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PREVALENCE OF EXTENDED SPECTRM β-LACTAMASE AND METALLO β-LACTAMASE GENES AMONG CLINICAL ISOLATES OF NONFERMENTING GRAM NEGATIVE BACTERIA

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ABSTRACT

Aim: *In Gram negative bacteria resistant to extended spectrum β-lactam antibiotics has been emerging rapidly worldwide over the last few years and has been ascribed predominantly to the production of extended spectrum β-lactamases (ESBLs) and Metallo β-lactamases (MBLs). Non-fermenters are very popular to produce extended spectrum β-lactamase and metallo β-lactamase. This study was designed to investigate the prevalence of different extended spectrum β-lactamases and Metallo β-lactamases encoding genes and drug resistant profiles among isolated non-fermenters.* ***Methods:*** *In this study non-fermenters were isolated from different clinical specimens like pus, stool and urine and were identified according to biochemical characteristics and 16s rRNA gene sequencing. Isolated non-fermenters were subjected for phenotypic and genotypic identification of extended spectrum β-lactamases and Metallo β-lactamases by using combined disk diffusion test, multiplex and simplex PCR amplifications.* ***Results:*** *Total 94 non-fermentative bacterial pathogens were identified with isolation rate of 24.86%. Pseudomonas aeruginosa and Acinetobacter baumannii were most predominant nonfermentative bacterial pathogens. Out of 94 isolates, 5 were found positive for both phenotypically and genotypically production of extended spectrum β-lactamases and metallo β-lactamases. Pseudomonas aeruginosa and Acinetobacter baumannii were having multiple β-lactamases genes name ^{bla}NDM, ^{bla}OXA-10 and ^{bla}SHV.* ***Conclusion:*** *Non-fermenters like Pseudomonas aeruginosa and Acinetobacter baumannii were having multiple β-lactamases genes like ^{bla}NDM, ^{bla}OXA-10 and ^{bla}SHV which highlight the emerging therapeutic challenge.*

Key words: Non-fermenters, ESBLs, MBLs, ^{bla}NDM-1

KEY WORDS

Gram negative bacteria, NDM-1

INTRODUCTION:

Heterogeneous group of aerobic non-spore forming Gram negative bacilli that either incapable of catabolized carbohydrates as source of energy or degrade them via oxidative rather than fermentative metabolic pathways are called non-fermenters. In Gram negative bacteria resistant to extended spectrum β-lactam antibiotics has been emerging rapidly worldwide over the last few years and has been ascribed predominantly to the production of extended spectrum β-lactamases (ESBLs) and Metallo β-lactamases (MBLs). Non-fermenters are very popular to produce extended spectrum β-lactamase and metallo β-lactamase [1]. *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

are two most common frequently isolated non-fermenters that produce ESBLs and MBLs [2, 3].

Generally TEM, SHV and CTX-M-type genes are responsible to encoded Extended-spectrum β-lactamases (ESBLs) which are resistance to 3rd generation cephalosporins and penicillins. [4, 5]. In case of metallo β-lactamases (MBLs) which are sorts of powerful enzymes known as carbapenemases responsible for resistance against penicillins and cephalosporins [6]. Four groups of these enzymes have been described in non-fermenters, including IMP, SIM-1, NDM-type and VIM like carbapenemases.

Utmost resistance in this group of organisms is mostly seen in patient with prolonged hospital stay in intensive care settings [7, 8]. ESBLs and MBLs have been associated with surgical site infections, catheter associated urinary tract infections and central venous catheter related bactremia to patients who are hospitalized. This study was designed to investigate the prevalence of ESBLs and MBLs encoding genes and drug resistant patterns among isolated non-fermenters.

MATERIALS AND METHODS:

The present study was conducted in department of microbiology Mrs. K.S.K College, Beed, Maharashtra India during Jan 2014 to Jun 2017. A total number of 378 clinical specimens were collect from different private hospitals, diagnostic centers, urologist and different unites of government hospital of Akola and Beed cities in Maharashtra, India. Identification of non-fermenters was carried out according to Koneman color atlas and text book of microbiology and 16s rRNA gene sequencing [9]. Isolated non-fermenters were subjected for antibiotic susceptibility test according to Kirby Bauer disc-diffusion method against antibiotics, Cefexime-5µg, Cefepime-30µg, Cefotaxime-30µg, Ceftazidime-30µg, Ceftizoxime-30µg, Imipenem-10µg, Meropenem-10µg.

