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Antibiotic Susceptibility Test of *Klebsiella pneumoniae* and *K. oxytoca* Isolated from Different Clinical Samples and Perform Random Amplified Polymorphic DNA among *K. pneumoniae*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the prevalence of MDR and ESBL producing *Klebsiella pneumoniae* and *Klebsiella oxytoca* and perform RAPD (Random amplified polymorphic DNA) by optimizing PCR among isolated ESBL producing *Klebsiella pneumoniae*.
Place and Duration of Study: This study was done to assess the prevalence of MDR and ESBL producing *Klebsiella pneumoniae* and *K. oxytoca* in urine, pus and sputum from March 2013 to April 2014 at KIST Medical College and PCR was performed at Nepal Academy of Science and Technology upto March, 2015.
Methodology: *K. pneumoniae* and *K. oxytoca* were isolated from urine, pus and sputum samples from KIST Medical College, Lalitpur, Nepal. Antibiotic susceptibility test was performed by using disk diffusion method. MDR isolates which were suspected as ESBL producers were confirmed by using double disk diffusion test and combined disk diffusion test for same isolates. Chromosomal

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DNA was isolated from ESBL producing *K. pneumoniae*. PCR was optimized by varying different reagents and visualized using gel electrophorosis. Under optimized condition chromosomal DNA was amplified, gel electrophoresis was performed and polymorphism was detected.

Results: The drug resistance pattern of *K. oxytoca* was high as compared to *K. pneumoniae*. In urine, pus and sputum samples the growth of both organisms was very much low i.e. 2.5% among total samples. 90% of *K. pneumoniae* isolated from urine were ESBL producers whereas only 42.85% of *K. pneumoniae* from pus and sputum were ESBL producers. *K. oxytoca* isolated from all samples were MDR as well as ESBL producers. Chromosomal DNA analysis of different antibiotic resistance pattern among *K. pneumoniae* showed higher the drug resistance lowers the polymorphism.

Conclusions: This study showed that higher the drug resistance lesser the polymorphism and greater the adaptability towards used antibiotics by production of antibiotic hydrolyzing enzymes. So, further study is required for the determination of polymorphism and drug resistance pattern correlation with organisms' adaptability to validate this concept.

Keywords: MDR; ESBL; DNA; PCR; RAPD.

1. INTRODUCTION

Among *Klebsiella* species *K. pneumoniae* and *K. oxytoca* are most commonly isolated members which are common pathogens that cause community or hospital acquired lobar pneumonia, wound infections, hospital acquired infections, meningitis, abscesses, urinary tract infections, diarrhea, liver abscesses septicemia. The genus *Klebsiella* includes *K. pneumoniae* subsp. *pneumoniae*, *K. oxytoca*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, *K. ornitholytica*, *K. planticola* and *K. terrigena* [1]. Therapeutic options for the infection with *K. pneumoniae* and *K. oxytoca* is limited not only due to production of ESBL, AmpC, carbapenemases etc but also by frequent co-resistance to other antibiotics [2]. It is expected that MDR *K. pneumoniae* and *K. oxytoca* will become more and more resistant to antimicrobials with the passage of time because of generation of new mutant strains [3]. The antimicrobial resistance is not only increasing morbidity and mortality but also great economic loss encompassing use of more expensive antibiotics to treat infection as well as threat of resistance to them [4].

K. pneumoniae and *K. oxytoca* are gram negative, oxidase negative, non-motile, encapsulated, lactose-fermenting facultative anaerobic, non-spore forming, rod shaped bacteria. These are frequently found in human faeces, sometimes pharynx and in environment. Clinically the majority of the human infections is caused by *K. pneumoniae* followed by *K. oxytoca*. Infections are common in very young, very old, invasive medical device user and immune-compromised patients or patients with chronic diseases [5].

Those organisms, which showed resistance to at least one agent in three or more antimicrobial categories, are considered multidrug resistant (MDR) bacteria [6].

