Original Article

DIFFERENTIATION OF COMMON GRAM NEGATIVE PATHOGENS BY PCR-RIBOTYPING

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ABSTRACT

Background: Gram negative bacteria especially members of family Enterobacteriaceae are among the most frequently isolated organisms from the clinical specimens. Rapid diagnosis of the pathogen in a clinical sample is always very important. Conventional methods are time-consuming. Among molecular techniques, PCR is very useful but unless very specific primers are used, non-specific amplifications are a problem.

Objectives: PCR-ribotying is a technique that gives very specific multiple bands by use of a single primer set. This study was designed to establish patterns for five common pathogens of Enterobacteriaceae, namely *Escherichia coli, Salmonella enterica serovar Typhi (Salmonella Typhi)*, *Proteus vulgaris, Klebsiella aerogenes,* and *Cirtobacter freundii* along with another very common and problematic gram negative pathogen *Pseudomonas aeruginosa*.

Results: Each species gave a specific ribotyping pattern. *Escherichia coli* gave four amplification products of 1200, 850, 800, and 700 bps. Four amplification products of different sizes were also observed in *Citrobacter freundii* (3000, 850, 700, and 580 bps), *Proteus vulgaris* (900, 800, 750 and 700 bps), and *Klebisella aerogenes* (3000, 870, 700 and 520 bps). More discrimination with five amplification products was seen in *Salmonella Typhi* (3000, 1200, 900, 850, and 700 bps). On the other side of spectrum was *Pseudomonas aeruginosa* only a single amplification product of 750 bps was observed.

Conclusion: PCR-ribotyping can very efficiently and specifically differentiate between opportunistic gram negative human pathogens.

KEY WORDS: Gram negative pathogens, Diagnosis, PCR-ribotyping.

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INTRODUCTION

The bacterial flora of human intestinal tract consists of a large variety of aerobic and anaerobic organisms. Majority are commensals with notable exceptions of Salmonella and Shigella. However, outside the intestine they assume the role of troublesome pathogens which are the most frequent causes of urinary tract infections, wound infections and various other types of infection. Most of these pathogens belong to family Enterobacteriaceae, but some others such as Pseudomonas species are also very important.

It has been reported that in the US, about 50% of all the urinary tract pathogens belong to the enteric group.[1] According to data from the US centers for disease control and prevention, about 30% of all identified nosocomial pathogens belong to this group.[2]

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Individualization of pathogenic strains is essential to study the association between clinical cases and possible sources of infection and for this purpose various typing methods have been devised. Conventional methods that include antibiotic resistance patterns, biochemical reactions, bacteriocin typing and phage typing are usually inefficient, time-consuming and expensive. Serotyping has been the reference method for strain characterization of certain organisms[3] but it fails to differentiate all isolates originating from different regions or sources.[4]

More recently, there has been an increasing interest in the application of molecular techniques to type bacterial pathogens. The techniques used include multilocus enzyme electrophoresis, biotyping, restriction endonuclease analysis, ribotyping, PFGE (pulse field gel electrophoresis), nucleotide sequence analysis, protein analysis and plasmid profiling. But all these techniques are technically demanding and expensive. PCR has been the most successful technique that is rapid and sensitive.

During last ten years, a new technique PCR-ribotyping has been widely used for differentiating bacteria up to species and in some cases even strain level. Sequencing of rRNA genes has been a very reliable method of characterizing bacterial species but it has not enough heterogeneity for further classification. PCR-ribotyping is based on the amplification of spacer regions or IVs (intervening sequences) between 16s and 23s RNA genes. The variability in length and number of copies provide means for classification of strains of different bacteria and mycoplasma.[5, 6] For example, in a study PCR-ribotyping allowed for the identification of seven serovars of Salmonellae.[7]

This technique has superceded the conventional PCR for confirmation of different species as it later may be complicated by non-specific amplifications and thus need supporting morphological and biochemical data.

