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Restriction Sites Annotation on *Klebsiella* 16s Ribosomal RNA using Selected Sticky End Cutting Endonucleases

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The 16S rRNA gene is composed of highly conserved, specie-specific sequences between different species of bacteria and the application of restriction endonucleases on the amplified 16S rRNA gene is a novel diagnostic tool in molecular characterization of bacterial isolates. In a study aimed at characterizing bacteria using some selected endonucleases, a total of 31 isolates collected from the stock culture in the Department of Medical Laboratory Science were re-identified using standard microbiological procedures as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Alcaligenes faecalis*. The isolates *were* subjected to antibiotic susceptibility testing to ascertain their susceptibility pattern using the agar disc diffusion method; followed by DNA extraction, purification, amplification, gel electrophoresis and sequencing. The Sequences were edited using trace edit, Blasted and Restriction site annotation was carried out using Geneious. Antibiotic susceptibility testing results showed that *K. pneumoniae* is susceptible to Streptomycin, Ciprofloxacin, Gentamicin, Nalidixic Acid, Pefloxacin, Septrin, Ofloxacin and resistant to Augmentin, Ampicillin, Cephalosporin. Restriction site annotation of the sequences revealed that Xma1 cuts *Klebsiella pneumoniae* and *Pseudomonas spp*. at position 499 while EcoR1cuts both *Klebsiella pneumoniae* and *Bacillus spp*. at position 561; showing that both Xma1 and EcoR1 cannot be used as restriction markers to differentiate between *Klebsiella pneumoniae* and *Pseudomonas spp*. and *Bacillus spp*. respectively.

Introduction

Klebsiella is a Gram negative, oxidase negative and non-motile rod-shaped bacteria with a prominent polysaccharide-based capsule or slime that can be used for serologic identification which presently have been replaced by molecular serotyping (Ryan and Ray, 2004; Brisse *et al*., 2004).They are facultative anaerobes and can grow optimally between 35 and 37°C at pH 7 and routinely found colonizing the nose, mouth and gastrointestinal tract as normal flora but can also act as opportunistic human pathogens (Ristuccia and Cunha Burke, 1984); and can be found in surface water, sewage, soil, plants, insects and animals (Bagley, 1985); with detection rates of 1 to 6% and 5 to 38% in stool and nasopharynx respectively (Davis and Matsen, 1974), but these rates change in hospital settings where the colonization rates is a function of the length of stay (Kloos and

Musselwhite, 1975). The principal reservoirs for transmission of *Klebsiella* in the hospital setting are gastrointestinal tract of patients and hands of hospital personnel (Montgomerie, 1979).. *Klebsiella* organisms can cause Pneumonia, Urinary Tract Infections, Septicemia, Meningitis, Diarrhea, Soft tissue infections (Podschun and Ullmann, 1998), Ankylosing Spondylitis, Spondyloarthropathies (Bagley, 1985); Pyogenic liver abscess, Donovanosis or Granuloma, Ozena and Rhinoscleroma (Janda and Abbott, 2006). Serotyping is the most widely used and it is based on capsular antigen division according to 77 internationally recognized capsule antigen scheme (Ørskov and Ørskov, 1984; Ørskov and Fife-Asbury, 1977). The combined use of biotyping and capsule typing enables the differentiation of a large number of bioserotypes (Rennie and Duncan, 1974). Phage typing of *Klebsiella* is useful mainly as a secondary method in combination with serologic testing (Johnson *et al*., 1992). Typing of *Klebsiella* with bacteriocins as an additional method to capsule typing. Bacteriocins are bactericidal substances produced by bacteria to inhibit the growth of other bacteria (Hall, 1971). Molecular typing methods include Plasmid profiles (Bauernfeind *et al*., 1993), Ribotyping (Bingen *et al*., 1994), multilocus enzyme analyses (Combe *et al*., 1994), and applications of pulsed-field gel electrophoresis (Arlet *et al*., 1994).

Endonucleases are enzymes that cleave the phosphodiester bonds within a polynucleotide chain and some, such as Deoxyribonuclease-1 cut DNA nonspecifically, producing a heterogeneous collection of fragments of varying sizes while restriction endonucleases or restriction enzymes cleave only at very specific nucleotide sequence (Restriction site) producing small set of homogenous fragments (Kobayashi, 2001). Three groups (Types I, II, III) of naturally occurring restriction endonucleases exist and all types of restriction enzymes recognize specific short DNA sequences but they differ in their recognition sequence, subunit composition, cleavage position and cofactor requirements (Sistla and Rao, 2004).

