Original Article

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Identification of *Klebsiella* species by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism along with Their Antibiotic Resistance Pattern

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ABSTRACT

Background: *Klebsiella species are closely related bacteria that can cause various multidrug resistant health care infections. The aim of the study was to detect different Klebsiella species by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) along with their antibiotic resistance pattern.*

Materials and methods: *This cross-sectional study was carried out in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh from January 2021 to December 2021.Total 340 different clinical samples were collected. Organisms were isolated and identified by culture, Gram staining and biochemical tests. PCR and RFLP were done to differentiate Klebsiella species. Antibiotic susceptibility pattern was determined by disc-diffusion method.*

Results: *Fifty-seven Klebsiella spp. were isolated and among them 56 (98.25%) were identified as K. pneumoniae and one (1.75%) as K. oxytoca by biochemical tests whereas PCR revealed that 55 (96.49%) were K. pneumoniae, one (1.75%) was K. ozaenae and one (1.75%) was K. oxytoca. Following HaeIII and taqIdigestion, all the K. pneumoniae and K. ozaenae demonstrated a single RFLP pattern whereas K.oxytoca demonstrated different pattern. Regarding antimicrobial resistance pattern, Klebsiella spp. showed higher resistance to ceftazidime and ceftriaxone (91.23%), cefepime (87.72%), aztreonam (68.42%),amikacin (63.16%) followed by imipenem (36.84%) and tigecycline (21.05%).*

Conclusion: *This study portrayed the antibiotic resistance pattern of Klebsiella species demanding the monitoring of strict antibiotic policy. PCR and RFLP may be suitable method to identify Klebsiella species.*

Key words: Antimicrobial susceptibility pattern; *Klebsiella species;* PCR; RFLP.

Introduction

The genus *Klebsiella* is a gram negative, lactose fermenting, non-motile, rod shaped, facultative anaerobic bacilli belonging to the *Enterobacteriaceae* family. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae,* the medically most important species of the genus.[1] It causes a wide range of infections including pneumonia, sepsis, urinary tract infection and wound infection.[2] It is the second most common cause of community and hospital acquired gram negative bloodstream infection.[3]

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K. oxytoca isolated from different clinical specimens mainly from the blood and respiratory secretions is gaining clinical significance in immunocompromised and debilitated patients.[4] *K. rhinoscleromatis* and *K. ozaenae* are associated with chronic diseases of the upper airways, rhinoscleroma and ozaena respectively.[5] Early diagnosis of these two rhinitis is crucial for treatment purpose, as diagnosis delay may have severe consequences.[6] Two more *Klebsiella species, K. quasipneumoniae* and *K. variicola* isolates are often misidentified as *K. pneumoniae* by routine microbiological tests and frequently cause severe life-threatening infections similar to *K. pneumoniae.*[7] Failure to distinguish these species is masking the true clinical significance of each species and their potential epidemiological specificities.[8]

Phenotypic identification is time consuming and may misidentify different *Klebsiella species* due to their close similarity. Genotypic methods have been performed by different typing techniques such as Polymerase chain reaction, Restriction fragment length polymorphism, Pulsed field gel electrophoresis, Multilocus sequence typing etc. However, PCR-RFLP assay represents an excellent complement to the conventional microbiological methods for rapid and species-specific diagnosis of *Klebsiella infection.*[9]

IAHS Medical Journal
Volume 08 Issue 01 June 2025; 26-31

Emergence of multidrug resistance among *Klebsiella species* is becoming a global public health concern. Widespread use of antibiotics in medical practice results in high rate of antibiotic resistance in developing countries.[10] Carbapenems are the beta-lactams of choice for the treatment of infections caused by ESBL producing bacteria.[11,12] Recently, carbapenemase, mainly, *K. pneumoniae* carbapenemase (KPC) and metallo-beta-lactamases (MBL) have become the more prevalent mechanisms for carbapenem resistant *Klebsiella pneumoniae* (CRKP) to hydrolyze cephalosporins, aztreonam and carbapenem.[13]

Along with *K. pneumoniae,* other species of this genus are also able to cause serious infections and delay in diagnosis of these infections may have serious outcome. There is lack of data regarding the prevalence of *Klebsiella* species other than *K. pneumoniae.* Therefore, this study has been carried out to detect different *Klebsiella* species by PCR and RFLP along with their resistance pattern.