The aim of present study was to establish a single step (single-primer set based) PCR-ribotyping reference pattern for molecular confirmation of common gram negative pathogens including members of family Entrobacteriacae and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Bacterial strains: Five isolates each of *Salmonella* Typhi, *Proteus vulgaris, Pseudomonas aeruginosa,* and *Escherichia coli*, *Klebsiella aerogenes* and *Citrobacter freundii* were taken from stock cultures of NIBGE collection. These isolates from urinary tract and wound infections had been identified and confirmed by conventional methods,[8] grown in Trypticase Soy Broth (TSB), and stored in TSB with 10% dimethylsulfoxide at –20°C.

Isolation and Purification of Bacterial Isolates: An aliquot of stock cultures was thawed and 100µL was inoculated in 3mL of Trypticase Soya Broth followed by overnight incubation at 37ºC. After 24 hours, a loopful from each tube was streaked on MacConkey agar plates and after overnight incubation at 37ºC, a single colony was picked for further studies.

PCR for confirmation of enteric bacteria: For confirmation by PCR different sets of primers targeting specific genes were used[9-13] (Table-I). The PCR conditions were as recommended by the respective authors.

PCR Ribotyping of Isolates: Two primers complementary to conserved regions of the 16s and 23s rRNA genes were synthesized on a Pharmacia KLB Gene Assembler Special. The sequences of P1 (5'-TTGTACACA CCGCCCGTCA-3') and P2 (5'-GGTACTTAGA TGTTTCAGTTC-3') have previously been described.[14]

PCR reaction mixture contained 1.5 mM $MgCl_2$, 50µM of each dNTP, 300 pmol of each primer, 4U of recombinant *Taq* polymerase (Fermentas) and 0.1µg/µl of DNA template. The thermal cycler conditions were 30 cycles each of 94°C for 1 minute, 50°C for 1 minute, and 72°C for1.5 minute followed by 5 minutes at 72°C.

Detection of PCR Products: A quantity of 40µl of amplified product mixed with 15µl of Bromophenol blue (tracking dye) was fractionated electrophoretically on 2.5% agarose gel for four hours. The voltage was kept constant at 150V.

Table-I: Primers used for identification of bacteria

Bacteria	Primers	Sequences (5′-3′)	Genes	Size (bps)	References
E. coli	EC-F	ATCACCGTGGTGACGCATGTCGC			
	EC-R	CACCACGATGCCATGTTCATCTGC	*uid A*	486	Heninger *et al* [9]
Ps.	PsA-F	GGGGGATCTTCGGACCTCA	*algD GDP*	915	Spilker *et al* [10]
aeruginosa	PsA-R	TCCTTAGAGTGCCCACCC	*mannose*		
S. Typhi	ST-F	TATGCCGCTACATATGATGAG	*fliC*	495	Song *et al*[11]
	ST-R	TTAACGCAGTAAAGAGAG			
P. vulgaris	PsV-F	GCTAATACCGCATAACGTTGC	*urease*	384	Kupfer *et al* [12]
	PsV-R	AACGCTTGCACCCTCGGTA			
K Kl. aerogenes	Kl-F	GACGCAGACCGAAATCGAACT	*gnd*	650	Brisse *et al*[13] &
Citrobacter freundii	Kl-R	CGGTTACGGCCAGTGGAATA			

After staining by ethidium bromide (0.5µg per ml), the gel was photographed by using Eagle Eye (Strategene, USA). PCR was performed in duplicate for each sample.

RESULTS

PCR for the confirmation of enteric bacteria: The results are shown in Fig 1. All isolates of *E. coli* were confirmed through PCR by targeting *uid A* gene (encoding glucuronidase). A single amplification product of 486 bps was obtained. *Salmonella* Typhi isolates were confirmed by targeting *fliC* gene by specific primers that produce a 363 bps product. For confirmation of *Proteus vulgaris* strains, PCR was performed to amplify *urease* gene to get a 385 bps fragment. The *algD GDP* mannose gene is specific for *Pseudomonsa aeruginosa.* Its presence was ascertained by PCR targeting a 956 bps fragment. Both *Citrobacter freundii* and *Klebsiella aerogenes* have a specific *gnd* gene. Primers were used to amplify a 650 bps region.

