which together have evolved the ability to cause a broad spectrum of human diseases. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease.

E. coli is one of the most thoroughly studied of all living things. It is a favorite organism for genetic engineering as cultures of it can be made to produce unlimited quantities of the product of an introduced gene. Several important drugs (insulin, for example) are now manufactured in *E. coli*.

Classification of *Escherichia coli*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Escherichia*
Species:	*E. coli*

Table 1

Classification of Genus *Klebsiella*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Klebsiella*

Table 2

Escherichia coli

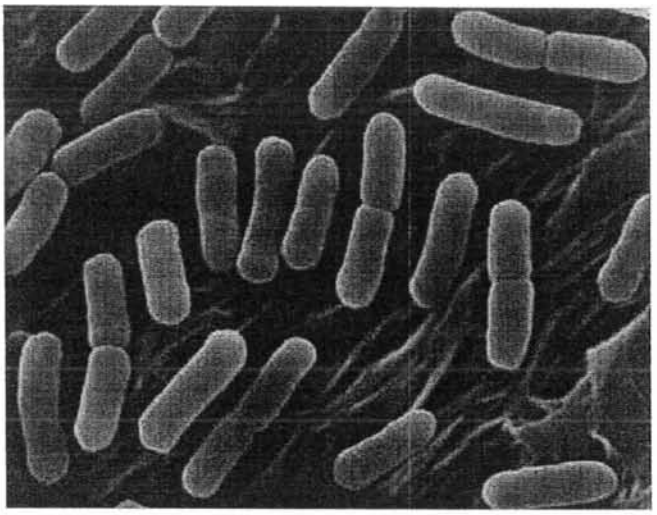

Figure 1. Cells of *Escherichia coli*.

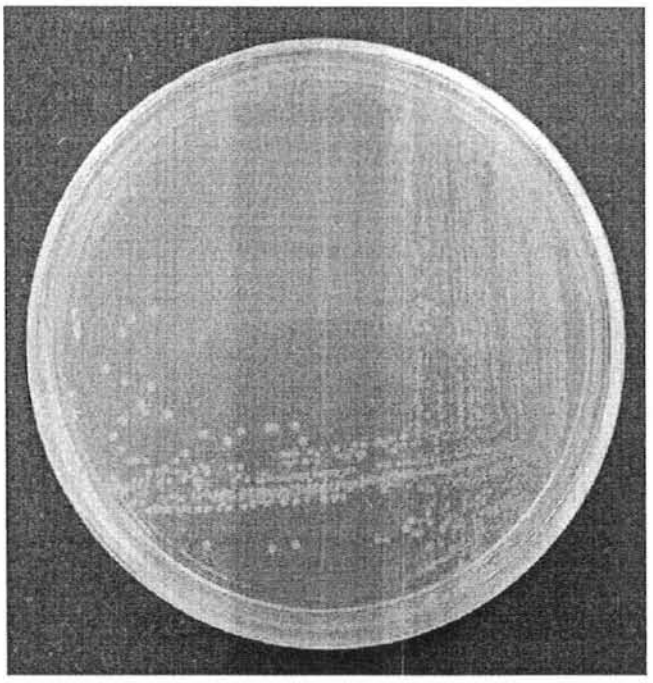

Figure 2. *Escherichia coli* on LB agar medium

Klebsiella

A genus of gram-negative, facultative anaerobic, rod-shaped bacteria whose organisms arrange singly, in pairs, or short chains. This genus is commonly found in the intestinal tract and is an opportunistic pathogen that can give rise to bacteraemia, pneumonia, urinary tract and several other types of human infections. Classification of *Klebsiella* genus is described in Table 2 on page 4. Cells of *Klebsiella* are shown in Figure 3 and culture on blood agar medium is shown in Figure 4 on page 7.

Klebsiella is among the five gram-negative pathogens most commonly encountered in hospital-acquired infections (Izard *et al.*, 1981), and *Klebsiella pneumoniae* is the most frequently occurring species, accounting for 75 to 86% of *Klebsiella* species reported (De La Torre, *et al.*, 1985; Hansen, D. S., Gottschau, A., and H. J. Kolmos. 1998; Watanakunakorn, C., 1991). Much more rarely encountered are *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis*, which have been retained as separate species because of their association with specific diseases (Podschun and Ullmann, 1998). Taxonomically, these two species are regarded as subspecies of *K. pneumoniae* based on DNA-DNA hybridization data (Ørskov, 1984). *Klebsiella oxytoca* is the other well-established species, accounting for 13 to 25% of isolates (Torre *et al.*, 1985; Hansen *et al.*, 1998; Watanakunakorn, 1991).

Klebsiella species are opportunistic bacteria, commonly found in the environment and in the intestinal flora of humans; they frequently cause nosocomial infections associated with high morbidity and sometimes mortality in hospitalized patients (Podschun and Ullman, 1998). Klebsiellae have also been commonly cultured from assorted internal tissues in marine mammals (Vedros *et al.*, 1982; Baker and McCann, 1989) and in a large range of wild birds, reptiles and terrestrial mammals (Aguirre *et al.*, 1994; Montgomery *et al.*, 2002; Steele *et al.*, 2005) but they have never been associated with mass mortality in a wild animal population.

Members of the family Enterobacteriacae, including *Klebsiella* species, can acquire plasmid-mediated extended-spectrum beta-lactamases (ESBLs) that provide resistance