16S ribosomal RNA (or 16S rRNA) is components of the 30S small subunit of prokaryotic ribosomes coded by the16S rDNA gene and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene (Woese and Fox, 1977). The 16S rRNA gene is highly conserved between different species of bacteria and archaea (Coenye and Vandamme, 2003) and it is also used for phylogenetic studies (Weisburg *et al*., 1991). There are sequences containing hypervariable regions having species-specific signature sequences used for the identification of bacteria (Clarridge, 2004). The 16S sequencing can also be handy in the reclassifying bacteria into completely new species, or even genera (Weisburg *et al*., 1991); used to describe new unculturable species; slow-growing bacteria; rare bacteria; bacteria with unusual phenotypic profiles and choice of antibiotics for treatment (Gray and Herwig, 1996).

This study aims to characterize *Klebsiella* using the 10 randomly selected endonucleases (Xma1, EcoR1, BspE1, Tfi1, Eae1, Eag1, Nco1, Tse1, Tfi1 and BsrG1) on the 16S rRNA gene

Materials and Methods

The Research was carried out in the Department of Medical Laboratory Science of Niger Delta University, Bayelsa State, Nigeria with organisms obtained from a Stock cultures from the Departmental Research Laboratory, which were processed by sub-

culturing on MacConkey and Blood Agar and incubated at 37^0C for 24 hours. The colonies were re-identified based on standard microbiological techniques (colonial morphology, Gram staining and biochemical characteristics).

The isolates were subjected to antibiotic susceptibility testing by agar disc diffusion method using commercially available discs. The antibiotic discs were placed on nutrient agar plate after the isolates were streaked on it. The plates were incubated at 37^0C for 24 hours and the results recorded.

DNA Extraction

DNA extraction from isolates was carried out using a ZR Fungal/Bacterial DNA MiniPrep$^{TM\cdot}$kit supplied by Inqaba Biotech, South Africa. Isolates were cultured on Luria-Bertani (LB) broth and incubated for 24 hours. After overnight culture, Bashing beads tubes were set up on a rack and labeled followed by pipetting 1,500µl of isolates in LB broth into respective bashing bead tubes and centrifuged at 14,000rpm for 30 seconds. The supernatant was removed and 750µl of Lysis buffer was added. The cells were bead bashed in the Cell Disruptor Genie ^{TM} for 5 minutes at maximum speed after which it was spun at 14,000 rpm for 1 minute. Then, 400µl of the supernatant was transferred to a Zymo-Spin ^{TM} IV Spin Filter which has its base snapped off in a collection tube and centrifuged at 7,000 rpm for 1 minute. About 1,200µl of Bacterial DNA Binding Buffer was added to the filtrate in the collection tube. Then, 800µl of mixture (400µl supernatant and 1,200µl bacterial DNA Binding Buffer) was transferred to a Zymo-Spin ^{TM} IIC Column in new collection tubes and centrifuged at 14,000 rpm for 1 minute. The flow through in collection tube was discarded and 800µl of mixture was transferred to a Zymo -Spin ^{TM} IIC Column in collection tubes and centrifuged at 14,000 rpm for 1 minute. About 200µl of DNA pre-wash Buffer was added to the Zymo-Spin ^{TM} IIC Column in a new collection tube and centrifuged at 14,000 rpm for 1 minute. Then, 500µl of Bacterial DNA Wash Buffer was added to the Zymo -Spin ^{TM} IIC Column and centrifuged at 14,000 rpm for 1 minute. Zymo -Spin ^{TM} IIC Column was transferred to a clean 1.5 ml micro centrifuge tube and 65µl of DNA Elution Buffer was added and centrifuged at 14,000 rpm for 30 seconds to elude the DNA. Finally, the bacterial DNA was quantified using a Nanodrop 1000.

