B-5483
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RESEARCH ARTICLE Indian Journal of Animal Research

Detection of Antimicrobial Resistance Genes in Pseudomonas Isolated from Poultry

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10.18805/IJAR.B-5483

ABSTRACT

Background: *P. aeruginosa* is one of the normal inhabitants and considered as an opportunistic organism of a*via*n spp. Under stressful conditions this organism may becomes pathogenic and induces a clinical picture with serious economic losses in the poultry industry.

Methods: A total of 150 samples were collected from birds, poultry farms and hatcheries for bacteriological isolation and identification of *Pseudomonas* at Genus level and *Pseudomonas aeruginosa* by phenotypic methods and PCR using species specific primers of gene *opr*L. Seven antimicrobial resistance genes (ARG) in *Pseudomonas* isolates were also detected using PCR.

Result: Out of 150 samples, only 10 (6.66%) samples yielded *Pseudomonas.* Out of total 10 *Pseudomonas* isolates, 07 (70%) were identified as *Pseudomonas aeruginosa* in PCR using *oprL* gene primers. Out of 10 *Pseudomonas* isolates, 1 (10%) isolate was positive for *bla*CTX-M gene, 2 (20%) for *tet*B and *bla*SHV gene, 3 (30%) for *tet*A and *Sul*1 gene. The most prevalent gene found in *Pseudomonas* isolates was *Mex*A as it was found in 9 (90%) isolates followed by *bla*TEM 5 (50%).

Key words: ARGs, PCR, *Pseudomonas aeruginosa*, *Pseudomonas.*

INTRODUCTION

Pseudomonas is a communal motif of environmental associated disease and causes a serious and extensive economic problem in all age groups in poultry farms and hatcheries (Fodor, 2007). The *Pseudomonas* is one of the ESKAPE group pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) and have capacity to “escape” from common antibacterial treatments (Omoya and Ajayi, 2016; Chakraborty *et al*., 2020). *P. aeruginosa* could adapt very easily to antibiotics and could quickly develop multidrug resistance (Sekhri *et al*., 2020). In relation to the burning problem of antimicrobial resistance, the low antibiotic susceptibility of *P. aeruginosa* against most antibiotics is worrisome.

This is mainly attributable to low permeability of the bacterial cellular envelopes, action of multidrug efflux pumps, mobile genetic elements and hydrolyzing enzymes. In addition to this intrinsic resistance, *P. aeruginosa* can get resistance by mutation either in chromosomally encoded genes or by the horizontal gene transfers of antibiotic resistance determinants. Better understanding and monitoring of AMR gene transfer could potentially help to limit the spread of AMR and prolong the life of new antibiotics (Strateva and Yordanov, 2009). The indiscriminate and injudicious use of antimicrobials in poultry is the major contributors in development of AMR in bacteria including *Pseudomonas* (Wibisono *et al*., 2020). Therefore, considering the above facts present research pursuit was undertaken with the objective of detection of some antimicrobial resistance genes *in Pseudomonas* isolates.

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How to cite this article: Khatri, R., Chhabra, D., Sikrodia, R., Gangil, R., Sharda, R. and Jogi, J. (2025). Detection of Antimicrobial Resistance Genes in Pseudomonas Isolated from Poultry. Indian Journal of Animal Research. 1-9. doi: 10.18805/IJAR.B-5483.

Submitted: 27-08-2024 **Accepted:** 19-02-2025 **Online:** 06-06-2025

MATERIALS AND METHODS

The work was carried out in the Department of Microbiology, Veterinary College, MHOW (M.P.) during October 2020 to October 2021.

Sample collection

Total 150 samples comprising of nasal exudate (n= 5) from the birds showing clinical signs of respiratory illness, cloacal swab (n=33), respiratory tract tissues (n= 8), liver (n=5) and heart (n=7) from dead birds, hatchery samples from egg incubators (n= 10), water (n=14) and yolk sacs of dead in shell embryos (n=37), samples from poultry farms: feed, water, soil and litter material (n=31) *were* collected from various organized and unorganized poultry farms and hatchery situated in and around Mhow and Indore cities for isolation and identification of *Pseudomonas* and *Pseudomonas aeruginosa.*

Isolation and Identification

The samples were first inoculated in BHI broth and incubated aerobically at 37°C for 24 hrs, followed by inoculation on basic and selective media and Gram staining for the presumptive identification of bacterial isolates as *Pseudomonas* by colonial and microscopic morphology (Collee *et al*.,1996). Further, the biochemical tests were carried out as per Barrow and Feltham (1993) and PCR using primers for species specific gene *opr*L.

Polymerase chain reaction (PCR) for species identification

Extraction of DNA

The *Pseudomonas* isolates were cultivated by inoculating in Brain heart infusion broth (Hi Media) and incubating at 37°C for 12-18 hours (overnight grown culture). DNA was extracted with the kit (GeneElute Bacterial Genomic DNA Kit).