β-lactamase are enzymes produced by microorganisms which hydrolyze the β-lactam ring of β-lactam antibiotics rendering then ineffective. β-lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactams [7]. Extensive use of broad spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* and development of multidrug resistance (MDR) strains that produce extended spectrum β-lactamase (ESBL) [8]. ESBLs are those enzymes, which can hydrolyze oxyimino-β-lactams, which include cefotaxime, ceftriaxone, ceftazidime and aztreonam (but not the cephamycins and carbapenems) and are inhibited by clavulanic acid [9]. ESBLs are chromosomal or Plasmid mediated β-lactamseses, which have mutated from the pre-existing broad-spectrum β-lactamseses TEM-1, TEM-2 and SHV-3 [10].

Arbitrary amplification of polymorphic DNA sequences, termed as Random amplified polymorphic DNA (RAPD) analysis or arbitrarily primed PCR (AP-PCR) typing is a technique that is being used in many epidemiological studies [11]. RAPD is economical, easier and faster but the reproducibility of this method is challenging. After optimization, RAPD has been suggested as a reliable, sensitive and reproducible assay for molecular typing of bacteria [12,13]. This

technique involves the amplification of random segments of genomic DNA by polymerase chain reaction, using short primer (4-10 bp) of arbitrary sequence [14,15]. The amplified region is RAPD locus and the variation is due to loss of RAPD loci [11]. This study was carried out to find out multidrug resistance and ESBL production of *K. pneumoniae* and *K. oxytoca*, and optimization of RAPD to find out relationship between antibiotic resistance and polymorphism among *K. pneumoniae* isolates.

2. MATERIALS AND METHODS

The study approved by ethical review board of Nepal Health Research council (NHRC), Nepal and was conducted in Microbiological Laboratory of KIST Medical College, and Nepal Academy of Science and Technology, Lalitpur from March 2013- April 2014 and PCR was performed up to March 2015. Written informed consents were obtained from all patients prior to their inclusion in study. A total of 801 samples including 580 urine, and 221 (pus and sputum) were processed.

2.1 Identification of Organisms

Urine (580), sputum (124) and pus (97) samples were collected from both inpatients and outpatients seeking treatment in KIST Medical College. Urine was inoculated aseptically on blood agar, and MacConkey agar, whereas sputum and pus were inoculated in Blood agar (BA), Chocolate agar (CA) and Mac-Conkey agar (MA) at 37°C for 24 hours. Urine samples were inoculated using 4 mm nichrome wire. In urine growth of 10^5 CFU/ml were regarded as significant bacteriuria. *K. pneumoniae* and *K. oxytoca* were identified based on cultural characteristics, morphological characterstics, and various Biochemical tests like Indole, Methyl red, Voges-proskauer and citrate, TSI [Triple sugar iron], O/F [Oxidation/fermentation], and urease. Indole negative *Klebsiella* isolates were identified as *K. pneumoniae* whereas positive isolates were identified as *K. oxytoca*. The mucoid and smooth colonies were stained by using india ink for the presence of capsule.

2.2 Antimicrobial Susceptibility Tests

Antimicrobial susceptibility testing was performed on *K. pneumoniae* and *K. oxytoca* following guidelines of Clinical and Laboratory Standard Institute 2012 [16]. The inoculums used for susceptibility testing was prepared in nutrient broth taking 5/6 colony of *K. pneumoniae* and *K. oxytoca* that matched to 0.5 McFarland standard (1.5 X 10^8 CFU/ml). Within 15 minutes, a sterile cotton swab was dipped into the inoculums suspension and pressed inside the wall of tube above the fluid level and inoculated at 60° over the dried surface of Muller-Hilton agar (MHA) plate. After 3-5 minutes antibiotic disc were applied and gently pressed down to ensure complete contact with agar. Those organisms, which showed resistance to at least one agent in three or more antimicrobial categories were considered multidrug resistant bacteria [6,17]. The antibiotic disc used for urine were ampicillin (10 mcg/disc), cotrimoxazole (25 mcg/disc), norfloxacin (10 mcg/disc), imipenum (10 mcg/disc), amikacin (30 mcg/disc) ofloxacin (5 mcg/disc), cefazoline (30 mcg/disc), nitrofurantoin (300 mcg/disc), cefotaxime (30 mcg/disc), ceftriaxone (30 mcg/disc), ceftazidime, gentamycin (10 µg). For pus and sputum ampicillin (10 mcg/disc), cotrimoxazole (25 mcg/disc), ciprofloxacin, amikacin (30 mcg/disc), imipenum (10 mcg/disc), cefotaxime (30 mcg/disc), ceftriaxone (30 mcg/disc), ceftazidime, gentamycin (10 µg) were used. Zone size measured in mm was tallied with interpretative criteria CLSI 2012 and isolates were identified as susceptible, intermediate and resistant. *K. Pneumoniae* ATCC 62003 was used as control strains [18].