Phenotypic determination of extended spectrum β-lactamases and metallo β-lactamases production:

Extended spectrum β-lactamase and metallo β-lactamase were determined by combined disk diffusion test.

Molecular characterization and identification of non-fermentative bacterial pathogens by 16S rRNA gene sequencing:

Organisms those are found phenotypically positive for both ESBLs and MBLs production were further subjected for 16S rRNA gene sequencing, where universal 16S rRNA primer was using to get complete genome information.

Molecular characterization of extended spectrum β-lactamases and metallo β-lactamases genes:

Organisms those are phenotypically positive for ESBLs and MBLs were further subjected for multiplex and simplex PCR assay (Bio-Rad USA) for characterizing the *bla* genes using the primers as mentioned Table 3.

Reaction condition for multiplex PCR assay of extended spectrum β-lactamases:

Reaction conditions were set as initial denaturation 94ºC for 5 minutes followed by 32 cycles of denaturation at 94ºC for 1 minute, annealing at 54ºC for 1 minute, extension at 72 ºC for 1 minute and final extension 72 ºC for 7 minutes.

Reaction condition for multiplex PCR assay of Carbapenemase Class-A genes:

Reactions conditions were set as initial denaturation 95ºC for 2 minutes followed by 34 cycles of denaturation at 95ºC for 20 sec, annealing at 52ºC for 30 sec, extension at 72 ºC for 1 minute and final extension 72ºC for 2 minutes.

Reaction condition for multiplex PCR assay of Carbapenemase Class-B genes:

Reactions condition were set according to primer manufacturers as initial denaturation 94ºC for 5 minutes followed by 34 cycles of denaturation at 94ºC for 30 sec, annealing at 53ºC for 1 minute, extension at 72 ºC for 1 minute and final extension 72 ºC for 5 minutes.

Gel electrophoresis:

After PCR amplification, amplified products were subjected for agarose gel electrophoresis (Bio-Rad USA) where different size DNA ladder were used to calculate PCR amplicon size.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by agar dilution method in Muller Hinton agar (Hi-media, Mumbai) against cefotaxime and meropenem.

Table 1: Oligonucleotides used as a primer in the study.

Type	Primer Pairs	Target	Sequence (5'-3')	Amplified product size (BP)	Gene Bank accessing number	Reference
ESBL	TEM-F TEM-R	*bla*TEM	5'ATGAGTATTCAACATTTCCG 3' 5'CTGACAGTTACCAATGCTTA3'	867	**NC_013120.1**	**[10]**
ESBL	SHV-F SHV-R	*bla*SHV	5'AGGATTGACTGCCTTTTTG3' 5'ATTTGCTGATTTCGCTCG3'	392	**GU211012.1**	**[11]**
ESBL	CTX-M-F CTX-M-R	*bla*CTX-M, 1-2-9 Group	5'CGCTTTGCGATGTGCAG3' 5'ACCGCGATATCGTTGGT33'	550	**X92506**	**[12]**
ESBL	OXA-10-F OXA-10-R	*bla*OXA-10	5'AAGAAACGCTACTCGCCTGC3' 5'CCACTCAACCCATCCTACCC3'	478	**NC_003292.1**	**[13]**
Carbapenemase Class-A	SME-F SME-R	*bla*SME	5'-AAC GGC TTC ATT TTT GTT TAG-3' 5'GCT TCC GCA ATA GTT TTA TCA-3'	831	**Z28968.1**	**[14]**
Carbapenemase Class-A	KPC-F KPC-R	*bla*KPC	5'-CAT TCA AGG GCT TTC TTG CTG C-3' 5'-ACG ACG GCA TAG TCA TTT GC-3'	538	**EU176011.1**	**[15]**
Carbapenemase Class-A	IMI/NMC-F IMI/NMC-R	*bla*IMI/NMC	5'- CCA TTC ACC CAT CAC AAC3' 5'- CTA CCG CAT AAT CAT TTG C-3'	440	**U50278.1**	**[16]**
Carbapenemase Class-B	IMP-F IMP-R	*bla*IMP	5'-TTG ACA CTC CAT TTA CDG-3' 5'-GAT YGA GAA TTA AGC CAC YCT-3'	139	**AF324464**	[17]
Carbapenemase Class-B	VIM-F VIM-R	*bla*VIM	5'-GAT GGT GTT TGG TCG CAT A-3' 5'- CGA ATG CGC AGC ACC AG-3'	390	**AF369871.1**	**[18]**
Carbapenemase Class-B	NDM-F NDM-R	*bla*NDM	5'GGG CAG TCG CTT CCA ACG GT-3' 5'- GTA GTG CTC AGT GTC GGC AT-3'	476	**JN656100.1**	**[18]**