16S rRNA Amplification

The 16S rRNA gene was amplified using a 9700 Applied Biosystem Thermal Cycler at final reaction volume of 25µl using a 27F and 1492R primers for 30 cycles. The PCR tubes were labeled and placed on an ice rack and 20µl of the PCR mix was pipette into each of the PCR tubes; PCR mix includes Dream Taq master mix (12.5µl), Forward Primer (0.4M), Reverse Primer (0.4M) and 6.7µl of Water. 5µl extracted DNA is added to the PCR mix. The PCR tubes was removed from the ice rack and loaded on the thermocycler after which the thermocycler was switched on, The PCR temperature conditions are set at 95^0C for 5 minutes, 95^0C for 30 seconds, 52^0C for 30 seconds, 72^0C for 30 seconds and 4^0C set at infinity. The PCR conditions were set to repeat for 30 cycles which lasted for about 121 minutes.

Agarose Gel Electrophoresis

The PCR product was resolved on 1% Agarose gel electrophoresis tinted with Ethidium bromide. The 1% agarose gel was prepared by dissolving 3 tablets of Agarose in 100ml of Tris Boris EDTA (TBE) and heated by microwave at 85^0C for 5 minutes until there was homogenous solution. After

allowing to cool, 3µl of Ethidium bromide was added and poured on the Electrophoretic cassette which had combs for the creation of wells after the gel has solidified. The solidified agarose is placed on an Electrophoretic tank, 5µl of the PCR product and 5µl of ladder was pipette from tubes into wells in the Agarose gel. The Electrophoretic machine was set up at 120V for 20 minutes and visualized with an Ultraviolet transilluminator. The sizes of the DNA were determined using a MassRuler high range DNA ladder. The PCR product was sequenced using the Big Dye Terminator Kit on a 3500 ABI Sequencer by Inqaba Biotech, South Africa.

Sequencing/ Endonuclease Site Annotation

The sequences were edited using Trace Edit, Similar sequences were downloaded from NCBI database using BLAST (Basic Local Alignment Search Tool) and downloaded sequences were aligned with the test isolate sequence using Clustal X. The restriction site annotation was carried out using Geneious.

Results and Discussion

Results captured in Table 1 show that isolates were identified based on the reactions observed. *Staphylococcus* is gram positive, coagulase and catalase positive ; *Escherichia coli* is gram negative, citrate negative, oxidase negative and indole positive; *Klebsiella* is gram negative and citrate positive, oxidase negative, indole negative while *Alcaligenes* and *Pseudomonas* is positive to oxidase and citrate and indole negative.

From Table 2, Ten isolates of *Escherichia coli* are susceptible to Streptomycin: 6(60%); Ciprofloxacin: 5(50%); Gentamicin: 5(50%); Nalidixic Acid: 1(10%); Pefloxacin: 4(40%); Augmentin: 0(0%); Septrin: 3(30%); Ampicillin: 2(20%); Cephalosporin: 2(20%); Ofloxacin: 7(70%). Nine isolates of *Pseudomonas* are susceptible to Streptomycin: 3(33%); Ciprofloxacin: 6(66%); Gentamicin: 4(44%); Nalidixic Acid: 3(33%); Pefloxacin: 7(77%); Augmentin: 1(11%); Septrin: 6(66%); Ampicillin: 2(22%); Cephalosporin: 2(22%); Ofloxacin: 7(77%). *Alcaligenes* with 1 isolate is susceptible to Streptomycin: 1(100%); Ciprofloxacin: 1(100%); Gentamicin: 0(0%); Nalidixic Acid: 1(100%); Pefloxacin: 1(100%); Augmentin: 0(0%); Septrin: 1(100%); Ampicillin: 0%; Cephalosporin: 1(100%); Ofloxacin: 1(100%). *Klebsiella* with 1 isolate is susceptible to Streptomycin: 1(100%); Ciprofloxacin: 1(100%); Gentamicin: 1(100%); Nalidixic Acid: 1(100%); Pefloxacin: 1(100%); Augmentin: 0(0%); Septrin: 1(100%); Ampicillin: 0(0%); Cephalosporin: 0(0%); Ofloxacin: 1(100%). *Staphylococcus* with 10 isolates is susceptible to Rifampicin: 4(40%); Amoxacillin: 2(20%); Streptomycin: 2(20%); Novobiocin: 0(0%); Chloramphenicol: 4(40%); Ciprofloxacin: 4(40%); Erythromycin: 3(30%); Levofloxacin: 2(20%); Gentamicin: 0(0%); Afloxacillin: 0(0%).