Materials and methods

This cross-sectional study was conducted in the Department of Microbiology of Dhaka Medical College, Bangladesh during January 2021 to December 2021. Urine, wound swab endotracheal aspirate blood and sputum samples of clinically suspected infected patients of Inpatient Department of Dhaka Medical College Hospital (DMCH) and samples received in the Microbiology Department for culture and sensitivity were included in this study irrespective of age, sex and antibiotic intake after taking written informed consent. Contaminated samples were excluded from this study.

Identification of *Klebsiella* species

Endotracheal aspirate, urine, sputum and wound swab samples were inoculated in Blood agar and MacConkey agar media followed by overnight incubation at 37°c. For blood samples, primary blood culture was done on Trypticase soy broth followed by subculture on Blood and MacConkey agar media. *Klebsiella* species were identified on the basis of their colony morphology, Gram staining and biochemical tests (Catalase, oxidase, urease, indole test, gas production, motility, lactose fermentation and citrate utilization).

Molecular method

Polymerase Chain Reaction (PCR) andRestriction Fragment Length Polymorphism (RFLP) were done for the detection of *Klebsiella* species.

DNA extraction was done by boiling method.

Table I Primers used for detection of *Klebsiella* species in this study[14-18]

Species	Gene		Sequence	Size (bp)
K. pneumoniae	16s-23s ITS	F	ATTTGAAGAGGTTGCAAACGAT	130
		R	TTCACTCTGAAGTTTTCTTGTGTTC	
K. oxytoca	PehX	F	GATACGGAGTATGCCTTTACGGTG	344
		R	TAGCCTTTATCAAGCGGATACTGG	
K. ozaenae	16s RNA	F	AGGCCTAACACATGCAAGTC	1413
		R	CCTCCCGAAGGTTAAGCTAC	
K. rhinoscleromatis	PhoE	F	GCGGCAGCGACTTCGCCGTA	209
		R	GTTCTGCGCTTTGTTGGCAAAC	
K. variicola	BlaLEN	F	CACGCTGCGYAAACTACTGACYGCGCAGCA	485
		R	AGAAGCATCCTGCTGTGCG	
K. quasipneumoniae	BlaOKP	F	GGCCGGTGAGCGGGGCTCA	348
		R	AGAAGCATCCTGTGCG	

The following cycling parameters were used:

Initial denaturation at 94ºC for one minute, the reaction was subjected to 32 cycles (Annealing at 57ºC for 45 seconds and elongation at 72ºC for one minute) followed by final extension at 72ºC for 10 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under UV light (Figure 1).

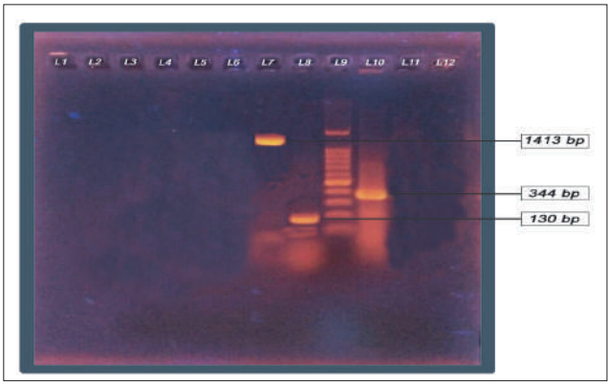

Figure 1 Photograph of gel electrophoresis of amplified DNA of Klebsiella species

Negative control (Lane 5) negative control of Escherichia coli ATCC 25922 (Lane 6) amplified DNA of 1413 bp for ribosomal 16s gene of *K. ozaenae* (Lane 7) DNA of 130 bp for 16s-23s gene of *K. pneumoniae* (Lane 8) 100 bp DNA ladder (Lane 9) and amplified DNA of 344 bp for pehX gene of *K. oxytoca* (Lane 10).

Procedure of RFLP analysis

Amplification in thermal cycler:PCR reaction consisted of preheat at 94°C for 10 minutes followed by 36 cycles of denaturation at 94°C for one minute, annealing at

IAHS Medical Journal
Volume 08 Issue 01 June 2025; 26-31

58°C for 45 seconds and extension at 72°C for two minutes. Primer sequence for gyrAgene 5'CGCG-TACTATACGCCATGAACGTA3' and 3'ACCGTTG-ATCACTTCGGTCAGG5' were used to yield PCR product of 441bp.[19]

All PCR amplicons of gyrA gene were digested with restriction enzyme Hae III and TaqI separately.