2.3 Phenotypic Characterization of ESBL

2.3.1 Screening for ESBL

Isolates resistant to ceftazidime, cefotaxime and ceftriaxone in antibiotic susceptibility test were identified as possible ESBL producers [19]. ESBL productions by these isolates were confirmed by confirmatory tests following CLSI 2012.

2.3.2 Confirmatory test for ESBL

The confirmation of ESBL production was done by two phenotypic confirmation methods.

2.3.2.1 Double disk diffusion [Double disk approximation/double disk synergy test (DDST)]

MHA plates were inoculated with 0.5 McFarland matched test inoculums. On inoculated plate amoxicillin-clavulanate (20 µg/10 µg) was placed at center and on sides ceftadizime (30 µg) and cefotaxime (30 µg) were placed 30 mm apart

from center to center. They were incubated at 37°C for 18-24 hrs. Those inoculums which exhibited an enhanced zone of inhibition, the synergism in between Amoxacillin/clavulanic acid and cefotaxime or ceftadizime was identified as confirmed ESBL producers. In doubtful cases distance between amoxicillin-clavulanate, ceftadizime and cefotaxime was reduced to 20 mm apart from center to center [20,21].

2.3.2.2 Combined disk method

On a MHA plate inoculated with possible ESBL producers, that matched to 0.5 McFarland, ceftazidime (30 µg) and ceftadizime with clavulanic acid and cefotaxime (30 µg) and cefotaxime with clavulanic acid (30 µg/10 µg were placed 30 mm apart center to center. Plates were incubated at 37°C for 18-24 hrs. An increase in zone of inhibition by ≥ 5 mm to ceftazidime clavulanic acid compared to that of ceftazidime alone and cefotaxime clavulanic acid with cefotaxime alone was interpreted as confirmed ESBL producers. The test was run parallel with *K. pneumoniae* ATCC 700603 ESBL producing isolate for quality control [18,20,21].

2.4 DNA Extraction

DNA was extracted in distilled water by direct boiling method. *K. pneumoniae* with multiple drug resistance were taken. They were cultured overnight on nutrient agar at 37°C. One loopful of each bacterial colonies was transferred to 1 ml distilled water and vortexed in microfuge tube sealed with O ring till homogeneously mixed. To inactivate bacterial cells tubes were submerged in a boiling water bath for 10 min. Inactivated bacterial suspensions were centrifuged for 5 min at 17,900 or 13000 g for 10 min. Supernatant was transferred to fresh microfuge tube and stored at fridge at -20°C for further use in PCR experiments [22,23].

2.4.1 Estimation of DNA

The quantification of DNA as well as quality assessment was carried out by spectrophotometrically by using Biophotometer (Eppendrof- AG 2231, Germany).

2.4.2 Gel electrophoresis

The quality of extracted DNA was assessed by electrophoresis in 1.2% agarose gel. The gel was stained with ethidium bromide (10 mg/ml) and poured in the slab with comb. After gelling, the comb was removed and placed in electrophorotic tank which was covered with 1x TAE buffer up to 1 mm. The DNA sample (10 µl) was prepared mixing with loading dye and loaded using micropipette. Current of 55 to 80 V was applied and after 2/3rd migration of loading dye in gel, the electricity supply was turned off and the gel was observed under UV chamber.

2.4.3 DNA purification

DNA was purified in according to Karimnasab et al. [22] Ausubel et al. [24], Chomczynski et al. [25].

2.4.4 Primers used

Four primers were used during optimization and all of then gave multiple distinct bands. Primers of 60-80% GC rich were used. Total PCR volume was made 25 µl by varying different concentrations of reagents. Primers used were as follows

RBA 2: 5'ACA GGG CTC T 3'
RBA 8: 5'ATC GGG TCG A3'
RBA 10: 5'CCC GCC TTC C 3'
RBA12: 5'CCG GCC TTAA 3'

2.4.5 PCR optimization

PCR reaction was performed in 25 µl reaction volume. RAPD-PCR optimization was done by varying different concentrations of isolated DNA, Buffer with $MgCl_2$, primers, Taq polymerase, dNTPs in accordance to Weeden et al. [26], Sambrok et al. [27] and Sijapati et al. [15].