RESULT:

Total 94 non-fermentative bacterial pathogens were identified with isolation rate of 24.86%. Out of 94 isolates 5 were found phenotypically positive for the production of both extended spectrum β-lactamases and metallo β-lactamases. They were subjected for 16S rRNA gene sequencing for further identification and were identified as *Pseudomonas aeruginosa*, *Acinetobaacter baumannii, Acinetobacter calcoaceticus, Alcaligenes faecalis* and *Pseudomonas sp.* results are recorded at table 2. Antibiotic susceptibility of ESBLs positive isolates were shown at table 3.

Findings of polymerase chain reactions:

After PCR amplification of resistant pathogens, it was observed that all 5 isolated nonfermenters were found positive to presence ^{bla}NDM gene where as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were having additional ^{bla}OXA-10 and ^{bla}SHV extended spectrum β-lactamase genes. Presence of ^{bla}NDM genes were confirmed by simplex PCR amplification. Results are recorded at table 4-6.

Determination of minimum inhibitory concentration MIC:

It was found that all resistant isolates were having MIC of ≥256 µg/ml against mention antibiotics.

Table 2: Sequence comparison and nomenclature of isolated non-fermenters

Sampled	Accession	Description	Total score	Query coverage	E value	Ident
1	KF984154.1	Pseudomonas aeruginosa strain CJ2 16S ribosomal RNA gene,	1109	98%	00	95%
2	LT594095.1	Acinetobacter baumannii Strain 3027STDY5784958 genome assembly, chromosome:1	350	96%	2e-92	79%
3	KT878384.1	Acinetobacter calcoaceticus strain PA 16S ribosomal RNA gene,	1615	99%	00	99%
4	KC478943.1	Alcaligenes faecalis Strain CD234 16S ribosomal RNA gene,	390	56%	1e-104	92%
5	KX231843.1	Pseudomonas sp. GT2 16S ribosomal RNA gene,	1234	55%	00	99%

Table 3: Antibiotic susceptibility of phenotypically ESBLs and MBLs positive isolates

Sl. No.	Name of Antibiotics	*Pseudomoas aeruginosa*	*Acinetobacter baumanni*	*Acinetobacter calcoacetis*	*Alcaligene sfaecalis*	*Pseudomonas sp.*
1.	Cefexime-5µg	R	R	R	R	R
2.	Cefepime-30 5µg	R	R	R	R	R
3.	Cefotaxime-30 µg	R	R	R	R	R
4.	Ceftazidime-30 µg	R	R	R	R	R
5.	Ceftizoxime-30µg	R	R	R	R	R
6.	Meropenem-10 µg	R	R	R	R	R

Table 4: Multiplex PCR amplification of extended spectrum β lactamases genes

			Isolated species				
Sl. No.	**Target Gene**	**Amplicon size (bp)**	***Pseudomonas aeruginosa***	***Acinetobacter baumanni***	***Acinetobacter calcoaceticus***	***Alcaligenes faecalis***	***Pseudomonas sp.***
1.	^{bla}OXA10	276	+	+	-	-	-
2.	^{bla}TEM	867	-	-	-	-	-
3.	^{bla}OXA-2	478	-	-	-	-	-
4.	^{bla}CTXM	550	-	-	-	-	-
5.	^{bla}SHV	392	+	+	-	-	-

Table 5: Multiplex PCR amplification of carbapenemase class A genes.

			Isolated species				
Sl. No.	**Target Gene**	**Amplicon size (bp)**	***Pseudomonas aeruginosa***	***Acinetobacter baumannii***	***Acinetobacter calcoaceticus***	***Alcaligenes faecalis***	***Pseudomonas sp.***
1.	^{bla}SME	831	-	-	-	-	-
2.	^{bla}KPC	538	-	-	-	-	-
3.	^{bla}IMI/NC	440	-	-	-	-	-

Table 6: Multiplex PCR amplification of carbapenemase class-B genes

			Isolated species				
Sl. No.	**Target Gene**	**Amplicon size (bp)**	***Pseudomonas aeruginosa***	***Acinetobacter baumanni***	***Acinetobacter calcoaceticus***	***Alcaligenes faecalis***	***Pseudomonas sp.***
1.	^{bla}NDM	476	+	+	+	+	+
2.	^{bla}VIM	390	-	-	-	-	-
3.	^{bla}IMP	139	-	-	-	-	-