Briefly, following restriction condition was used:Ten (10) µl PCR reaction mixture,18 µl Nuclease free water, 2 µl 10X buffer R and 2 µl Restriction enzyme were mixed gently and spinned down for a few seconds. The incubation was done at 37°C for HaeIII and at 65°C for TaqI for 2 hours followed by overnight at room temperature. HaeIII was heat inactivated by incubation at 80°C for 20 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under UV light (Figure 2).

Figure 2 Photograph of TaqIRFLP analysis of amplified DNA of 441 bpgyrA gene of *Klebsiella pneumoniae* (Lane 5) gyrA gene of *Klebsiella ozaenae* (Lane 6) gyrA gene of *Klebsiella oxytoca* (Lane 8) hundred bp DNA ladder (Lane 7) negative control (Lane 3) negative control of Escherichia coli ATCC 25922 (Lane 4)

Antimicrobial susceptibility test

All bacterial isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer modified disc-diffusion technique using Mueller-Hinton agar plates following Clinical and Laboratory Standard Institute (CLSI) guideline 2021 and United States Food and Drug Administration (FDA) guideline for tigecycline. Escherichia coli ATCC 25922 strain was used as control strain to assess the performance of the method.[20,21]

Statistical analysis was performed with SPSS software, versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp.). Continuous data that were normally distributed were summarized in terms of the mean, standard deviation, median, minimum, maximum and number of observations, Categorical or discrete data were summarized in terms of frequency counts and percentages.

All procedures of the present study were carried out in accordance with the principles for human investigations (i.e. Helsinki Declaration) and with the ethical guidelines of the Institutional research ethics. This research protocol was approved by the Research Review Committee and Ethical Review Committee of Dhaka Medical College (Reference number: MEU-DMC/ERC/2021/144).

Results

Table II Different *Klebsiella* species identified by culture, biochemical tests and PCR (N = 57)

Klebsiella species	Culture and biochemical tests n (%)	PCR n (%)
K. pneumoniae	56 (98.25)	55 (96.49)
K. ozaenae	0 (0.00)	1 (1.75)
K. oxytoca	1 (1.75)	1 (1.75)
Total (N)	57 (100.00)	57 (100.00)

N = Total number of *Klebsiella* species

n= Total number of positive cases.

Out of 226 culture positive samples, 57 (25.22%) were identified as *Klebsiella* species. Among the 57 *Klebsiella spp.* 56 (98.25%) were identified as *K. pneumoniae* and one (1.75%) as *K. oxytoca* by biochemical tests whereas by PCR, 55 (96.49%) were identified as *K. pneumoniae,* one (1.75%) as *K. ozaenae* and one (1.75%) as *K. oxytoca*.

Table III RFLP pattern of *Klebsiella* species

Klebsiella species	Fragment size (bp) following *HaeIII* digestion	Fragment size (bp) following *TaqI* digestion
K. pneumoniae	175, 129, 92, 45	197, 142, 93
K. ozaenae	175, 129, 92, 45	197, 142, 93
K. oxytoca	175, 174, 92	197, 142

Following *HaeIII* and *taqI* digestion of *gyrA* gene, all the *K. pneumoniae* and *K. ozaenae* demonstrated a single RFLP pattern whereas *K. oxytoca* demonstrated different pattern.

IAHS Medical Journal
Volume 08 Issue 01 June 2025; 26-31

Table IV Antibiotic resistance pattern of isolated *Klebsiella spp.* (n =57)

Antimicrobial Drugs	*K. pneumoniae* N=55 n (%)	*K. ozaenae* N= 1 n (%)	*K. oxytoca* N= 1 n (%)	Total n= 57 n (%)
Amikacin	36 (65.45)	0 (0.00)	0 (0.00)	36 (63.16)
Amoxiclav	46 (83.64)	1 (100.00)	1 (100.00)	48 (84.21)
Cefepime	48 (87.27)	1 (100.00)	1 (100.00)	50 (87.72)
Ceftazidime	50 (90.91)	1 (100.00)	1 (100.00)	52 (91.23)
Cefuroxime	49 (89.09)	1 (100.00)	1 (100.00)	51 (89.47)
Ceftriaxone	50 (90.91)	1 (100.00)	1 (100.00)	52 (91.23)
Ciprofloxacin	41 (74.55)	0 (0.00)	0 (0.00)	41 (71.93)
Piperacillin/tazobactam	32 (58.18)	1 (100.00)	0 (0.00)	33 (57.89)
Aztreonam	38 (69.09)	1 (100.00)	0 (0.00)	39 (68.42)
Imipenem	21 (38.18)	0 (0.00)	0 (0.00)	21 (36.84)
Meropenem	23 (41.82)	0 (0.00)	0 (0.00)	23 (40.35)
Tigecycline	12 (21.82)	0 (0.00)	0 (0.00)	12 (21.05)

Among the 55 isolated *K. pneumoniae,* 90.91% were resistant to ceftazidime and ceftriaxone, followed by 74.55% to ciprofloxacin, 69.09% to aztreonam, 65.45% to amikacin, 38.18% to imipenem and 21.82% to tigecycline. *K. ozaenae* was resistant to cephalosporins, amoxiclav, piperacillin-tazobactam and aztreonam and *K. oxytoca* was resistant to cephalosporins and amoxiclav.