PCR Ribotyping: The results are shown in Fig-II and summarized in (Table-II). PCR-Ribotyping of *Escherichia coli* gave 4 amplification products of 1200, 850, 800, and 700 bps. As in *E.coli,* four amplification products were observed in *Citrobacter freundii* but the pattern was different. The bands were of 3000, 850, 700, and 580 bps. A different pattern with 4 amplification products was seen in *Proteus vulgaris* as bands of 900, 800, 750 and 700 bps were observed. *Klebisella aerogenes* also produced 4 amplification products having a different pattern with bands of 3000, 870, 700 and 520 bps. More discrimination with 5 amplification products was observed with *Salmonella* Typhi. The sizes were 3000, 1200, 900, 850, and 700 bps. On the other side of spectrum with *Pseudomonas aeroginosa* only a single amplification product of 750 bps was observed.

DISCUSSION

During last few decades the strength of molecular biology has revolutionized biological sciences. The molecular techniques have opened a new chapter for characterization of different bacteria. These methods can discriminate to a better degree than phenotypic methods and improve our knowledge of genetic and epidemiological relationships.[15]

Restriction fragment length polymorphism (RFLP) has given promising results and even phage types can be subdivided,[16] but this technique is expensive, laborious and time-consuming. PFGE is extremely discriminatory and can be successfully used in epidemiological investigation of *S.* Typhi and other enteric bacteria outbreaks[17, 18] but it has same disadvantages as RFLP. Sequencing of rRNA genes has been a very reliable method of characterizing bacterial species but it is technically demanding and expensive.

Table-II: PCR Ribotyping Pattern of Enteric Bacteria

Organism	Sizes of amplification products (bps)
Escherichia coli	1200, 850, 800, 700
Salmonella Typhi	3000, 1200, 900, 850, 700
Klebsiella aerogenes	3000, 870, 700, 520
Proteus vulgaris	900, 800, 750, 700
Citrobacter freundii	3000, 850, 700, 580
Pseudomonas aeruginosa	750

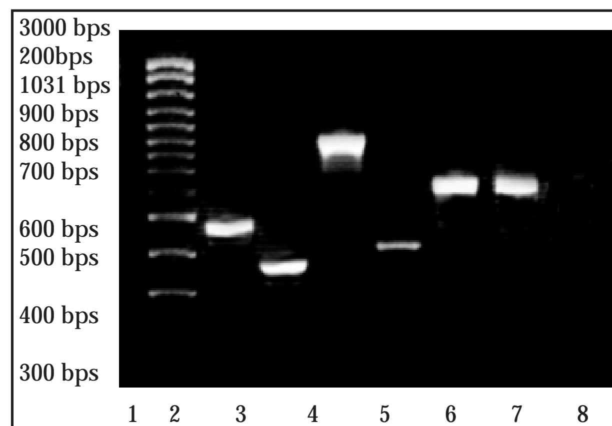

Fig-I: *Lane 1:* Molecular weight marker (SM0323S) showing 3000, 2000, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp in descending order.
Lane 2: Amplification product of 486 bps produced by targeting of *uid* gene of *E.coli.*
Lane 3: Amplification product of 363 bps produced by targeting of *fliC* gene of *S.* Typhi.
Lane 4: Amplification product of 486 bps produced by targeting of *algD GDP* manose gene of *Pseudomonas aeruginosa.*
Lane 5: Amplification product of 385 bps produced by targeting of *urease* gene of *Proteus vulgaris.*
Lane 6: Amplification product of 650 bps produced by targeting of *gnd* gene of *Klebsiella aerogenes.*
Lane 7: Amplification product of 650 bps produced by targeting of *gnd* gene of *Citrobacter freundii*
Lane 8: Negative control with no template DNA.