DNA **Concentration used (ng) for each RAPD reaction:** 10, 20, 30, 40, 50, 60, 70, 80 90, 100 1

X **Buffer with 1.5 mM $MgCl_2$ (µl):** 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5

dNTPs Concentration 2.5 mM (µl) : 0.5 ,1, 1.5, 2, 2.5, 3, 3.5, 4,

Primer concentration (10picomole/ µl) Diluted in 100 µl sterile water (µl): 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4

Taq polymerase concentration (U):0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5

2.4.5.1 PCR programs used

(a) 4 min of initial denaturation at 94°C, denaturation at 94°C for 45 sec, 1 min

annealing at 37°C, and extension at 72°C for 2 min, for 10 cycles followed by denaturation at 94°C for 45 sec, annealing at 37°C for 1 min, extension at 72°C for 2 min and final extension for 10 min followed by 30 cycles [27].

(b) 3 minute of initial denaturation at 95°C, 40 cycles of denaturation at 95°C for 1 min, annealing at 34°C for 2-minute extension at 72°C and the final extension at 72°C for 5 min [28].

(c) 1 minute of initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 minute a, annealing at 36°C for 1 min, 2 minute extension at 72°C followed by 72°C of final extension for 7 minutes [29].

3. RESULTS AND DISCUSSION

3.1 Antibiotic Susceptibility Pattern of *K. pneumoniae* and *K. oxytoca*

Among 801 samples, 580 were urine whereas 221 were pus and sputum. Samples were collected from patients between 6 months to 90 years of both sexes. A total of 10 *K. pneumoniae* were isolated from 580 urine samples. They were 100% resistant to ampicillin, 70% to cefazoline, nitrofurantoin, ofloxacin, 90% to cefotaxime, 90% to ceftriaxone and 80% to ceftazidime. The most effective drug was found to be imipenem followed by amikacin. Refer Table 1.

Among 2 urinary isolates of *K. oxytoca*, the AST pattern showed that they were 100% resistant to all antibiotics tested except imipenem and amikacin in which they were 100% susceptible. They were resistant to all third generation cephalosporins used.

3.2 Antibiotic Susceptibility Pattern of *K. pneumoniae* and *K. oxytoca* Isolated from Pus and Sputum

Among 221 samples of (97) pus and (124) sputum 3.16% (7/221) were *K. pneumoniae* and only one was *K. oxytoca.* These samples were tested for antibiotic susceptibility profile. 100% of isolates were resistant to Ampicillin, cefazoline, cefepime, cefotaxime, ceftriaxone and ceftadizime followed by 85.7% to ciprofloxacin and cotrimoxazole. The most effective antibiotic was gentamycin (57% sensitive) followed by imipemen and amikacin (28.2%). Refer Table 2.

Only one *K. oxytoca* was isolated from sputum among all the samples. It was resistant to all antibiotics except imipenem and amakacin. Hence, was identified as MDR.

3.3 ESBL Pattern among MDR Isolates *K. pneumoniae* and *K. oxytoca* from Urine

Both DDST and combined disk diffusion tests were performed for ESBL confirmation. Reproducible result was obtained during repetition. Among 12 isolates (*K. pneumoniae* (10) and *K. oxytoca* 2), 11 isolates were MDR in which 9 were *K. pneumoniae* and 2 were *K. oxytoca*. All MDR *K. pneumoniae* and *K. oxytoca* were ESBL producers.

3.4 ESBL Pattern among MDR Isolates *K. pneumoniae* and *K. oxytoca* from Pus and Sputum

By following both methods ESBL were detected. All isolates were MDR. Among 8 isolates 7 were *K. pneumoniae* and one was *K. ocytoca*. Among 7 *K. pneumoniae*, 3 isolates showed ESBL production whereas 4 did not. Only one *K. oxytoca* was isolated during study period that was ESBL producers.