Table 7: Simplex PCR amplification of ^{bla}NDM genes

			Isolated species				
Sl. No.	**Target Gene**	**Amplicon size (bp)**	***Pseudomonas aeruginosa***	***Acinetobacter baumanni***	***Acinetobacter calcoaceticus***	***Alcaligenes faecalis***	***Pseudomonas sp.***
1.	^{bla}NDM	476	+	+	+	+	+

DISCUSSIONS:

In the present study *Pseudomonas aeruginosa* was the most predominant non-fermentative bacterial pathogens and a major cause of hospital acquired infections and known to cause a wide spectrum of life-threatening diseases. Non-fermentative bacterial pathogens are resistant to almost all commonly available antibiotics with limited treatment options. ^{bla}*NDM*-1 was the most predominant resistance genes found in all resistant non-fermentative bacterial pathogens. The presence of ^{bla}OXA-10 and ^{bla}SHV together with ^{bla}NDM-1 in *Pseudomonas aeruginosa* and *Acinetobacter baumanni* reported in this study indicates the spreading of ESBls and MBLs carrying organisms in India.

This finding of NDM-1-producing non-fermentative bacterial pathogens and presence of more than one *bla* genes in carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* highlights the emerging therapeutic challenge. The implementation of strict antimicrobial policies and infection control programs may help to prevent the rapid spread of these non-fermentative resistant bacterial pathogens.

CONCLUSION:

To the best of our knowledge this is the first report from this region to detect *Pseudomonas aeruginosa* and *Acinetobacter baumannii*that were having multiple β-lactamases genes name ^{bla}NDM, ^{bla}OXA-10 and ^{bla}SHV. The widespread use and more often the misuse of

antimicrobial drugs has led to a general rise in the emergency of resistant bacteria. This study advocates execution of suitable antimicrobial policy and infection control management in hospital environments so as to prevent horizontal spread of these resistant pathogens within different host range.

REFERENCES:

