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Genetic Variability of Uropathogenic Strains of *Klebsiella Pneumoniae* in Diabetic Women

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Abstract

Objective: To study the genetic variability of uropathogenic strains of *Klebsiella pneumonia*e in diabetic patients.

Materials and methods: Klebsiella pneumoniae strains isolated from five diabetic patients with UTI were collected and transported to laboratory and tentatively confirmed through various morphological, biochemical and plating techniques using standard procedures. Plasmid DNA was then isolated from all strains and subjected to RFLP analysis after restriction digestion with BamH1.

Results: All the strains were found to be morphologically similar and capable of utilizing glucose. Biochemical analysis revealed that the five strains of *Klebsiella pneumonia*e were positive for catalase and indole test and negative for MP-VP coagulase, H_2S production and gas production. RFLP pattern of five *Klebsiella pneumonia*e stains revealed the existence of 4 distinct clones.

Conclusion: The above findings in the present study suggest the possibility of emergence of new strains of *Klebsiella pneumoniae*.

Key words: *Klebsiella pneumoniae*, diabetes, UTI, RFLP**.**

1. INTRODUCTION

Diabetes is a pathological condition in which the metabolism of blood glucose is abnormal because of insulin receptor defect. Glucose level in blood is elevated with subsequent excretion in urine. High glucose level in blood can cause glycation of various cellular proteins. Formation of advanced glycation end products has been implicated in various diseases (Basta *et al.*, 2004). Patients with diabetes have been reported to have an increased susceptibility to infections. Any increase in blood glucose level above 200mg/dl results in impaired leukocyte function, thus increasing the risk for development of urinary tract infections, ulcers, and pneumonia. Such infections may be asymptomatic but is potentially life threatening (Bagdade *et al.*,1978)

Urinary Tract Infection (UTI) is an infection that affects part of the urinary tract. When it affects the lower urinary tract it is known as a simple *cystitis* (a bladder infection) and when it affects the upper urinary tract it is known as *pyelonephritis* (a kidney infection). Symptoms from a lower urinary tract infection include painful urination and either frequent urination or urge to urinate (or both), while those of pyelonephritis include fever and flank pain in addition to the symptoms of a lower UTI. In the elderly and the very young, symptoms may be vague or nonspecific. The main causal agent of

both types is *Escherichia coli*, *Klebsiella pneumoniae*, however other bacteria, viruses or fungi may rarely be the cause.

Klebsiella pneumoniae is a Gram - negative, non – motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacteria with a prominent polysaccharide capsule. This capsule encases the entire cell surface, accounts for the large appearance of the organism on gram stain , and provides resistance against many host defense mechanisms (Podschun,R & Ulman U, 1998). The size ranges from 0.3-1.0 mm in width and 0.6-6.0 mm in length. *Klebsiella pneumoniae* is commonly found in gastrointestinal tract and hands of hospital personnel. It is clinically the most important member of the Klebsiella genus of *Enterobacteriaceae.* The organism grows at temperature between 12 to 43^0C for 30 minutes. They may survive drying for months and when kept at room temperature, culture remains viable for many weeks (Janda *et al.*, 2006).

Genetic diversity among the strains are common events that can lead to formation of new emerging strains with antibiotic resistance capacity to varying extends. This diversity could be determined by various techniques one among which is RFLP (restriction fragment length polymorphism) (Botstein *et al.*, 1980). Restriction fragment length polymorphisms (RFLP) are variation in DNA fragment banding patterns of electrophoreses restriction digests of DNA form different individuals of a species. Often due to the presence of a restriction enzyme cleavage site at one place in the genome in one individual and the absence of that specific site at one place in the genome in one individual and the absence of that specific site in another individual. Restriction fragment length polymorphism or RFLP analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. The resulting restriction fragments are separated according to their lengths using gel electrophoresis (Thorai Shinawi, 2010). RFLP can be used to trace inheritance patterns, identify specific mutations, and for other molecular genetic techniques. Restriction enzymes are proteins isolated from bacteria that recognize specific short sequences of DNA and cut the DNA at those sites. The normal function of these enzymes in bacteria is to protect the organism by attacking foreign DNA, such as viruses.