Klebsiella

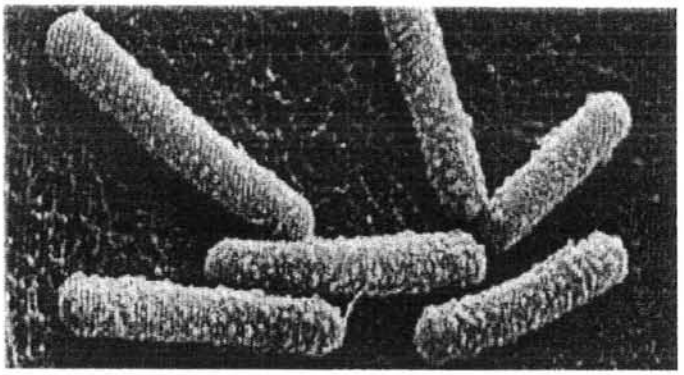

Figure 3. Cells of *Klebsiella*

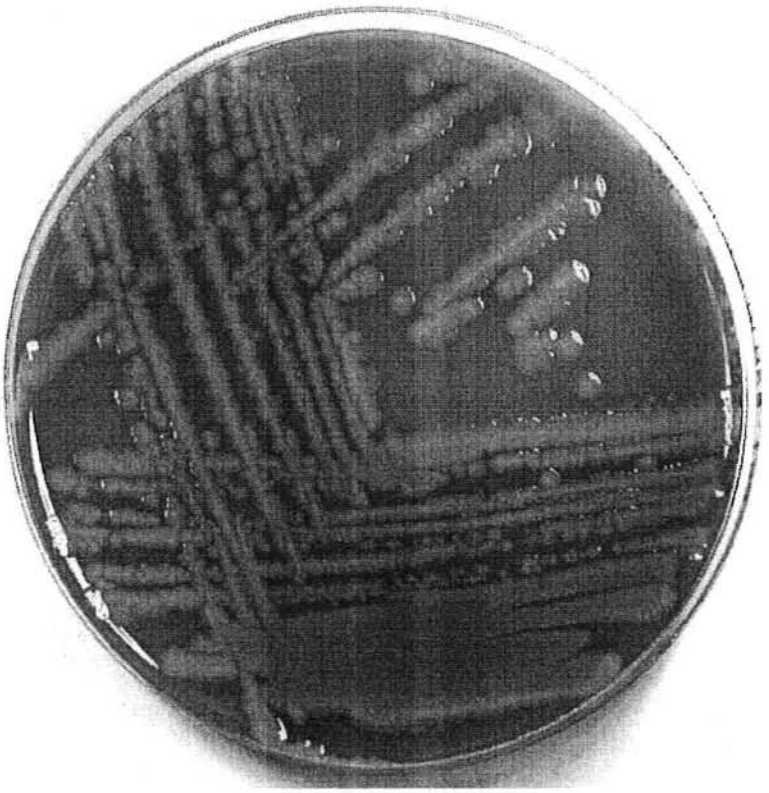

Figure 4. *Klebsiella* on Blood agar medium

not only to penicillins but also to broad-spectrum cephalosporins, monobactams, and cephamycins (Hindler *et al.,* 1994). The acquisition of such extended resistance is characteristic of Enterobacteriacae in the human environment and has become a key feature in the investigation of epidemic situations in hospitals (Gouby *et al.*, 1994; Hindler *et al*, 1994). Pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments is a highly discriminatory whole-genome DNA-typing method for bacterial sub-typing and has frequently been used to characterize ESBLs-producing *K. pneumoniae* strains involved in nosocomial infections and outbreaks (Gouby *et al.*, 1994).

Proteus

Bacteria of the genus *Proteus* are widely distributed in nature and are important human opportunistic pathogens that cause wound and urinary tract infections (Kotelko 1986; RoÂzalski *et al.*, 1997). These can result in serious complications, including formation of kidney and bladder stones, catheter obstruction, acute pyelonephritis, and bacteraemia (Mobley and Warren, 1987). Classification of *Proteus* genus is described in Table 3 on page 9. Cells of *Proteus* are shown in Figure 5 and culture on agar medium is shown in Figure 6 on page 10.

Much has been written about the taxonomy of *Proteus* since the original publication by Hauser in 1885 who established the genus (Hauser, 1885). Currently, the genus *Proteus* consists of species (*P. mirabilis, P. penneri, P. vulgaris, P. myxofaciens and P. hauseri*) and three unnamed genomospecies 4, 5 and 6 (O'Hara *et al.*, 2000). *Proteus* rods are widespread in the environment and make up part of the normal flora of the human gastrointestinal tract. *Proteus* ranks third (after *Escherichia* and *Klebsiella*) as the cause of uncomplicated cystitis, pyelonephritis and prostatitis, particularly, in hospital-acquired cases (Stamm, 1999). *P. mirabilis* accounts for approximately 3% of nosocomial infections in the United States where, together with *P. penneri*, it may play a role in some diarrhoeal diseases (MuÈller, 1986). Recently, it has been suggested that *P. mirabilis* may play an ethiopathogenic role in rheumatoid arthritis (Wilson *et* al., 1997)

Classification of Genus *Proteus*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Proteus*

Table 3

Classification of Genus *Pseudomonas*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Pseudomonadales
Family:	Pseudomonadaceae
Genus:	*Pseudomonas*

Table 4

Proteus

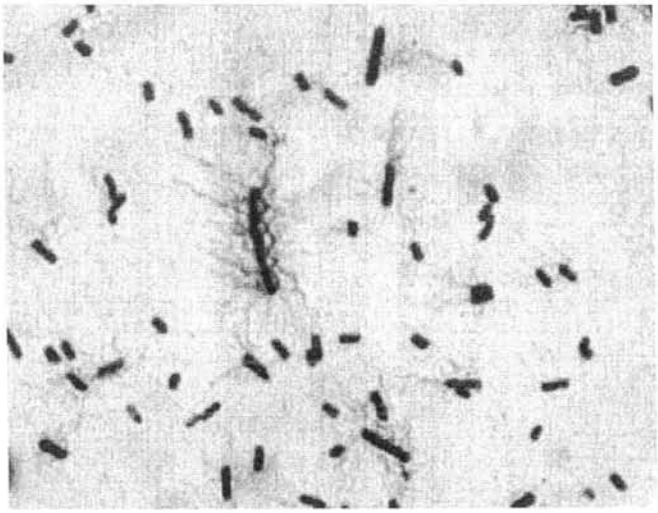

Figure 5. Cells of *Proteus*

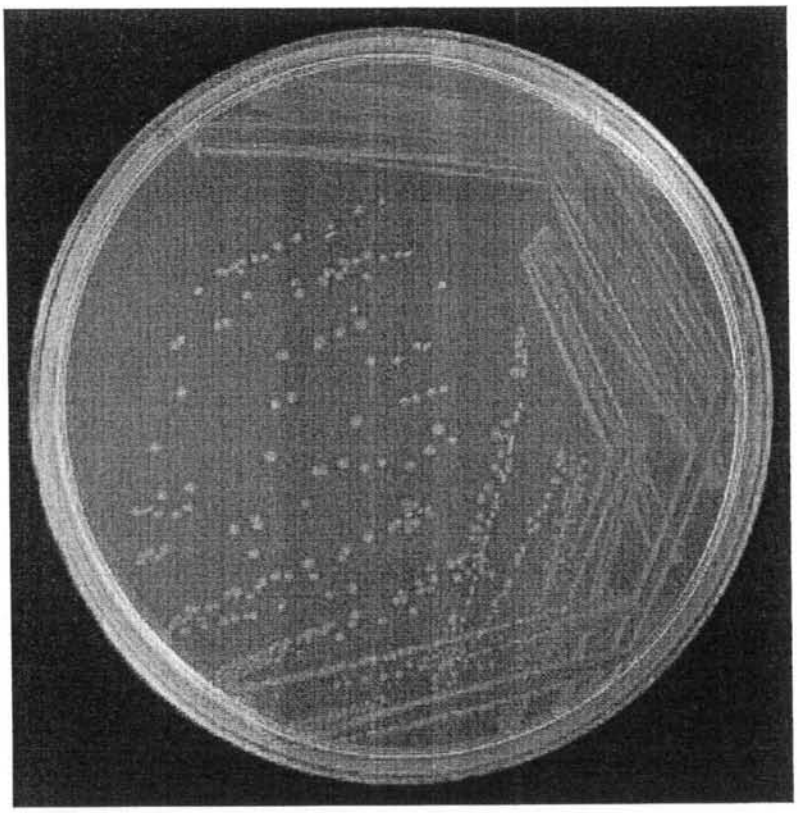

Figure 6. *Proteus* on LB agar medium

Pseudomonas

Pseudomonas is a gram-negative rod that belongs to the family Pseudomonadaceae. More than half of all clinical isolates produce the blue-green pigment pyocyanin. Often, *Pseudomonas* has a characteristic sweet odor. These pathogens are widespread in nature, inhabiting soil, water, plants, and animals (including humans). Classification of *Pseudomonas* genus is shown in Table 4 on page 9. Cells of *Pseudomonas* are shown in Figure 7 and culture on agar medium is shown in Figure 8 on page 12. The genus *Pseudomonas* contains more than 140 species, most of which are saprophytic. More than 25 species are associated with humans.