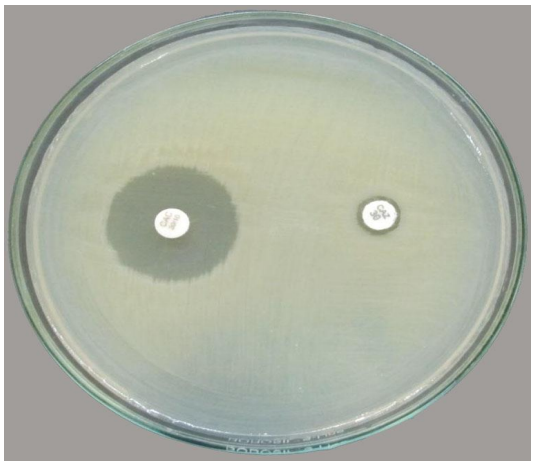


Fig. 1. Confirmatory test for ESBL by using ceftazidime and ceftazidime with clavulanic acid

3.5 DNA Extraction Protocol

Three solutions (TE buffer with RNase, TE buffer, and Distilled water) were used for the extraction of DNA. DNA was extracted by direct boiling method. DNA extracted in distilled water was found more in quantity and gave good bands

then other two solutions. The absorbance ration of DNA at A_{260}/A_{280} was in between 1.7 to 2 in distilled in almost samples in distilled water. Extracted DNA was then purified and used for RAPD.

3.6 Optimization of RAPD-PCR Reaction

For RAPD-PCR, numbers of parameters like isolation of DNA, template DNA concentration and its purification, Taq DNA polymerase concentrations, dNTP mix concentration, Random primer concentration, Taq polymerase assay buffer with $MgCl_2$ need to be optimized as it is highly sensitive to reactions. Cycling parameters are most influencing factors, if changed it can lead to non-reproducible result [26]. Optimized RAPD-PCR reactions and cycling parameter are shown below in Table 3.

S. N	PCR parameters	Tested range (Concentration/ RAPD)	Optimized at
1	DNA concentration (ng)	10, 20, 30, 40, 50, 60, 70, 80, 90, 100	20 ng
2	dNTP mix (2.5 mM each) µl	0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5	1.5 µl
3	Random primer (ng) 100 ng/ µl	50, 75, 100, 125, 150, 175, 200, 250	100 ng
4	Taq polymerase buffer with $MgCl_2$ (1X) µl	0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5,	2.5 µl
5	Taq polymerase (U)	0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5	1.5 U

Table 1. AST pattern of *K. pneumoniae* isolated from urine

K. pneumoniae	Antibiotic susceptibility pattern (%/ No)												
(10) Pattern	AMP	COT	NX	NA	CZ	NIT	IPM	AK	OF	CTX	CTR	CAZ	GEN
Sensitive	-	60 (6)	50 (5)	60 (6)	30 (3)	30 (3)	90 (9)	70 (7)	33.0 (3)	10 (1)	10 (1)	(2)	60 (6)
Intermediate	-	-	-	-	-	-	-	-	-	-	-	-	-
Resistant	*100 (10)*	40 (4)	50 (5)	40 (4)	70 (7)	70 (7)	10 (1)	30 (3)	70 (7)	90 (1)	90 (1)	80 (2)	40 (4)

Note: Amp:-Ampicillin, CIP:-Ciprofloxacin, COT:-Cotrimoxazole, NX:-Norfloxacin, NA:-Nalidixic Acid, CZ:-Cefazoline, NIT:-Nitrofurantoin, IPM:- Imipenem, Ak:- Amikacin, OF:- Ofloxacin, CTX:-Cefotaxime, CTR:-Ceftriaxone, CAZ:-Ceftazidime, GEN:-Gentamycin.