2. MATERIALS AND METHODS

2.1 Collection of bacterial strains

The uropathogenic strains of *Klebsiella pneumoniae* were collected from the urine samples of diabetic women referred to Vivek Laboratories, Nagercoil, Kanyakumari District, Tamil Nadu, India based on the blood glucose levels. The collected strains were designated as ASKM 1/ MA1, ASKM 2/ MA2, ASKM 3/ MA3, ASKM 4/ MA4 and ASKM 5 /MA5 and then subjected to the following tests.

2.2 Morpholological & biochemical characterization of *Klebsiella pneumoniae* (Cappuccino *et al.*, 1996)

2.2.1 Gram staining

Gram staining technique was performed by the procedures described by The bacterial cultures were heat fixed on a clean glass slide and differential staining was performed to ascertain whether the bacterial cultures were Gram positive or Gram negative based on their cell wall composition.

2.2.2 Motility test

The motility test is performed to the find the presence of flagella which is the external appendage used for movement of the organism. The pure culture of bacterial strains were inoculated in the tyrptic soy broth and inoculated at 37^0C for 24-48 hours. A drop of the broth was placed on a clean microscopic slide and covered with a cover slip; the slide was viewed under the microscope to observe the motility.

2.2.3 Indole production test

The pure culture of bacterial strains were inoculated in the tryptone broth and inoculated at 37^0C for 24-48 hours. After incubation, 10 drops of kovac's reagent was added directly to the culture tube. The immediate formation of cherry red ring at the top of the broth indicates positive result for indole test whereas the absence of cherry red indicates negative result.

2.2.4 MR-VP Test

MR-VP broth was prepared and sterilized. The bacterial culture was inoculated into MR-VP broth and incubated at 37^0C to 28 hours. After incubation, 5 drops of methyl red indicator was added to one set of tubes and 0.6ml of VP reagent 1 was added followed by 0.2 ml of reagent II to other set of tubes. The result were noted.

2.2.5 Citrate utilization test

This test was used to determine the ability of an organism to utilize sodium citrate as its carbon. Bacteria that grow in this medium turn the bromo-thiomol blue indicator from green to blue. Pure cultures of bacteria were inoculated in the Simmon citratae medium and the tubes were inoculated at 37^0C for 24-28 hours. After incubation, appearance of blue colour indicates positive results whereas green colour signifies negative to citrate utilization.

2.2.6 H_2S Production test

H_2S can be produced by the reduction of organic sulphur present in the aminoacid cysteine a componentof peptone in the medium. In the presence of cysteine desulfurase, the cysteine loses the sulphur atom which is then reduced by the addition of H_2 from water to form H_2S. The SIM (Sulphide Indole Motility medium) agar deep tubes were inoculated with the isolated bacterial cultures to the depth of one cm and incubated at 37^0C for 24-48 hours. The tubes were then observed for the production of H_2S.

2.2.7 Carbohydrate fermentation

This test was recommended for identification of an unknown bacterial species. The acid, alkali or gas production results in a visible change in the inoculated broth. Bacterial cultures were beef extract and peptone. Phenol red indicator was added to record the production of acid and Durham's tubes were dropped into the broth to ascertain the production of gas. The tubes were incubated at 37^0C for 24 hours. If the tubes were yellow with no gas production the results was recorded as acid production. If the inverted vial contains gas and the tube is yellow, the result was recorded as both gas and acid production by the bacterium.

2.2.8 Catalase test

This test is used to detect the presence of catalase enzyme by the decomposition of H_2O_2 to release oxygen and water.

A small amount of bacterial culture was placed on a microscopic slide containing a drop of H_2O_2. It was then mixed with the help of applicator stick. If there were bubbles of froth formation the organism was reported as catalase positive and if not, the organism was reported as catalase negative.