Figure 1 captures the Agarose gel electrophoresis of 16S rRNA gene of bacterial isolates using a 1kb ladder to show the band sizes of 1500bp of PCR product.

Figure 2 shows EcoR1 cuts retrieved control *Klebsiella*, *Klebsiella* (test) and *Bacillus* at position 561. From its cut pattern it can be deduced that EcoR1 is not a good restriction marker to differentiate between *Klebsiella* and *Bacillus* while Xma1cuts retrieved control *Klebsiella* and *Pseudomonas* at position 499, this shows that Xma1 is not a good marker to differentiate between *Klebsiella* and *Pseudomonas*. Xam1 cuts *Klebsiella* and *Pseudomonas* at position 499 while EcoR1 cuts *Klebsiella* and *Bacillus* at position 561. This leads to the inference that Xam1 isn't a

good restriction marker to differentiate between *Klebsiella* and *Pseudomonas* and EcoR1 to differentiate between *Klebsiella* and *Bacillus.*

The study concurs with Ryan and Ray (2004) that *Klebsiella pneumoniae* is a Gram negative, rod shaped organism and is also in agreement with Cheesbrough (2010) that *K. pneumoniae* is Citrate positive, Oxidase and Indole negative; *Staphylococcus aureus* is a Gram positive cocci and also Catalase and Coagulase positive; *Alcaligenes faecalis* is a Gram negative rod that is citrate and oxidase positive ; *Pseudomonas aeruginosa* is a Gram negative rod, citrate and oxidase positive and *Escherichia coli* a Gram negative rod is indole positive.

The only *Klebsiella isolate* compared to other isolates had an overall high antibiotic susceptibility but the high susceptibility to Gentamicin (100%) and Ciprofloxacin (100%) is not in agreement with 17.39% and 41.3% respectively of Ullah *et al*., 2009 and 35.4% and 62.5% respectively of Abdullah *et al*., 2013.

The antibiogram of the 10 *E. coli* isolates showed low susceptibility of 20% to Ampicillin which is similar to 11.6% reported by Niranjan *et al*., 2014 and 10.3% reported by Duredoh *et al*., 2012. However, Susceptibility of Ciprofloxacin and Gentamicin showed a 50% susceptibility which is close to 70% and 53.6% respectively as reported by Duredoh *et al*., 2012.

The antibiogram of *Pseudomonas* with 9 isolates reveal susceptibility to Streptomycin 3(33%); Ciprofloxacin 6(66%); Gentamicin 4(44%); Nalidixic acid 3(33%); Pefloxacin 7(77%); Augmentin 1(11%); Septrin 6(66%); Ampicillin 2(22%); Cephalosporin 2(22%) and Ofloxacin 7(77%). The percentage susceptibility is in agreement with studies by Golshani *et al*., 2012 with Gentamicin having 40%.

Table.1 Showing biochemical reactions of bacterial isolates

Isolate names	Gram stain	Catalase	Coagulase	Indole	Citrate	Oxidase
E. coli	-	NA	NA	+	-	-
Pseudomonas	-	NA	NA	-	+	+
Staphylococcus	+	+	+	NA	NA	NA
Klebsiella	-	NA	NA	-	+	-
Alcaligenes	-	NA	NA	-	+	+

KEY +: Positive **; -:** Negative**; NA:** Not Applicable

Table.2 Showing susceptibility pattern of the bacterial isolates

Isolate Names (N/S)	**RD**	**AMX**	**S**	**NB**	**CH**	**CPX**	**E**	**LEV**	**CN**	**AFX**	**NA**	**PEF**	**AU**	**SXT**	**PN**	**CEP**	**OFX**
	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)	S(%)
E. coli	NA	NA	6(60)	NA	NA	5(50)	NA	NA	5(50)	NA	1(10)	4(40)	0(0)	3(30)	2(20)	2(20)	7(70)