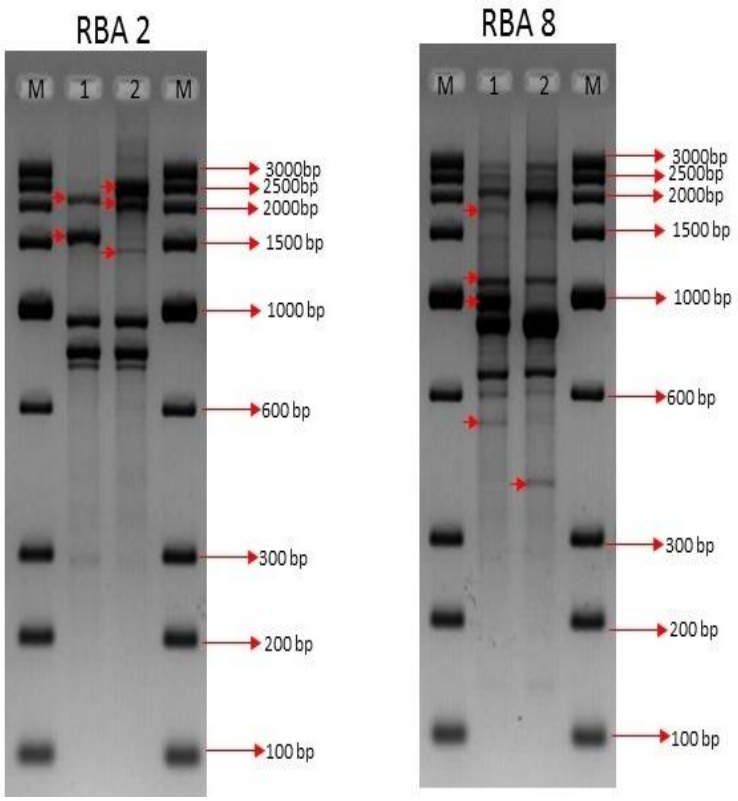
Table 2. Antibiotic susceptibility testing pattern of *K.pneumoniae* isolated from pus and sputum

K. pneumoniae	Antibiotic susceptibility pattern in percentage										
(7)	Amp	CIP	COT	CZ	CPM	IPM	Ak	CTX	CTR	CAZ	GEN
Sensitive	-	14.3 (1)	14.3 (1)	-	-	28.2 (2)	28.2 (2)	-	-	-	57 (4)
Intermediate	-	-	-	-	-	-	-	-	-	-	-
Resistant	*100 (7)*	85.7 (6)	85.7 (6)	100 (7)	100 (7)	71.8 (5)	71.8 (5)	100 (7)	100 (7)	100 (7)	43 (3)

Note: Amp:-Ampicillin, CIP:-Ciprofloxacin, COT:-Cotrimoxazole, CZ:-Cefazoline, IPM:- Imipenem, Ak:- Amikacin, CTX:-Cefotaxime, CTR:-Ceftriaxone, CAZ:-Ceftazidime, GEN:-Gentamycin

Table 3. PCR cycling parameters

94°C	94°C	38°C	72°C	94°C	45°C	72°C	72°C	4°C
5 min	45 sec	1 min	2 min	45 sec	1 min	2 min	10 min	∞
	10 cycles			35 cycles				



Figs. 2 and 3. RAPD of two samples using RBA 2 and RBA 8 respectively (red arrow shows polymorphisms)

Fig 2: ***Lane 1:*** *RAPD of sample 195 with RBA 2 primer,* ***Lane 2:*** *RAPD of sample 205 with RBA 2 primer*
Fig 3: ***Lane 1*** *RAPD sample of 195 with RBA 8 primer,* ***Lane 2*** *RAPD sample of 205 with RBA 8 primer*