2.2.9 Coagulase test

Coagulase is an enzyme which exists in two forms, bound coagulase and free coagulase. Bound coagulase is detected by the slide test whereas the free coagulase is detected by the tube coagulase test. In the slide coagulase test the culture was smeared to form a smooth suspension in a microscopic slide. To this a loop full of blood plasma of human/rabbit was added and then they were mixed thoroughly and viewed for clumping. In the tube coagulase test, mixture nutrient broth and plasma were taken and to this mixture the pure culture was inoculated and viewed for clumping.

2.3 Conformation of *Klebsiella pneumoniae* in Mac Conkey's agar

The collected strains of *Klebsiella pneumoniae* after biochemical characterization were subjected to confirmation in selective Mac Conkey's agar media by streak plate technique.

2.4 Plasmid DNA isolation

Inoculated a 5ml liquid culture with the bacterial strain of interest and grown in conditions appropriate for that strain until the culture was saturated 1.5 ml of the culture was spined in micro centrifuge for 2 minute, the supernatant was discarded. Re suspended the pellet in 567 µl TE buffer by repeated pipetting. Added 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K TO get a final concentration of 100µg/ml proteinase K in 0.5% SDS. Mixed thoroughly and incubated 1hour at 37^0C.Added 100µl of 5M Nacl and mixed thoroughly. Added 80µl of CTAB/Nacl solution. Mixed thoroughly and incubated at 65^0C for 10 minutes. Added approximately equal volume (0.7-0.8ml) of phenol/chloroform/isoamyl alcohol. Mixed thoroughly and centrifuged for about 4.5 minutes in a micro centrifuge. Removed aqueous viscous supernatant to a fresh micro centrifuge tube, leaving the interphase behind. Added an equal volume of chloroform/isoamyl alcohol extract thoroughly and spined in a micro centrifuge for 5 minutes. Transferred a supernatant to a fresh tube. Added 0.6µl of isopropanol, to precipitate the nucleic acids. Shaken the tube back and froth until a stringy white DNA precipitate became clearly visible. At this point it was possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it on to the end of micropipette that has been heat sealed and bent in a Bunsen flame. Alternatively the precipitate has been pelleted by spinning briefly at room temperature. DNA was washed with 70% ethanol to remove residual CTAB and respined 5 minutes at room temperature to repellet it. Removed the supernatant and dried pellet in a lysophilizer. Dissolved the pellet in 100µl TE buffer. The isolated plasmid DNA from the bacterial strains was then loaded in 0.8% agarose for checking the quality (Sambrook *et al.*, 1989).

2.5 RFLP Analysis

Restriction endonuclease used for DNA digestion was BamHI. The reaction mixture for RFLP analysis was prepared by mixing plasmid DNA, restriction enzyme& 1x assay buffer. The reaction mixture was incubated at 37^0C for 1-3 hours to enable digestion followed by inactivation of enzyme by gently raising DNA of 1kb size and visualized under UV light after staining with ethidium bromide.

3. RESULTS AND DISCUSSION

3.1 Blood glucose level of diabetic patients.

The blood glucose levels of all the refered patients exceeded above 200 mg/dl which was above the normal range.

Table 1: Blood Glucose Levels Of Diabetic Patients

PATIENTS	SEX	AGE	BLOOD GLUCOSE LEVEL (Mg/dl)
Patient A	Female	53	210
Patient B	Female	40	260
Patient C	Female	52	290
Patient D	Female	38	310
Patient E	Female	45	280

3.2 Morphololological & biochemical characterization of *Klebsiella pneumoniae* strains

The five clinical strains of *Klebsiella pneumoniae* viz ASKM 1, ASKM 2, ASKM 3, ASKM 4, ASKM 5 were found to be Gram negative and were non-motile in nature. Biochemical analysis revealed that the five strains of *Klebsiella pneumoniae* were positive for catalase and indole test and were negative for MR-VP,coagulase, H_2S production and gas production. Sugar fermentation test revealed that all the strains were positive for glucose whereas for sucrose and arabinose the strains