Since many years it is well-known that the genus *Pseudomonas* is phylogenetically extremely diverse and in fact comprises many different genera. Already in 1973, Palleroni *et al.*, (1973) described five major subdivisions within this genus. The number of species studied was considerably extended by De Vos and De Ley a few years later but basically they found the same five major groupings (De Vos and De Ley, 1983).

Most Pseudomonads known to cause disease in humans are associated with opportunistic infections. These include *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. cepacia*, *P. stutzeri*, *P. maltophilia*, and *P. putrefaciens*. Only two species, *P. mallei* and *P. pseudomallei*, produce specific human diseases: glanders and melioidosis. *Pseudomonas aeruginosa* and *P. maltophilia* account for approximately 80 percent of pseudomonads recovered from clinical specimens. Because of the frequency with which it is involved in human disease, *P. aeruginosa* has received the most attention. It is a ubiquitous free-living bacterium and is found in most moist environments. Although it seldom causes disease in healthy individuals, it is a major threat to hospitalized patients, particularly those with serious underlying diseases such as cancer and burns. The high mortality associated with these infections is due to a combination of weakened host defenses, bacterial resistance to antibiotics, and the production of extracellular bacterial enzymes and toxins.

The pathogenesis of Pseudomonal infections is multifactorial and complex. *Pseudomonas* is both invasive and toxigenic. The 3 stages, according to Pollack (2000), are (1) bacterial

Pseudomonas

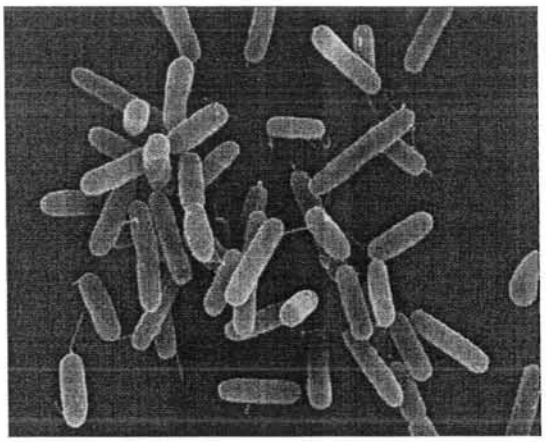

Figure 7. Cells of *Pseudomonas*

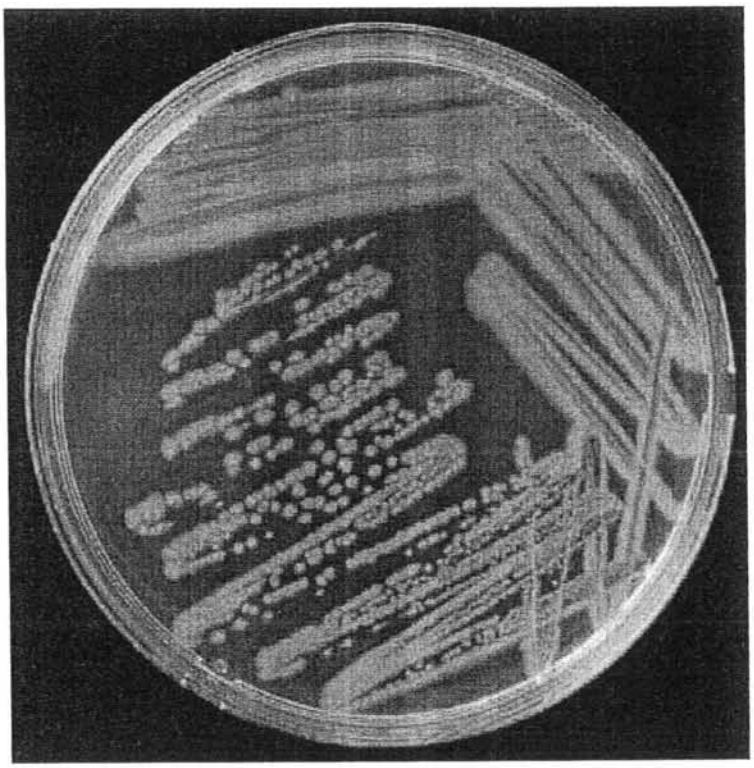

Figure 8. *Pseudomonas* on LB agar

attachment and colonization, (2) local infection, and (3) bloodstream dissemination and systemic disease. The importance of colonization and adherence is most evident when studied in the context of respiratory tract infection in patients with cystic fibrosis and in those that complicate mechanical ventilation. Production of extracellular proteases adds to the organism's virulence by assisting in bacterial adherence and invasion.

Pseudomonas aeruginosa has become an important cause of infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week. It is a frequent cause of nosocomial infections such as pneumonia, urinary tract infections (UTIs), and bacteraemia. Pseudomonal infections are complicated and can be life threatening.

Pseudomonas aeruginosa causes various diseases. Localized infection following surgery or burns commonly results in a generalized and frequently fatal bacteraemia. Urinary tract infections following introduction of *P. aeruginosa* on catheters or in irrigating solutions are not uncommon. Furthermore, most cystic fibrosis patients are chronically colonized with *P. aeruginosa*. Interestingly, cystic fibrosis patients rarely have *P. aeruginosa* bacteraemia, probably because of high levels of circulating *P. aeruginosa* antibodies. However, most cystic fibrosis patients ultimately die of localized *P. aeruginosa* infections. Necrotizing *P. aeruginosa* pneumonia may occur in other patients following the use of contaminated respirators. *Pseudomonas aeruginosa* can cause severe corneal infections following eye surgery or injury. It is found in pure culture, especially in children with middle ear infections. It occasionally causes meningitis following lumbar puncture and endocarditis following cardiac surgery. It has been associated with some diarrheal disease episodes. Since the first reported case of *P. aeruginosa* infection in 1890, the organism has been increasingly associated with bacteraemia and currently accounts for 15 percent of cases of Gram-negative bacteraemia. The overall mortality associated with *P. aeruginosa* bacteraemia is about 50 percent. Some infections (e.g., eye and ear infections) remain localized; others, such as wound and burn infections and infections in leukemia and lymphoma patients, result in sepsis. The difference is most probably due to altered host defenses.