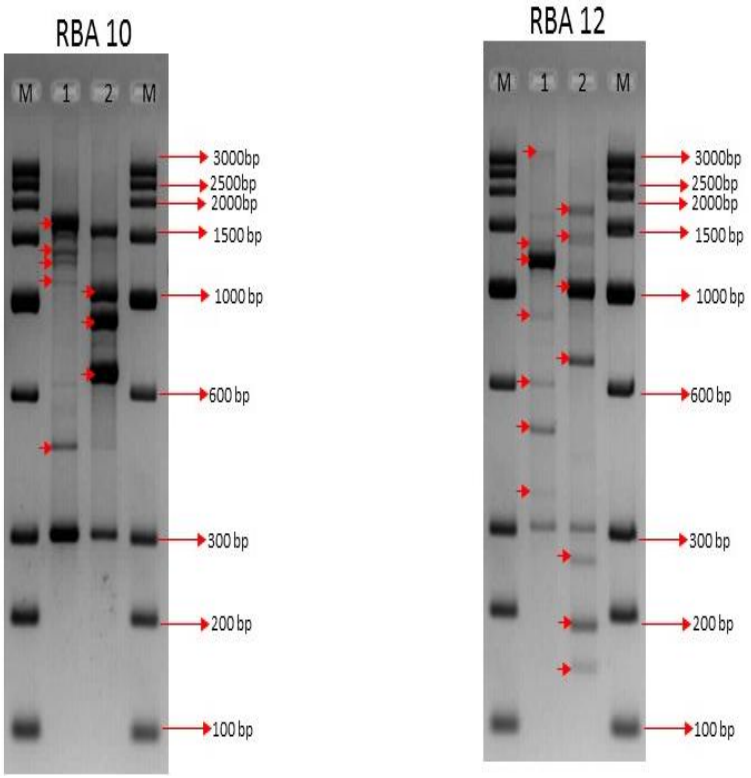
3.7 PCR Programs Optimized at which Talley with Sambrook et al. [28]

The polymorphic DNA bands obtained after of RAPD-PCR using 4 primers is shown below. Only two samples of *K. pneumoniae* 195 and 205 were selected for RAPD-PCR. Both isolates differed in their antibiotic susceptibility pattern. Isolate 195 was susceptible to most antibiotics tested and 205 was resistant to the used antibiotics.

4. DISCUSSION

K. pneumoniae and *K. oxytoca* are most commonly encountered organisms in clinical medicine causing a wide range of clinical conditions from relatively mild to serious, sometimes life-threatening conditions that can lead to death. In this study both *Klebsiella* spps were isolated from different age groups and from both male and female. Among total of 20 isolates 19 (95%) of the isolates were MDR. The drug resistance pattern of *K. oxytoca* was high as compared to *K. pneumoniae*. In urine, pus and sputum samples the growth of both organisms was very much low i.e. 2.5% among total samples. Although these organisms are most frequently isolated from these samples, the less growth percentage may due to previous exposure of patients to used antibiotics that hindered their growth or dominance of organisms growth.

In this study *a* total of 10 *K. pneumoniae* were isolated from 580 urine samples. Among the 10 *K. pneumoniae* isolated, they were 100% resistant to ampicillin, 70% to cefazoline, nitrofurantoin, ofloxacin, 80% to cefotaxime, 50% to ceftriaxone and 60% to ceftazidime. The most effective antibiotic was imipenem (90%) followed by amikacin (7%). Among 2 isolates of *K. oxytoca* of urine, 100% isolates were resistant to all antibiotics tested except imipenum and amikacin in which they were susceptible. Nine of



Figs. 4 and 5. RAPD of two samples using RBA 10 and RBA 12 respectively (red arrow shows polymorphisms)

Fig 4: Lane 1: *RAPD of sample 195 with RBA 10 primer,* ***Lane 2:*** *RAPD of sample 205 with RBA 10 primer,*
Fig 5: Lane 1 *RAPD sample of 195 with RBA 12 primer,* ***Lane 2*** *RAPD sample of 205 with RBA 12 primer*

ten (90%) of *K. pneumoniae* isolated from urine were MDR whereas cent-percent *K. oxytoca* was MDR. *K. pneumoniae* from urine were resistant to cefotaxime, ceftadizime and ceftriaxone. This indicates that CTX, SHV, TEM genes are homogeneously distributed. *K. oxytoca* which was highly resistant to all antibiotics except imienem and amikacin was found ESBL producers. Similar finding has been reported in a study conducted in TUTH where 100% of *Klebsiella* spps isolates were MDR [30].

Among 221 samples of pus and sputum, 7 (41.17%) were *K. pneumoniae* and only one was *K. oxytoca.* All isolates were MDR. 100% of isolates were resistant to Ampicillin, cefazoline, cefepime, cefotaxime, ceftriaxone and ceftadizime followed by 85.7% to ciprofloxacin and cotrimoxazole. The most effective drug was gentamycin (57% sensitive) followed by imipemen and amikacin. This indicates that gentamycin can be a drug for people infected by *K. pneumoniae* and *K. oxytoca* from samples pus and sputum. In the similar study conducted in India, only four druges Amikacin (88.9%), Galtifloxacin (77.8%), Gentamycin (57.8%) and to chloramphenicol (55.6%) were effective against *K. pneumoniae* [31].