MA2 and MA3 alone were found positive. Lactose was found utilized by all the strains except ASKM 5 while dextrose was unutilized by all the five strains. The presence of mucoid colonies in the streaked Mac Conkey's agar plate tentatively confirmed that all the collected strains were *Klebsiella pneumoniae*. The morphological and biochemical tests for the five strains were carried out and the results were represented in the table 2.

Table 2: Morphololological & Biochemical Characterization Of *Klebsiella Pneumoniae Strains.*

Morphological and Biochemical test.	**ASKM 1**	**ASKM 2**	**ASKM 3**	**ASKM 4**	**ASKM 5**
Colony morphological	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Gram staining	-	-	-	-	-
Motility	-	-	-	-	-
Catalase	+	+	+	+	+
Coagulase	-	-	-	-	-
Citrate	+	+	+	-	-
MR-VP	-	-	-	-	-
Indole	+	+	+	+	+
H_2S Production	-	-	-	-	-
Gas production	-	-	-	-	-
Glucose	+	+	+	+	+
Sucrose	-	+	+	-	-
Lactose	+	+	+	+	-
Arabinose	-	+	+	-	-
Dextrose	-	-	-	-	-

3.3 Plasmid DNA isolation and RFLP pattern of *Klebsiella pneumoniae* strains

Plasmid DNA was isolated from the five clinical strains of *Klebsiella pneumoniae* and the DNA was separated in agarose (0.8%) gel electrophoresis to assess their purity. The isolated DNA samples of five bacterial strains were subjected to RFLP analysis after restriction digestion using BAM H1. The plasmid DNA of *Klebsiella pneumoniae* strains ASKM 3 and ASKM 5 was cut with BAM H1 enzyme which produced two bands in which both of them were monomorphic. The *Klebsiella pneumoniae* strain ASKM 1 produced a single band which was polymorphic. The *Klebsiella pneumoniae* strains ASKM 2 and ASKM 4 produced a single band which was monomorphic. RFLP pattern of five *Klebsiella pneumoniae* strains revealed the existence of 4 distinct strains which was evident from the banding pattern identified (Table 3, 4 and Fig 1).

Table 3: Rflp Pattern Of *Klebsiella Pneumoniae* Strains.

	TOTAL NUMBER OF BANDS				
ENZYME	ASKM 1	ASKM 2	ASKM 3	ASKM 4	ASKM 5
BAM H1	1	1	2	1	4

Table 4: Monomorphic And Polymorphic Banding Pattern Of *Klebsiella Pneumoniae*

LANES	**ASKM 1**		**ASKM 2**		**ASKM 3**		**ASKM 4**		**ASKM 5**	
	M	**P**	**M**	**P**	**M**	**P**	**M**	**P**	**M**	**P**
LANE 1	0	0	1	0	1	0	1	0	1	0