Plasmids

Many procaryotes contain plasmids in addition to their chromosomes (Bainbridge and Typas, 1984; Stanisich, 1988) (Figure 9 on page 15 shows the plasmids of *E. coli*). Such plasmids are normally circular (exceptions are the linear forms found in *Streptomyces rochei*, *Borrelia* species, and *Thiobacillus versutus* (Barbour and Garon, 1987; Hirochika *et al.*, 1984; Wlodarczyk and Nowicka, 1988) and range between a few and several hundred kilobases (e.g., p15A from *Escherichia coli* is 2.2 kilobases (kb) long (Chang and Cohen, 1978), pZA2 from *Zymomonas anaerobia* is 1.7 kb long (Yoon and Pack, 1987), and megaplasmids of lithoautotrophic bacteria can be about 700 kb long (Hogrefe and Friedrich, 1984). Most plasmids are cryptic, but often they provide their host with new phenotypic characteristics (Stanisich, 1988; Trevors *et al.*, 1987). Plasmids represent an important factor in bacterial evolution, they enable rapid short-term adaptation of bacteria to changing environmental conditions; they confer gene amplification; and they can be transferred within one or between many species. Normally, plasmids are nonessential to their hosts, conferring only an energy burden that can slow cell growth (Cheah *et al.*, 1987; Zund and Lebek, 1980). However, plasmids can be stably maintained in a bacterial population even under nonselective conditions (de Bernandez and Dhurjati, 1987; Nordstrom and Hansen, 1984; Summers and Sherratt 1984). Strategies such as overreplication (Campbell, 1981), partition modes (Austin, 1988; Mann, 1985), killing of plasmid-free segregants (Gerdes *et al.*, 1986; Hiraga, *et al.*, 1986; Rasmussen *et al.*, 1987), infectious conjugal transfer (Levin, 1986; Lundquist and Levin, 1986), and surface (entry) exclusion against conjugative entrance of additional, related deoxyribonucleic acid (DNA) molecules (van der Hoeven, 1985; van der Hoeven, 1986) contribute to the maintenance of plasmids in a bacterial population. Some plasmids occur in only one or a few copies per cell; others occur in several copies (Novick, 1987; Thomas and Helinski, 1979). The maintenance of low-copy-number plasmids requires a tighter regulation of replication and of segregation than does maintenance of multicopy plasmids (Mann, 1985; Scott, 1984).

Plasmids often contain genes or gene cassettes that confer a selective advantage to the bacterium harboring them, e.g., the ability to make the bacterium antibiotic resistant.

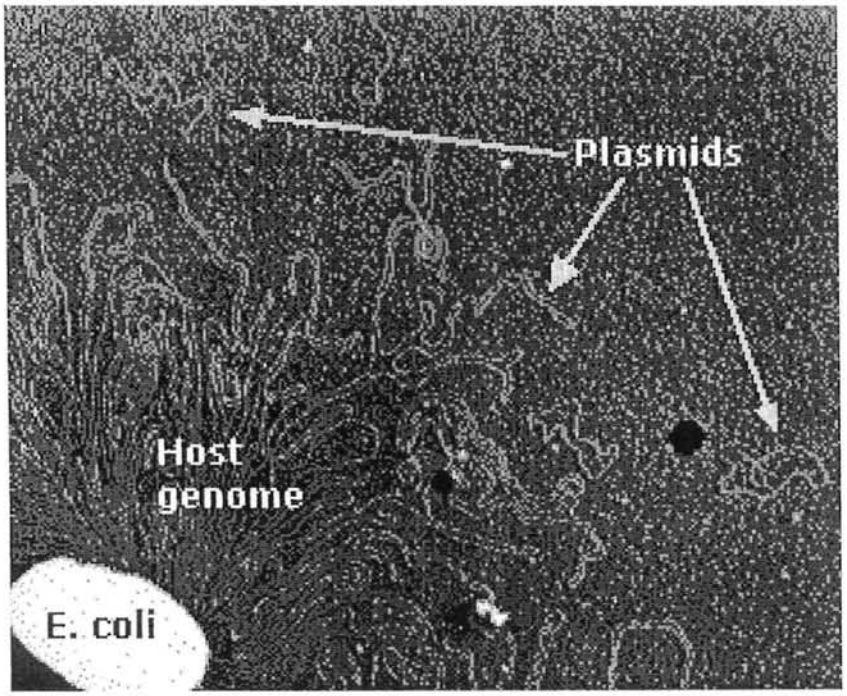

Figure 9. Electron micrograph of Plasmids of *E. Coli*

Every plasmid contain at least one DNA sequence that serves as an origin of replication or *ori* (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA.

The nature of the plasmid shape aids in replication, being much easier to replicate in bacteria (separate from chromosomal DNA) than a linear strand. It also makes biochemical sense that the plasmid is circular and attached to itself.

Types of plasmids

One way of grouping plasmids is by their ability to transfer to other bacteria. Conjugative plasmids contain so called tra-genes, which perform the complex process of conjugation, the sexual transfer of plasmids to another bacterium. Non conjugative plasmids are incapable of initiating conjugation, hence they can only be transferred with the assistance of conjugative plasmids, by accident. An intermediate class of plasmids are mobilizable, and carry only a subset of the genes required for transfer. These plasmids can parasitize another plasmid, transferring at high frequency in the presence of a conjugative plasmid.

An obvious way of classifying plasmids is function. There are five main classes.

1. Fertility-(F) plasmids, which contain tra-genes. They are capable of conjugation.
2. Resistance-(R) plasmids, which contain genes that can build a resistance against antibiotics or poisons. Historically known as R-factors, before the nature of plasmids was understood.
3. Col-plasmids, which contain genes that code for (determine the production of colicines, proteins that can kill other bacteria.
4. Degradative plasmids, which enable the digestion of unusual substances, e.g., toluene of salicylic acid.
5. Virulence plasmids, which turn the bacterium into a pathogen.

Plasmids can belong to more than one of these functional groups. Plasmids that exist only as one or few copies in each bacterium are, upon cell division, in danger of being lost in

one of the segregating bacteria. Such single copy plasmids have systems which attempt to actively distribute a copy to both daughter cells.

Some plasmids include an addiction system or "postsegregational killing system" (PSK). These plasmids produce both a long lived poison and a short lived antidote. Daughter cells that retain a copy of the plasmid survive, while a daughter cell that fails to inherit the plasmid dies or suffers a reduced growth-rate because of the lingering poison form the parent cell. This is an example of plasmids as selfish DNA.

Applications of Plasmids

Plasmids serve as important tools in genetics and biochemistry labs, where they are commonly used to multiply (make many copies of) or express particular genes. There are many plasmids that are commercially available for such uses. Initially, the gene to be replicated is inserted in a plasmid. There plasmids contain, in addition to the inserted gene, one or more genes capable of providing antibiotic resistance to the bacterium that harbors them. The plasmids are next inserted into bacteria by a process called transformation, which are then grown on specified antibiotic(s). Bacteria which took up one or more copies of the plasmid then express (make protein from) the gene that confers antibiotic resistance. This is typically a protein which can break down any antibiotics that would otherwise kill the cell. As a result, only the bacteria with antibiotic resistance can survive, the very same bacteria containing the genes to be replicated. The antibiotic(s) will, however, kill those bacteria that did not receive a plasmid, because they have no antibiotic resistance genes. In this way the antibiotic(s) act as filter selecting out only the modified bacteria. Now these bacteria can be grown in large amounts, harvested and lysed to isolate the plasmid of interest.