Among 7 *K. pneumoniae* isolates 3(42.85) were ESBL producers. As compared to that of urine, *Klebsiella* isolates isolated from pus and sputum were more resistant to commonly used antibiotics. One *K. oxytoca* was isolated which was ESBL producer. Carbapenems are the treatment of choice for serious infections caused by ESBLs, but the prevalence of carbapenemase producing *K. pneumoniae* and *K. oxytoca* is increasing day by day. Similar study has been done in Sudan where 68.8% of the isolated *K. pneumoniae* were ESBL producers whereas 28.6% *K. oxytoca* were ESBL producers [32]. The present study shows that gentamycin can be a choice of drug for *K. pneumoniae* isolated from pus and sputum followed by imipenem and amikacin. This may be due to prevalence of carbapenemase producing *K. pneumoniae* (KPC).

The antibiotic resistance is increasing rapidly. ESBL cases are profoundly predominant among *Klebsiella* spp. ESBL producers are sensitive to carbapenems. Increase in Carbapenamases producing *Klebsiella* spps are creating the treatment problem complicated with morbidity, mortality as well as great economic loss. *K. pneumoniae* producing carbapenemase are resistant to carbapenem leaving a little choice of antibiotics. The best therapeautic agents against KPC are polymyxins, tigecycline and less frequently some aminoglycosides [33]. Hence, continuous monitoring systems and effective infection control measures to prevent the rapid and worldwide spread of MDR and ESBLs are necessary to avoid treatment failure in different patients. The early detection and use of complete course of suitable antibiotics can reduce antibiotic resistance.

Clinical isolate 195 showed resistance to ampicillin, nitrofurantoin, cefazoline,ceftadizime, cefoperazone, cefotaxime, ceftriaxone and sensitive to gentamycin, ciprofloxacin, cotrimoxazole, norfloxacin, nalidixic acid, ofloxacin, imipenem and amikacin. Another clinical isolate, 205 which was sensitive to only one drug imipenem. Both were ESBL producers. Isolates with wide range of variation in antibiotic susceptibility and resistance cases was taken to know genetic differences between 2 isolates of *K. pneumoniae*. Sample 195, which was more sensitive many antibiotics, showed more polymorphism than that of sample 205 which was sensitive only to imipenem. The amplified region is RAPD locus and the variation is due to loss of RAPD loci [34]. Two isolates belongs to 2 different clusters showing that they were not genetically related. This is also evidenced by the antibiotic susceptibility pattern. Due to optimization, though the experiment was repeated it gave reproducible result. This research showed that higher the antibiotic resistance pattern, lower the polymorphism. All tested parameters for RAPD-PCR like template DNA, Primer, Taq polymerase, dNTPs, temperature, PCR programs, Taq polymerase assay buffer which were optimized during study and produced clear, multiple and scoreable bands. Hence, this method can be used for further research in molecular epidemiology and genetic diversity in *K. pneumoniae* in Nepal.

5. CONCLUSION

Out of 801 samples of urine pus and sputum 17 *K. pneumoniae* and 3 *K. oxytoca* were found. Of 17 *K. pneumoniae* 16 were MDR, in which 16 were also possible ESBL producers and 12 were confirmed as ESBL producers. Out of 3 *K. oxytoca* isolated all were confirmed as ESBL producers. *K. oxytoca* was highly resistant to antibiotics used. All 3 were MDR and confirmed as ESBL producers. Imipinem, amikacin and gentamycin are the choice of drugs but resistance of these drugs is also found prevalent so it can be problematic in future days. Higher polymorphic organisms have their more genetic originality so may be lesser the antibiotic resistance but further research is required. The primers used in this study can be used for further study in molecular comparison among drug resistance, their diversity, molecular epidemiology, and to find the comparative and evolutionary trend of drug resistance. The used PCR program and different reagents concentration can be a basis for further study of *K. pneumoniae* in genetic level to compare antibiotic resistance pattern and polymorphism.

ETHICAL APPROVAL

This research was approved by Nepal Health Research Council ethical review board Nepal. Written consent was taken from the patients during this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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