Table.3 Showing endonuclease activity of isolates

Restriction Enzymes	Test	1	2	3	4	5	6	7	8	9	P	B	Fragment Size	Number of Fragment
	(*Klebsiella spp.*)													
Xma1	+	+	+	+	+	+	+	+	+	+	+	-	499, 161	2
EcoR1	+	+	+	+	+	+	+	+	+	+	-	+	561, 99	2
BspE1	-	-	-	-	-	-	-	-	-	-	+	+		
Tfi1	-	-	-	-	-	-	-	-	-	-	-	+	249	2
Eae1	-	-	-	-	-	-	-	-	-	-	-	+	195	2
Eag1	-	-	-	-	-	-	-	-	-	-	-	-		
Nco1	-	-	-	-	-	-	-	-	-	-	-	-		
Tse1	-	-	-	-	-	-	-	-	-	-	-	-		
Tfi1	-	-	-	-	-	-	-	-	-	-	-	-		
BsrG1	-	-	-	-	-	-	-	-	-	-	-	-		

+: Positive
-: Negative
1-9: Retrieved Control *Klebsiella sp.*

Fig.1 Agarose electrophoresis of 16S rRNA gene of the various isolates (1500bp). 1-6 represents the bands for the different isolates while L represents 1kb ladder.

1 2 3 4 4 5 6 L

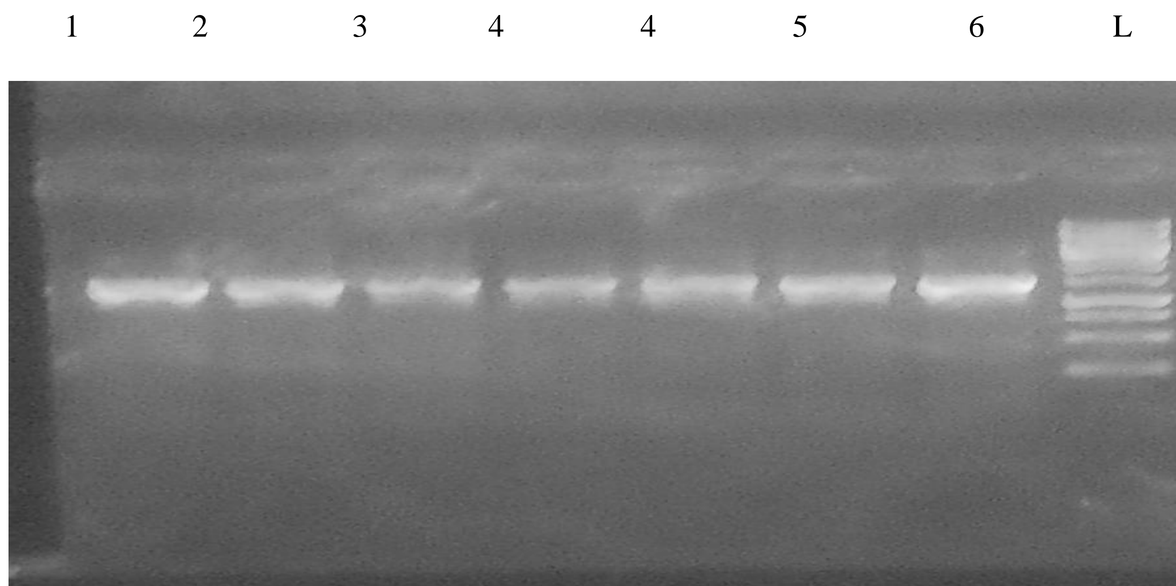
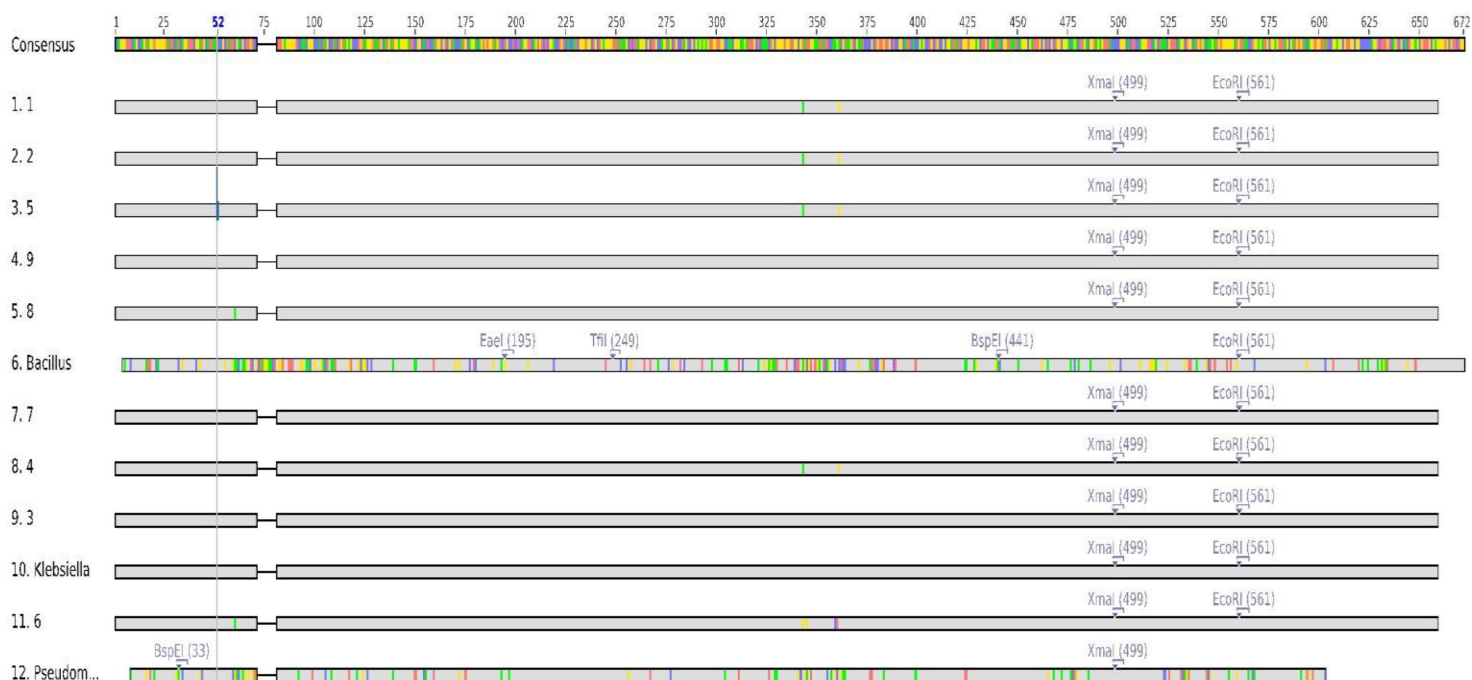