Another major use of plasmids is to make large amounts of proteins. In this case you grow the bacteria containing a plasmid harboring the gene of interest. Just as the bacterium produces proteins to confer its antibiotic resistance, it can also be induced to produce large amounts of proteins from the inserted gene. This is a cheap and easy way of mass-producing a gene or the protein it then codes for, i.e. insulin or even antibiotics.

Restriction Mapping

Restriction mapping is the process of obtaining structural information on a piece of DNA by the use of restriction enzymes.

Restriction Enzymes

Restriction enzymes are enzymes that cut DNA at specific recognition sequences called "sites." They probably evolved as a bacterial defense against DNA bacteriophage. DNA invading a bacterial cell defended by these enzymes will be digested into small, non-functional pieces. The name "restriction enzyme" comes from the enzyme's function of restricting access to the cell. A bacterium protects its own DNA from these restriction enzymes by having another enzyme present that modifies these sites by adding a methyl group. For example, *E. coli* makes the restriction enzyme EcoRI and the methylating enzyme EcoRI methylase. The methylase modifies EcoRI sites in the bacteria's own genome to prevent it from being digested. Restriction enzymes are endonucleases that recognize specific 4 to 8 base regions of DNA.

Restriction enzyme, EcoRI, used in present study recognizes the following six base sequence.

5' . . . G-A-A-T-T-C . . . 3'

3' . . . C-T-T-A-A-G . . . 5'

After cleavage,

5' . . . G A-A-T-T-C . . . 3'

3' . . . C-T-T-A-A G . . . 5'

Restriction enzyme, HindIII, used in present study recognizes the following six base sequence.

5' . . . A-A-G-C-T-T . . . 3'

3' . . . T-T-C-G-A-A . . . 5'

After cleavage,

5' . . . A A-G-C-T-T . . . 3'

3' . . . T-T-C-G-A A . . . 5'

Applications of Restriction Mapping:

In molecular biology, restriction maps are used to determine the relationships between two different species at the molecular level. Because so many fragments are obtained from the genome of a cell, restriction mapping is more practical for comparing smaller segments of DNA, usually a few thousand nucleotides long. Several laboratories have used restriction maps to compare mitochondrial DNA (mtDNA) for eukaryotic organisms (as opposed to prokaryotes), which is relatively small. There is the added benefit that mtDNA changes by mutation about ten times faster than the nuclear genome, which makes it possible to sort out phylogenetic relationships between very closely related species, or even between different populations of the same species. Making restriction maps is a routine lab activity that is necessary for any type of cloning project. Maps are a common way for labs to archive information about entire libraries of plasmid constructs.

Restriction map information is important for many techniques used to manipulate DNA. One application is to cut a large piece of DNA into smaller fragments to allow it to be sequenced. Genes and cDNAs can be thousands of kilobases long (megabases - Mb); however, they can only be sequenced 400 bases at a time. DNA must be chopped up into smaller pieces and subcloned to perform the sequencing. Also, restriction mapping is an easy way to compare DNA fragments without having any information of their nucleotide sequence.

Restriction maps have become powerful research tools in molecular biology by helping the location of genetic markers. The distance between two restriction sites can be calculated experimentally by gel electrophoresis.

Objectives of present study

- To isolate and determine the sizes of plasmids of local gram negative clinical isolates.
- To find out the restriction sites in isolated plasmids.
- To evaluate the extent of similarities with international strains.

MATERIALS AND METHODS

MATERIALS AND METHODS

Bacterial Isolates

Clinical isolates of gram negative bacteria were collected from Pakistan Institute of Medical Sciences (PIMS). These included *Escherichia coli, Pseudomonas* Species, *Klebsiella* Species and *Proteus* Species.

Isolation of Pure Culture:

Pure culture was obtained by re-plating the isolates on Agar medium.

Media Preparation:

The medium was prepared by dissolving 3.1g Nutrient Agar (Britania Laboratories) in 100ml of distilled water, heated to dissolve all agar and then autoclaved at 121°C at 15psi pressure for 30 minutes. Then the medium was pored in sterilized Petri plates and a single bacterial colony was picked by a sterilized loop and was streaked on the Agar medium and incubated at 37°C in an oven overnight.

Preparation of Liquid Media:

Broth medium was prepared by dissolving 8g Nutrient Broth (Britania Laboratories) in 1L water, autoclaved at 121°C at 15psi pressure for 30 minutes. After cooling the Ampicillin was dissolved to make a final concentration of 6mg/L. Then the 4ml of the medium was transferred to 10ml test tubes. A single colony of bacterial isolate was picked up by a sterilized inoculating loop and was inoculated in medium. Then medium was incubated for 16 hours in a shaker at 37 °C, the speed of shaker was set at 125rpm.

Plasmid Isolation:

- Plasmid isolation was performed by using QIAGEN QIAprep Miniprep kit.
- 4ml of culture was transferred to a Microcentrifuge tube and centrifuged at 14,000 rpm for 5 minutes, supernatant was discarded to get cell pellet.
- The pelleted cells were re-suspended in 250μl of P1 buffer (RNase A and LyseBlue reagent added). Cells were re-suspended completely by vortexing.

- 250μl of P2 buffer was added and mixed thoroughly and immediately by inverting the tubes 4-6 times until the solution became viscous and clear and homogenously blue colored and let it stand for 5 minutes.
- 350μl of N3 buffer was added and mixed immediately by inverting the tubes 4-6 times, until the blue color disappears.
- It was then centrifuged for 5 minutes at 14,000 rpm to pellet cell debris and chromosomal DNA.
- The supernatant was transferred to the supplied QIAGEN QIAprep spin column by pippetting.
- It was centrifuged for 1 minute and flow through was discarded.
- 500μl of PB buffer was added to spin column, centrifuged for 1 minute and flow through was discarded.
- 750μl of PE buffer was added to spin column and centrifuged for 1 minute and flow through was discarded, again centrifuged for one minute to completely remove the buffer and flow through was discarded.
- The spin column was transferred to Microcentrifuge tube, 50μl of EB buffer was added to the center of column to elute the plasmid DNA. It was incubated for 1 minute at room temperature and then centrifuged for 1 minute.
- The column was discarded and purified plasmid DNA was stored at −20°C.

Characterization

Plasmid DNA of the Isolates was characterized by using following techniques

Restriction Digestion

Digestion with Eco R1

44μl of distilled water was placed in Microcentrifuge tube, then 5μl of 10X buffer EcoR1 was added, then 1μl of enzyme EcoR1 was added and centrifuged for 1 minute at 10,000 rpm to ensure that all the reagents are mixed at the bottom of tube. The mixture was incubated for 1.5 hours at 37°C. Then enzyme was deactivated by heating in a water bath at 60°C for 20 minutes.

Digestion with Hin dIII

4μl of distilled water was placed in Microcentrifuge tube, then 5μl of 10X buffer R was added, then 1μl of miniprep DNA was added then, 1μl of enzyme Hin dIII was added and centrifuged for 1 minute at 10,000 rpm to ensure that all the reagents are mixed at the bottom of tube. The mixture was incubated for 1.5 hours at 37°C. Then enzyme was deactivated by heating in a water bath at 65°C for 20 minutes.