1. Gupta V., Chhina D., Kaur A., Incidence of metallo-β-lactamase (mbl) producing nonfermenters isolated from respiratory samples in icu patients. Int Jr of Pharma and Bio Sci, 4(2):580-585, (2013).
2. Gupta R., Malik A., Rizvi M.,Ahmed M., Presence of metallo-beta-lactamases (MBL), extended-spectrum beta-lactamase (ESBL) & AmpC positive non-fermenting Gram-negative bacilli among Intensive Care Unit patients with special reference to molecular detection of bla_{CTX-M} & bla_{AmpC} genes. Indian Jr Med Res, 144(2): 271–275, (2016).
3. Alan TF., Goudarzil H., Hashemil A., Fallah F., Doustdar F., Bostan H., Detection of *bla*NDM, *bla*DIM, *bla*IMP, *bla*VIM and *blaCTX-M-15* beta-lactamase Genes among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Strains Isolated from Two Hospitals of Tehran, Iran. Novelty in Biomedicine, 4(4):153-158 (2016).
4. Safari M., Nejad ASM., Bahador A., Jafari R., Alikhani MY., Prevalence of ESBL and MBL encoding genes in Acinetobacter baumannii strains isolated from patients of intensive care units (ICU). Saudi Jr of Biological sci, 22(4):424-429 (2015).
5. Pfeifer Y., Wilharm G., Zander E., Wichelhaus TA., Gottig S., Hunfeld KP., Seifert H., Witte W., Higgins PG., Molecular characterization of ^{bla}NDM-1 in an *Acinetobacter baumannii* strain isolated in Germany in 2007. Jr of Antimicrob Chemother, 66(9):1998-2001 (2011).
6. Bush K., New beta-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin Infect Dis 32(7):1085-1089(2001) .
7. Du Bois SK., Marriott MS., Amyes SG., TEM-and SHV-derived extended spectrum beta-lactamses: relationship between selection, structure and function. Jr Antimicrob Chemother 35 (1):7-22 (1995).
8. Clark NM., Patterson J., Lynch JP., Antimicrobial resistance among gram negative organisms in the intensive care unit. Curr Opin Crit Ca 9(5):413-23 (2003).
9. Winn W Jr. Allen S. Janda W. Koneman E. Procop G. Schreckenberger P. Koneman's Color Atlas and textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams and Wilkins Company, 305–391 (2006).
10. Bert F., Branger C., Zechovsky NL., Identification of PSE and OXA β-lactamase in *Pseudomonas aeruginosa* using PCR-restriction fragment length polymorphism. Antimicrob Chemother 50(1):11-18 (2002).
11. Colom K., Perez J., Alonso R., Aranguiz AF., Larino E., Cisterna R., Simple and reliable multiplex PCR assay for detection of ^{bla}TEM, ^{bla}SHV and ^{bla}OXA-1 gene in *Enterobacteriaceae.* FEMS Microbiol Lett, 223(2):147-151(2003).
12. Lee S., Park YJ., Kim M., Lee HK., Han K., Kang CS., Kang MW., Prevalence of Ambler class Ambler class A and D β-lactamases among clinical isolates of *Pseudomonas aeruginosa* in Korea. Antimicrob Chemother 56(1):122–127(2005).
13. Naas T., Vandel L., Sougakoff W., Livermore DM., Nordmann P., Cloning and Sequence Analysis of the Gene for a Carbapenem-Hydrolyzing Class A β-Lactamase, Sme-1, from *Serratia marcescens* S6. Antimicrob Agents Chemother 28(6):1262-1270 (1994).
14. Nass T., Cuzon G., Villegas MV., Lartigue MF., Quinn JP., Nordmann P., Genetic structures at the origin of acquisition of the beta-lactamase ^{bla} KPC gene. Antimicrob Agents Chemother 52(4):1257-1263 (2008).
15. Rasmussen BA., Bush K., Keeney D., Yang Y., Hare R., O'Gara C., Characterization of IMI-1 betalactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloaceae*. Antimicrob Agents Chemother 40(9):2080–2086 (1996).
16. Yum JH., Yi K., Lee H., Young D., Lee K., Kim JM., Rossolini GM., Chong Y., Molecular characterization of metallo-beta-lactamase-producing *Acinetobacter baumannii* and *Acinetobactergenomo species* 3 from Korea: identification of two new integrons carrying the bla(VIM-2) gene cassettes. Antimicrob Chemother 49(5):837-840 (2002).
17. Tsakris A., Pournaras S., Woodford N., Palepoy MF., Babini GS., Douboyas J., Livemore DM., Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece.J Clin Microbiol 38(3):1290-1292 (2000).
18. Young D., Toleman MA., Giske CG., Cho HS., Sundman K., Lee K., Walsh TR., Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 53(12):5046-5054 (2009).