Fig.2 Restriction site annotation of various bacterial isolates.



The antibiotic pattern showed that *Alcaligenes* with 1 isolate is susceptible to Ciprofloxacin 1(100%) and Gentamicin 0(0%) which the results disagrees with 58.9% and 69.1% susceptibility to Gentamicin and Ofloxacin respectively as recorded by Mordi *et al*., (2013).

The antibiogram showed that *Staphylococcus* with 10 isolates is susceptible to Chloramphenicol 4(40%); Ciprofloxacin and Gentamicin 0(0%) but this disagrees with the work carried out by Hoerlle and Brandelli, 2009 with Gentamicin 96.3%, Chloramphenicol 93% and Ciprofloxacin 63.7%.

Factors such as temperatures, incubation period, pH of agar medium and the size of bacterial inoculums can affect antibiotics susceptibility and may be responsible for the different results documented by various authors (Marina *et al*., 2008).

Amplification of the 16S rRNA region of the isolates and subsequent agarose gel electrophoresis using a MassRuler high range DNA ladder revealed that bands were successfully separated showing the 1500bp length.

The study also revealed that among the sticky end restriction enzymes, which includes Xma1, EcoR1, BspE1, Tfi1, Eae1, Nco1, Tse1, BsrG1, BspH1 and Eag1 used in this study, EcoR1 cuts *K. pneumoniae* and *Bacillus spp.* at position 561 while Xma1 cuts *K. pneumoniae* and *Pseudomonas spp*. at position 499. This infers that both EcoR1 and Xma1 aren't good restriction markers to identify *K. pneumoniae.* However, BspE1cuts *Pseudomonas* sp. And Bacillus sp. at position 33 and 441 respectively and Tfi1and Eae1cuts Bacillus sp. at position 249 and 195 respectively. Eag1, Nco1, Tse1, Tfi1, BsrG1 were unable to cut any of the bacterial isolates.

In conclusion, he study revealed that *Klebsiella pneumoniae* cannot be identified/ characterized by restriction endonucleases used in this study which includes: Xma1, EcoR1, BspE1, Tfi1, Eae1, Nco1, Tse1, BsrG1, BspH1 and Eag1 due to its ability to cut various bacteria at the same position in the 16S rRNA gene.

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