Mixed Digestion

44μl of distilled water was placed in Microcentrifuge tube, then 5μl of 10X buffer Tango was added, then 1μl of miniprep DNA was added, then 1μl of enzyme Hin dIII and 1μl of enzyme Eco R1 was added and centrifuged for 1 minute at 10,000 rpm to ensure that all the reagents are mixed at the bottom of tube. The mixture was incubated for 1.5 hours at 37°C. Then enzymes were deactivated by heating in a water bath at 65°C for 20 minutes.

Agarose Gel Electrophoresis

The evaluation of the isolated DNA and digested DNA was done by agarose gel electrophoresis. This technique is a standard method that separates and identifies DNA fragments according to their molecular weights. Agarose gel was prepared as 1% in 1X TBE buffer by boiling the solution in a microwave oven. Various concentrations provide different resolution capacities, higher concentrations being more specific in separating shorter fragments. To avoid degradation of DNA coloring dye ethidium bromide was added to final concentration of 0.5 mg/ml after the agarose solution cooled down to less than 50°C. Warm agarose was then cast and comb was positioned into the solution. After the polymerization of gel, the gel alongwith cast was placed into an electrophoresis tank containing 1X TBE buffer and the comb was gently removed from the gel to form the wells for loading the DNA samples.

7μl of DNA sample was mixed with the 3μl of bromophenol blue loading buffer and loaded into the well using a micropipette. In one appropriate well 5μl of Ladder (Fermetas 1kb #SM0313) was loaded. When an electric supply was applied, DNA molecules ran towards the anode due to negatively charged PO_4 groups at DNA backbone. Electric supply was applied at 100Volts, 500mA for 1.5 hours. When

electrophoresis is completed, DNA bands could be visualized under UV light and documented.

(TBE 10X) Tris Borate EDTA

218g Tris base, 110g Boric acid and 9.3g EDTA were added in a flask containing 1.9L distilled water mixed thoroughly pH adjusted at 8.3 using NaOH and volume made up to 2L.

RESULTS

RESULTS

Figure 10 on page 26 shows plasmids of *Klebsiella* in lanes 1 and 2 and plasmids of *Protues* in lanes 4 and 5 however in lane 3 is DNA marker of known size (Generuler Fermentas 1kb # SM0313, sizes of all bands of generuler are shown in Figure 14 page 30). Lane 1 and 2, by comparison with generuler show that *Klebsiella* has five different plasmids of sizes 10k, 6k, 5k, 3k and 2k base pairs. Lane 4 and 5 show that *Protues* has 3 different plasmids of sizes nearly 50k, 30k and 18k base pairs.

In Figure 11 page 27, lane 1 shows plasmids of *Klebsiella* (Control). Lane 2 shows restriction products of *Klebsiella* with EcoR1. Lane 3 shows restriction products of *Klebsiella* with HindIII. Lane 4 shows restriction products of *Klebsiella* with EcoR1 and HindIII (Mixed digestion). Lane 5 shows DNA marker (Generuler Fermentas 1kb #SM0313). Lane 6 shows restriction products of *Protues* with EcoR1, lane 7 shows restriction products of *Protues* with HindIII, lane 8 shows restriction product of *Protues* with EcoR1 and HindIII (Mixed digestion) lane 9 shows plasmids of *Protues* (Control). Lane 2 shows similar bands as shown in lane 1. Lane 3 shows the bands of sizes 6k, 5k, 2k, and 0.5k base pairs. Lane 4 shows similar bands as shown in lane 3. Lane 6 shows three bands of sizes nearly 14k, 10k and 8k base pairs. Lane 7 shows a thick band corresponding to size 14k to 18k base pairs. Lane 8 shows three bands of sizes10k, 6k and 4k. Results of *Klebsiella* are tabulated in Table 5 and of *Protues* are tabulated in Table 6 on page 31.

In Figure 12 page 28, lanes 1 and 2 show plasmids of *Escherichia coli*. Lanes 8 and 9 show plasmids of *Pseudomonas*. Lane 5 shows DNA marker (Generuler Fermentas 1kb #SM0313). Lanes 1 and 2 show that *Escherichia coli* has five different plasmids of sizes 14k, 3.5k, 1.8k, 1.2k and 1k base pairs. Lanes 8 and 9 show that *Pseudomonas* has five different plasmids of sizes nearly 20k base pairs and 10k, 4.5k, 2k and 1.6k base pairs.

In Figure 13 page 29, lane 1 shows plasmids of *Escherichia coli* (Control). Lane 2 shows restriction products of *Escherichia coli* with EcoR1. Lane 3 shows restriction products of *Escherichia coli* with HindIII, lane 4 shows plasmids of *Pseudomonas* (Control), Lane 5 shows restriction products of *Escherichia coli* with EcoR1 and HindIII (Mixed digestion), lane 6 shows restriction products of *Pseudomonas* with EcoR1, lane 7 shows DNA marker (Generuler Fermentas 1kb #SM0313), lane 8 shows restriction products of *Pseudomonas* with HindIII, lane 9 shows restriction product of *Pseudomonas* with EcoR1 and HindIII (Mixed digestion), lane 2 shows bands of five different sizes 14k, 3.5k, 2.2k, 1.8k, and 1.6k base pairs. Lane 3 shows similar bands as in lane 1. Lane 5 shows similar bands as in lane 2. Lane 6 shows 6 different bands of sizes 16k, 7k, 4.5k, 2.5k 2k and 0.85k base pairs. Lane 8 shows 6 different bands of sizes 5k, 4.5k, 3.5k, 2.8k, 2.5k and 2k base pairs. Lane 9 shows 4 different bands of sizes 4k, 3.5k, 2.5k, and 2.2k base pairs. Results of *Escherichia coli* are tabulated in Table 7 and of *Pseudomonas* are tabulated in Table 8 on page 32.

Lane # 1 2 3 4 5

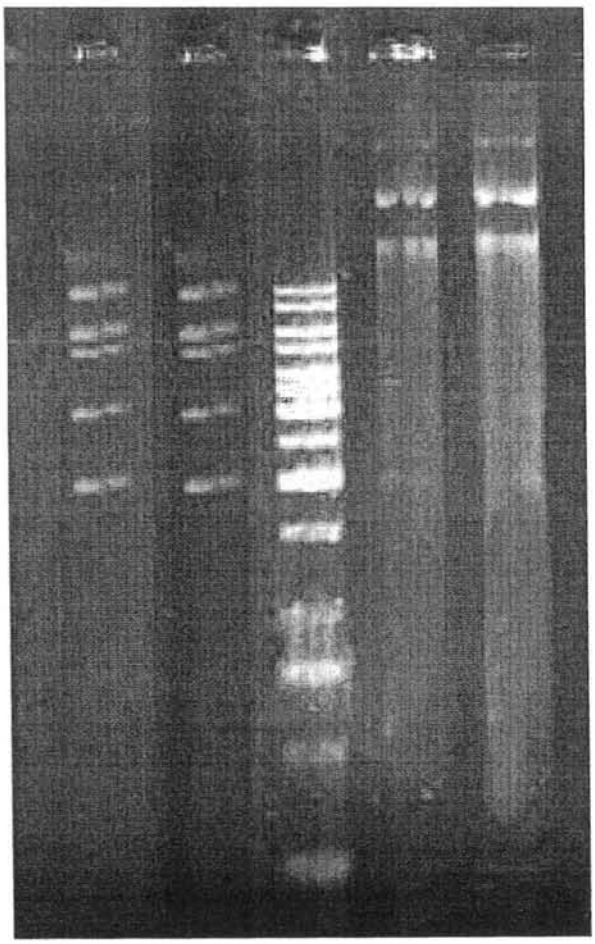

Figure 10

Lane # 1 and 2: Plasmids of *Klebsiella*

Lane # 3: Generuler (Fermentas 1kb #SM0313)