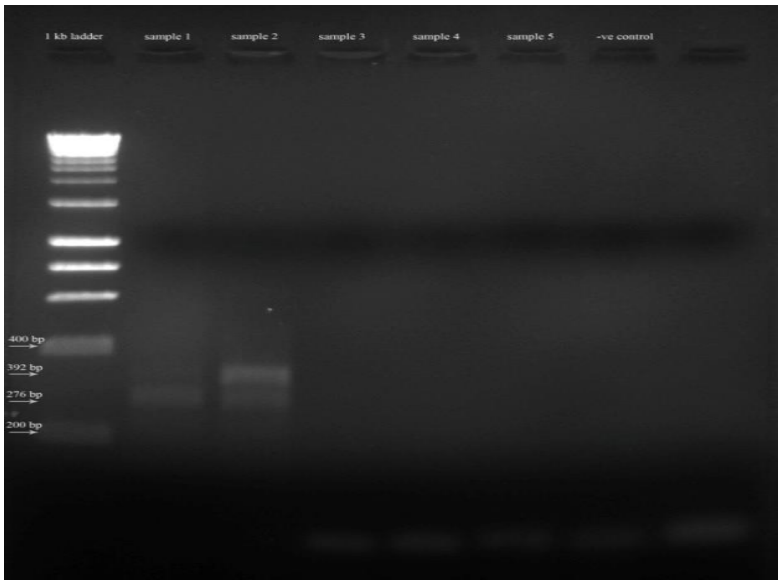

Fig 1: Multiplex PCR amplification of ESBLs genes

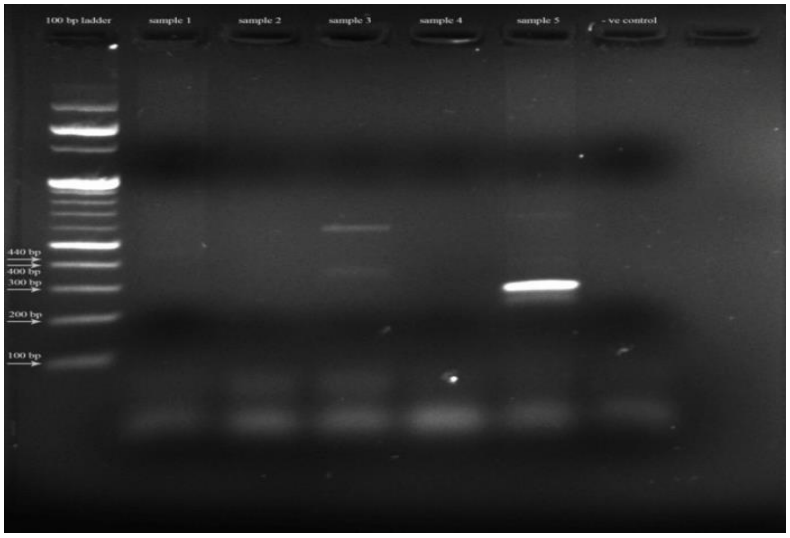

Fig2: Multiplex PCR amplification of carbapenemase class A

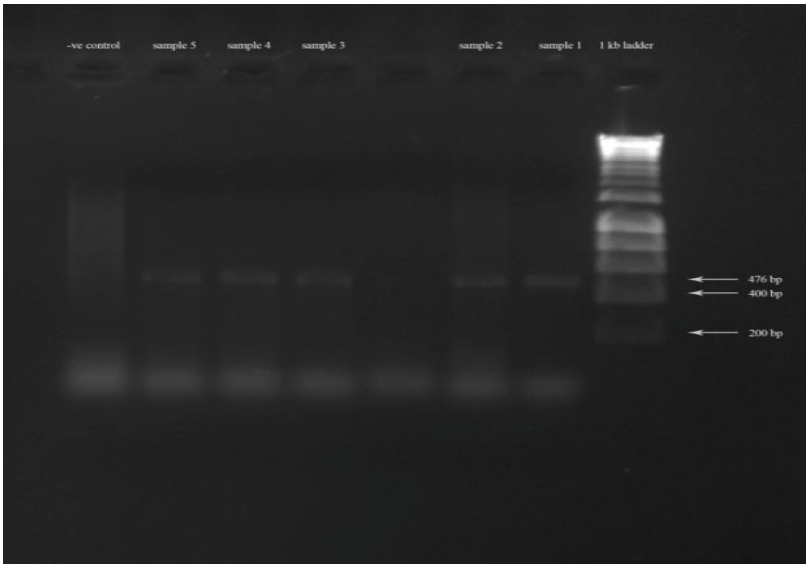

Fig 3: Multiplex PCR amplification of carbapenemase-B

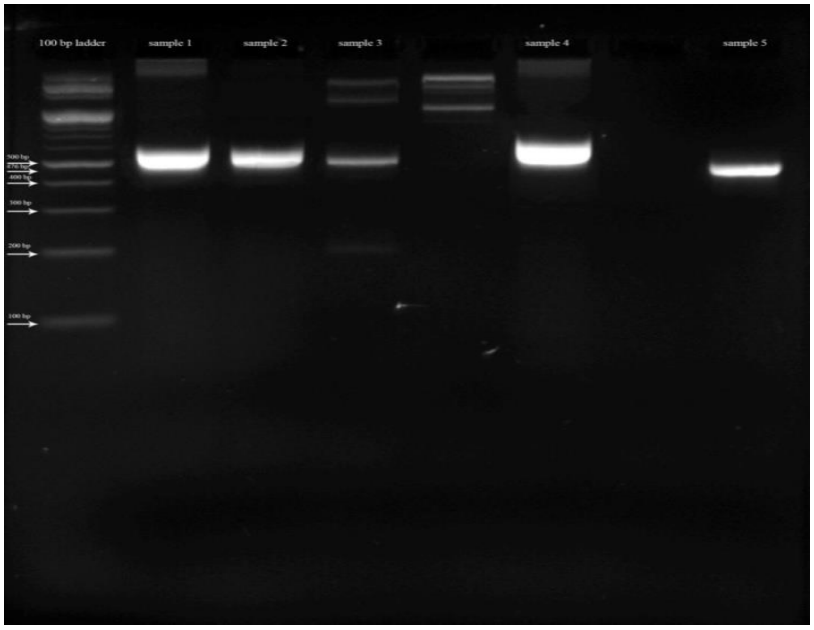

Fig 4: Simplex PCR of NDM genes

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