LANE 2	0	0	0	0	1	0	0	0	1	0
LANE 3	0	1	0	0	0	0	0	0	0	0
LANE 4	0	0	0	0	0	0	0	0	0	1
LANE 5	0	0	0	0	0	0	0	0	0	1

M- Monomorphic band; **P**- Polymorphic band

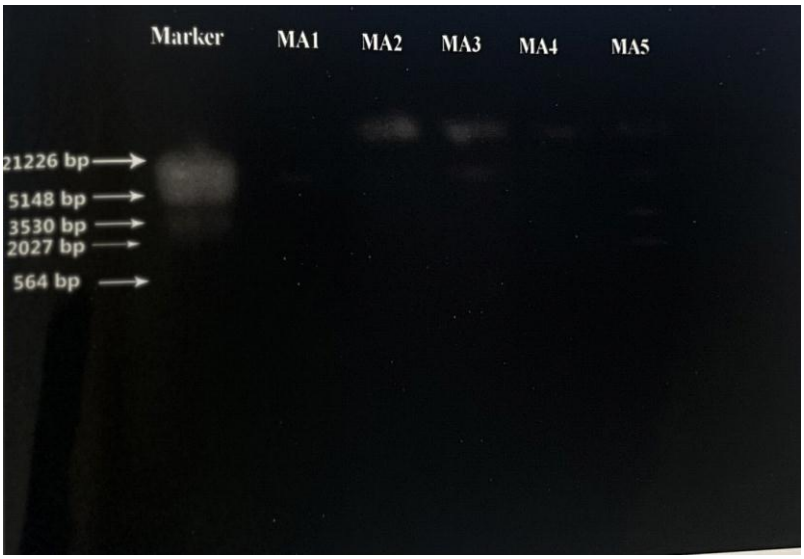

Fig: Rflp Pattern Of Klebsiella Pneumoniae Strains

DISCUSSION

Klebsiella pneumolniae causes a wide variety of diseases in both humans and animals, among which UTI is one of the common disease in women causing health issues during pregnancy and other condition. In the present work, morphological and biochemical characterization all the collected strains revealed similarity and capable of utilizing glucose which they differed from one another in their capacity to utilize other sugars. This clearly indicates that as all the strain are capable of utilizing glucose, they are potent pathogens capable of infecting urinary tract of diabetic patients. The microbiological and genomic features of *Klebsiella pneumoniae* strains isolated from renal transplant patients with asymptomatic bacteriuria in Europe was reported by Magdalena (2020). The marked difference in the RFLP pattern of *Klebsiella pneumoniae* strains observed in the present study also suggested distinct clinical features of strains that should be recognized to treat the infections appropriately. Meritxell *et al*. (2018) discovered high genetic variability among the *Klebsiella pneumoniae* bacteremic isolates and suggested their intrapatient endogenous origin. Golam *et al.* (2018) introduced PCR-RFLP as a simple, selective and cost effective method for determining genetic variability in *K. pneumonia* clinical isolates based on their investigation. They also reported that age could be a factor to concern among women regarding restriction pattern of DNA which was also observed in the banding pattern of present results. Bahareh *et al.* (2022) done the molecular characterization and genotyping of *K. pneumoniae* strains isolated from clinical cases in Iran and reported the presence of *K. pneumoniae* strains belonging to diverse clones. Zeinab *et al.* (2022) conducted bacterial identification of *K. pneumoniae* isolates from Iran according to standard microbiological tests. The strains were further identified to poses point mutation in gyrA gene through PCR-RFLP technique. RFLPs can be used as genetic markers which are often used to follow the inheritance of DNA through families which in simple terms specify polymorphism that was successfully determined in the present study. High degree of variability among *P. teres* isolates existed was identified by RFLP (Chien, 2010). In contrast to this genetic similarity of *K. pneumoniae* isolated from Covid-19 patients was assessed by Asma *et al.* (2021). They also suggested that

determining the characteristics of these isolates will help medical specialist in effective infection management practises. The present study was also focussed on finding effective medication against UTI starins of *K. pneumoniae* in diabetic women of middle aged group referred to hospitals of Kanyakumari District, based on genotyping studies.

CONCLUSION

The findings of the present suggest the possibility of emergence of new strains of *Klebseilla pneumoniae*. This could be a factor of reckon in future and the scientific community should identify the conserved regions in the antigenic determinants of *Klebseilla pneumoniae* to design a common drug to compact these organisms so as to avoid its related complications in diabetic patients. Further the study can be extended to learn the antibiotic resistance pattern of *K. pneumonia*. Moreover, genetic makeup of specific patients can also be analyzed on the other side to design personalized medicines which will be more and more effective in treating infections. The results could also pave the tactic for further research in the detection of *K. pneumoniae* from UTI infected women.

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