Discussion

Klebsiella have been considered as one of the major opportunistic pathogens that cause a range of clinical diseases including nosocomial pneumonia, urinary tract infections and bacteremia in immunocompromised human.[22] The increasing rate of multidrug resistant *K. pneumoniae* is a major challenge in hospital settings.[23]

In this study, 25.22% *Klebsiella* species were identified. Sodhi et al.[24] in India reported the prevalence of *Klebsiella* species as 21.33%.The increasing prevalence rate of *Klebsiella* among clinical patientsis alarming as 24% and 19.72% prevalence rates were reported from northeastern and southeastern part of Bangladesh.[25,26] These findings are in accordance with the present study.

In the present study, among 57 isolated *Klebsiella* species, 56 (98.25%) were identified as *K. pneumoniae* and one (1.75%) as *K. oxytocaby* biochemical tests. By PCR, 55 (96.49%) were identified as *K. pneumoniae,* one (1.75%) as *K. oxytoca* and one (1.75%) as *K. ozaenae* which was misidentified biochemically as *K. pneumoniae. K. ozaenae* could not be differentiated from *K. pneumoniae* as they often present similar biochemical patterns[27].

A study in South Africa reported that 83.7% were *K. pneumoniae* followed by 14.4% were *K. oxytoca.*[28] Previous study in Dhaka Medical College Hospital reported that, 44 (95.65%) were identified as *K. pneumoniae* and 2 (4.35%) as *K. oxytoca.*[29] Another study in India by Singh reported the prevalence of *K. pneumoniae* as 96.48% and *K. oxytoca* as 3.52%. This similarity may be attributed to the fact that these two studies were conducted in same geographic areas.

In the present study, *K. pneumoniae* and *K. ozaenae* could not be differentiated by RFLP as both yielded similar sized bands. However, *K. oxytoca* could be differentiated as it yielded different patterns of bands. These findings are in accordance with the study of Brisseet al.[30] *K. ozaenae* can be differentiated by using genes other than gyrA gene. However, digestion of other genes by restriction enzymes was not done in the present study.

In this study, *K. pneumoniae* seemed to be highly resistant to cephalosporins, aztreonam, amikacin and ciprofloxacin. These findings can be correlated with previous studies in DMCH who reported that 90.67% *K. pneumoniae* were resistant to ceftriaxone, followed by 72% to amikacin, 70.45% to aztreonam and 88% to ciprofloxacin.[31] High resistance to cephalosporins and quinolone could be due to extensive use of these drugs in past few years.[32] Besides, antibiotic resistance in Bangladesh and developing countries commonly occurs due to inappropriate antibiotic use, over prescribing, unethical practices of health professionals, unqualified drug sellers offering alternative drugs when the prescribed drugs are unavailable.[33]

In the present study, 38.18% *K. pneumoniae* were resistant to imipenem which is in agreement with the study by Indrajith et al. who also reported that 38% *K. pneumoniae* showed resistance to imipenem.[34] However, A previous studyin DMCH showed 20.45% resistance.[29] Probably, the frequency of imipenem resistance is increasing in Bangladesh due to irrational use of antibiotic and also due to horizontal transfer of plasmid encoded genes responsible for carbapenemase production.

Limitation

All other species of *Klebsiella* could not be detected due to time and resource constraints.

Conclusion

Among the isolated *Klebsiella* species, *K. pneumoniae* was the most common organism causing various infections. Increasing resistance to commonly used antibiotics found in this study is alarming. Antibiotic stewardship programme should be implemented to reduce resistance and to mitigate the spread of multidrug resistant strains.

IAHS Medical Journal
Volume 08 Issue 01 June 2025; 26-31

Recommendation

Amplification and digestion of gene other than gyrAcan be done to differentiate *K. pneumoniae* from *K. ozaenae.*

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Disclosure

The authors declare no conflict of interest.

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IAHS Medical Journal
Volume 08 Issue 01 June 2025; 26-31

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