Lane # 4 and 5: Plasmids of *Protues*

Lane # 1 2 3 4 5 6 7 8 9

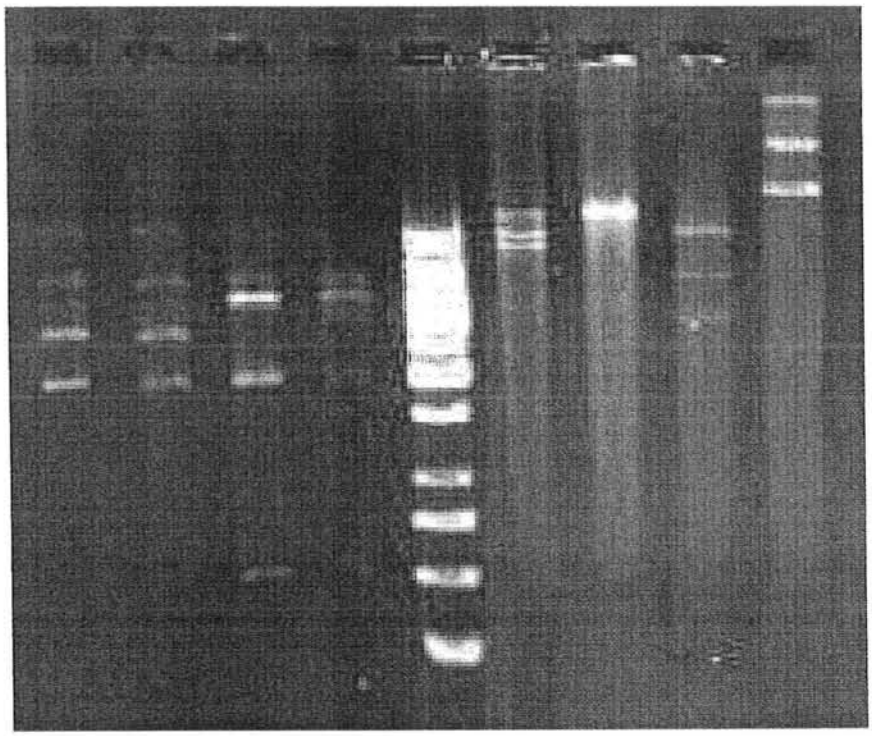

Figure 11

Lane # 1: Plasmids of *Klebsiella*

Lane # 2: EcoR1 cut of *Klebsiella*

Lane # 3: HindIII cut of *Klebsiella*

Lane # 4: Mixed (EcoR1 and HindIII) cut of *Klebsiella*

Lane # 5: Generuler (Fermentas 1kb #SM0313)

Lane # 6: EcoR1 cut of *Protues*

Lane # 7: HindIII cut of *Protues*

Lane # 8: Mixed (EcoR1 and HindIII) cut of *Protues*

Lane # 9: Plasmids of *Protues*

Lane # 1 2 3 4 5 6 7 8 9

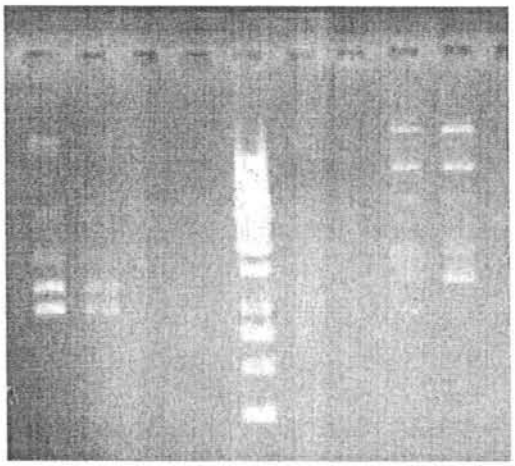

Figure 12

Lane # 1and 2: Plasmids of *Escherichia coli*

Lane # 5: Generuler (Fermentas 1kb #SM0313)

Lane # 8 and 9: Plasmids of *Pseudomonas*

Lane # 1 2 3 4 5 6 7 8 9

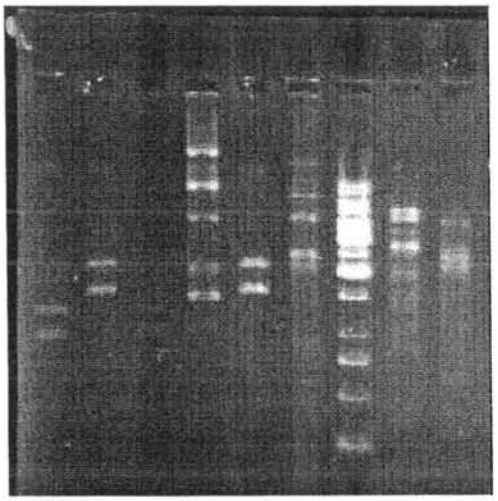

Figure 13

Lane # 1: Plasmids of E. *coli*

Lane # 2: EcoR1 cut of E. *coli*

Lane # 3: HindIII cut of E. *coli*

Lane # 4: Plasmids of *Pseudomonas*

Lane # 5: Mixed (EcoR1 and HindIII) cut of E. *coli*

Lane # 6: EcoR1 cut of *Pseudomonas*

Lane # 7: Generuler (Fermentas 1kb #SM0313)

Lane # 8: HindIII cut of *Pseudomonas*

Lane # 9: Mixed (EcoR1 and HindIII) cut of *Pseudomonas*

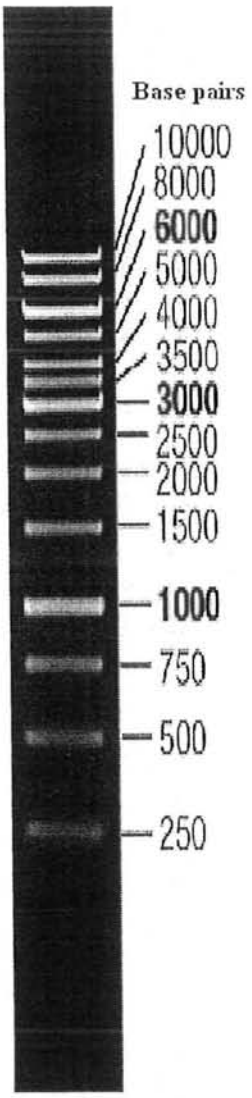

Figure 14

Sizes of different bands of Fermentas Generular 1kb
#SM0313

Klebsiella

Description	**No. of bands**	**Sizes in kilo base pairs**
Original Plasmids	5	10, 6, 5, 3, 2
Digestion with EcoR1	5	10, 6, 5, 3, 2
Digestion with HindIII	4	6, 5, 2, 0.5
Mixed Digestion	4	6, 5, 2, 0.5