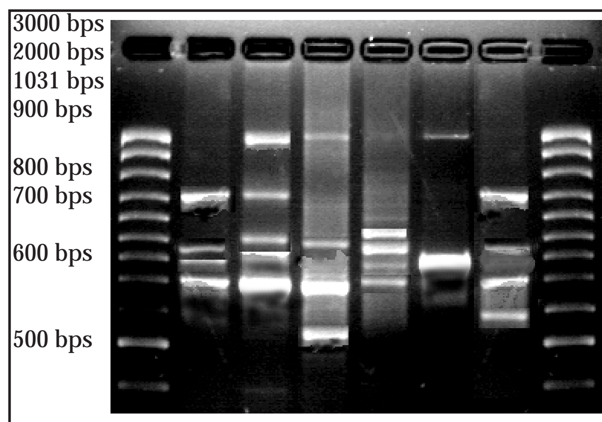

Fig-II: *Lane 1, 8:* Molecular weight marker (SM0323S, Fermentas)
Lane 2: Amplification products of 1200, 850, 800, 700 bps produced *E.coli.*
Lane 3: Amplification products of 3000, 1200, 900, 850, 700 bps produced by *S.* Typhi.
Lane 4: Amplification products of 3000, 870, 700, 520 bps produced by *Klebsiella aerogenes.*
Lane 5: Amplification products of 900, 800, 750, 700 bps produced by *Proteus vulgaris.*
Lane 6: Amplification product of 750 bps produced by *Pseudomonas aeruginosa.*
Lane 7: Amplification products of 3000, 850, 700, 580 bps produced by *Citrobacter freundii.*

The most revolutionary technique has been PCR and it can be used effectively by targeting signature sequences of the target DNA. However, it is usually difficult to find very specific primers that have a single target and do not give non-specific amplifications. This problem can be overcome by use of PCR-ribotyping that is based on exploiting the polymorphism in the 16s-23s intergenic spacer regions. A pattern of multiple bands is produced which is very discriminatory and can be used for confirmation of bacteria not only up to species level but in many cases even beyond.[5, 6]

The present study was designed to establish a new more reliable PCR-ribotyping based identification scheme for common gram negative pathogens including five members of family *Enterobacteriaceae* and *Pseudomonas aeruginosa* that could provide rapid and reliable results.

Our results (Fig-II) showed that these bacteria provide discrete PCR-ribotyping patterns with number of bands varying from one in case of *Pseudomonas aeruginosa* to five in case of *S.* Typhi isolates. *Escherichia coli* gave five discrete amplification products ranging from 700 bps to 1200 bps. As in *E.coli*, four amplification products were observed in *Citrobacter freundii* but the pattern was different and the product size ranged from 580 bps to 3000 bps. A different pattern with four amplification products ranging from 700 t0 900 bps was seen in *Proteus vulgaris. Klebisella aerogenes* also produced four amplification products having a different pattern with bands of 520 to 3000 bps. More discrimination with five amplification products was observed with *Salmonella Typhi.* The product size ranged from 700 to 3000 bps. On the other side of spectrum with *Psedumonas aeurogenosa* only a single amplification product of 750 bps was observed. We used five isolates of each species and results were similar within a species.

The main advantage this technique has over conventional methods is that it can provide results in 24 hours whereas routine culture followed by biochemical tests need 36-48 hours. In addition, ambiguous results which can confuse the diagnosis such as variable level of H_2S and gas production in TSI medium. Further analysis takes more time and becomes rather expensive.

Its advantage over conventional PCR is that instead of setting up reactions for each species or multiplexing them by use of six pairs of primers, a single primer is used. Because more discrimination is available so chances of false-negative results due to non-specific amplification are reduced considerably as well.

We conclude that this technique is superior to conventional methods and PCR in terms of better reliability and speed and can be used for diagnostic and epidemiological purposes.

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