Table 5: Number of bands and their sizes of original plasmids of *Klebsiella* and their restriction products in Agarose gel electrophoresis

Protues

Description	**No. of bands**	**Sizes in kilo base pairs**
Original Plasmids	3	50, 30, 18
Digestion with EcoR1	3	14, 10, 8
Digestion with HindIII	1	14~18
Mixed Digestion	3	10, 6, 4

Table 6: Number of bands and their sizes of original plasmids of *Protues* and their restriction products in Agarose gel electrophoresis

Escherichia coli

Description	**No. of bands**	**Sizes in kilo base pairs**
Original Plasmids	5	14, 3.5, 1.8, 1.2 1
Digestion with EcoR1	5	14, 3.5, 2.2, 1.8, 1.6
Digestion with HindIII	5	14, 3.5, 1.8, 1.2 1
Mixed Digestion	5	14, 3.5, 2.2, 1.8, 1.6

Table 7: Number of bands and their sizes of original plasmids of *Escherichia coli* and their restriction products in Agarose gel electrophoresis

Pseudomonas

Description	**No. of bands**	**Sizes in kilo base pairs**
Original Plasmids	5	20, 10, 4.5, 2, 1.6
Digestion with EcoR1	6	16, 7, 4.5, 2.5, 2 0.85
Digestion with HindIII	6	5, 4.5, 3.5, 2.8 2.5, 2
Mixed Digestion	4	4, 3.5, 2.5, 2.2

Table 8: Number of bands and their sizes of original plasmids of *Pseudomonas* and their restriction products in Agarose gel electrophoresis

DISCUSSION

DISCUSSION

The purpose of present study is restriction mapping of plasmids of different clinical isolates of gram negative bacteria.

Klebsiella showed bands of plasmids ranging in size from 2 to 10 kb. As it gave similar results with digestion with EcoR1 hence it shows that there is no restriction site for this enzyme on any of the plasmids of *Klebsiella*. When it was restricted with HindIII 10kb plasmid was divided into 2 fragments of equal length of 5kb, however the 3kb fragment was divided into 3 fragments of unequal lengths. Two are of 0.5 and one of 2.5 kb. Hence it can be inferred that three of the HindIII restriction site are located at 3kb plasmid and two at 10 kb plasmid. As there was no cutting site for EcoR1 on any of the plasmids hence mix digestion gave the same banding pattern as in case of EcoR1 alone.

Protues showed 3 bands and its digestion with EcoR1 results in the production of a different banding pattern which is due to breakdown of the plasmids into fragments of almost same length. As only 3 fragments were obtained but as these are of smaller size as compared to the others found in original. Hence it can be concluded that all 3 plasmids were digested into smaller fragments and all of the bands which were of equal length they stayed at same location showing only one band. Digestion product of HindIII is only one thick band inferred that HindIII produced fragments ranging from 14 to 18 kb but as there is very minute difference so all of the bands gathered together thus giving a very thick and a bright band. With mixed restriction digestion, different lighter bands of 4, 6 and 10kb. However the bigger EcoR1 restricted fragment was cleaved into smaller fragments, it is due to the presence of restriction sites for HindIII within these fragments.

E. coli gave 5 bands of sizes ranging from 1kb to 14kb. Digestion with EcoR1 shows that there is no site for EcoR1 in plasmids of sizes 1.8, 3.5 and 14kb, while plasmids of sizes 1 and 1.2kb, both have only one site for EcoR1 because they seem to be heavier after digestion and remained behind the location as was in undigested sample. This is due to

change in conformation after cleavage at one site and plasmids became linear from circular and remained behind the original location.

Restriction digestion of *E. coli* with HindIII gave all similar bands as in original, it can be due to the fact that there is no restriction site for HindIII in any of the plasmids of *E. coli*. This was further confirmed by similar results to that of EcoR1 digestion when *E. coli* was restricted with both the enzymes i.e. mixed digestion.

In *Pseudomonas* digestion with EcoR1 the plasmid of 20k base pairs was cleaved to give three fragments of nearly 16k, 2.5k and 2k base pairs so it has three cut sites for EcoR1. Plasmid of size was cleaved to give two fragments one of nearly 7k base pairs and the other of 2.5k (because the band of 2.5k base pairs is much bright) base pairs so it has two cut sites for EcoR1. The plasmid of size 4.5k base pairs was at the original location so it has no cut site for EcoR1. Plasmid of 2k base pair was at its original location (This band is also bright) so it no cut site for EcoR1. Plasmid of size 1.6k base pairs was cleaved to give the two fragments of equal sizes of nearly 0.85k base pairs. With HindIII two plasmids of sizes 20k and 10k base pairs were cleaved to give different fragments of sizes 5k 2.8k and 2.5k base pairs. Plasmid of size 4.5k base pairs is probably cleaved to give fragments of different sizes of probably 2.5k and 2k base pairs because there is no band in the region of 4.5k base pairs in the lane of mixed digestion. Plasmid of size 2k base pairs is at its original location so it has no cut site for HindIII. A smear appeared in the region ranging from 0.5k to 1.5k base pairs, showing the presence of different fragments within this region. It is due to the presence of many restriction sites among plasmids of pseudomonas. Fragments made by cleavage of plasmid of size of 1.6k base pairs probably lye in this region. In mixed digestion, the fragments of 16k and 7k base pairs which were produced by digestion with EcoR1 are further cleaved by HindIII to produce smaller fragments. 16k base pair fragment cleaved to give 4 fragments of equal sizes i.e. 4k base pairs and 7k base pairs fragment cleaved to give two fragments of equal sizes i.e. 3.5k base pairs. Plasmid of 4.5k base pairs cleaved to give two fragments of sizes 2.5k and 2k base pairs, band of 2k is not clearly visible due to sample dilution. Fragment of

size 2.2k base pairs is at its original location as is in lane of restriction product of EcoR1, so it has no cut site for HindIII.

Interpreting these gels is not always straightforward. The DNA contains impurities so will tend to yield a more "smeared" banding throughout the lanes. The smear contains pieces of bacterial DNA as well as RNA and plasmid DNA. It is also confusing to see in the lanes of uncut plasmid DNA as many as three bands when you expect only one. This occurs because plasmid DNA can exist in three forms: supercoiled plasmid DNA (tight circle), relaxed plasmid DNA (floppy circle) and linear plasmid DNA. All these forms have the same molecular weight, but they migrate at different rates through the gel matrix: the supercoiled DNA runs fastest (farthest from well); the floppy circle runs slowest (closest to well); the linear form moves faster than floppy relaxed circular form but slower than supercoil – hence, in between the two circular forms.

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