Thermocyclic conditions for *opr*l gene

The PCR was standardized for the detection of *Pseudomonas aeruginosa* specific *opr*L using published primers (Farghaly *et al*., 2017). The nucleotide sequences of the forward and reverse primers for amplification of 504 bp product of *opr*L gene were as forward ATGGAAATGC TGAAATTCGGC and reverse CTTCTTCAGCTCGACGC GACG. The PCR reaction was optimized for amplification by adding 12.5 µl of Master mix, 1.0 µl of forward and reverse primer (20 pm/µl) each, 5 µl of DNA Template (150-200 ng) and the reaction was made up to 25 µl by using 5.5 µl nuclease free water. The negative control consisted of sterile water. PCR was performed in thermocycler (Eppendorf Research, Germany) for identification of *Pseudomonas aeruginosa* targeting *opr*L gene. The protocol included 35 cycles of initial denaturation at 96°C for 5 minutes, denaturation at 96°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplified products were electrophoresed in 1.5% agarose gel (in TBE buffer), stained with ethidium-bromide solution. The resolution of bands in the gel was visualized by a UV transilluminator (Alpha-Innotech) and digitally recorded by gel documentation system (Alphalmager, USA).

Polymerase chain reaction for antimicrobial genes

The PCR was standardized for the detection of seven antimicrobial genes in *Pseudomonas* using published primers (Table 1).

Thermocyclic condition fo Thermocyclic condition for identification of AMR genes

PCR was performed for identification of antimicrobial genes of *Pseudomonas* species (Table 2).

Gene sequencing

For the sequence analysis, the PCR product of two genes (*opr*L and *Mex*A) were got sequenced from Eurofins (Bengaluru).

RESULTS AND DISCUSSION

Isolation and Identification of *Pseudomonas* spp.

Out of 150 samples collected for bacteriological isolation and identification of *Pseudomonas* and *Pseudomonas aeruginosa* by phenotypic and genotypic methods, only 10 (6.66%) samples yielded *Pseudomonas.* All the 10 isolates producing characteristic colonies with greenish pigmentation, fruity odour on nutrient agar and greenish yellow colonies, sweet odour on Pseudomonas base agar and Gram negative rods were suggestive of *Pseudomonas.* The results of biochemical tests were in agreement with the standard book reference and other reports (Barrow and Feltham, 1993; Abd El- Tawab *et al*., 2014, Shahat *et al*., 2019).

Ten isolates of *Pseudomonas* were further tested and positive amplification of 504 bp fragment specific for the *opr*L gene was disclosed only in 7 (70%) isolates and was thus confirmed as *Pseudomonas aeruginosa* (Plate 1).

Table 1: Details of primers used for molecular characterization of ARGs of *Pseudomonas.*

Target gene	Antimicrobial resistance	Primer sequence	Product size (bp)	Reference
*bla*CTX- M	Beta-lactam	F: GTGCAGTACCAGTAAAGTTATGG R: CGCAATATCATTGGTGGTGCC	538	Adesoji *et al.,* 2015
*bla*SHV	Beta-lactam	F: TCGGGCCGCGTAGGCATGAT R: AGCAGGGCGACAATCCCGCG	628	Gundran *et al.,* 2020
*bla*TEM	Beta-lactam	F: TTGGGTGCACGAGTGGGTTA R: TAATTGTTGCCGGGAAGCTA	506	Gundran *et al.,* 2020
*Mex*A	Beta- actam	F: CGACCAGGCCGTGAGCAAGCAGC R: GGAGACCTTCGCCGCGTTGTCGC	316	Farghaly *et al*., 2017
*Sul*1	Sulfa	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822	Shehata *et al.,* 2016
*tet*A	Tetracycline	F: TTGGCATTCTGCATTCACTC R: GTATAGCTTGCCGGAAGTCG	494	Adesoji *et al.,* 2015
*tet*B	Tetracycline	F: CAGTGCTGTTGTTGTCATTAA R: GCTTGGAATACTGAGTGTAA	773	Van *et al.,* 2008

Many researchers have made attempts to develop molecular methods especially PCR for the detection of *P. aeruginosa* (Nikbin *et al.,* 2012). In this study the size of amplicon for the gene of interest was 504 bp. The reports which used same set of primer and same PCR amplified fragment size to confirm *Pseudomonas aeruginosa* strain genotypically were Abd El- Tawab *et al*. (2014); Elsayed *et al*. (2016); Bakheet and Torra (2020) and Soha *et al*. (2021).

Identification of *P. aeruginosa* can be achieved by amplification of a peptidoglycan associated lipoprotein (*opr*L) gene (species-specific gene). It encodes a protein in the inner and outer membranes and is essential for the invasion of epithelial cells and indicate also the pathogenic potential of these isolates (Elsayed *et al.,* 2016).

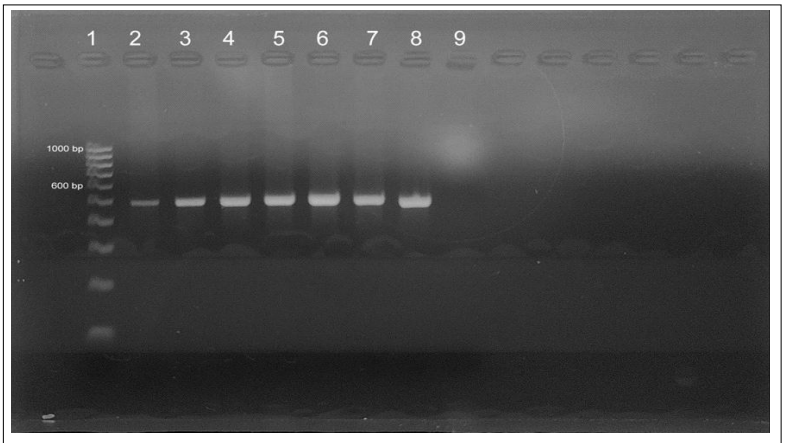
Detection of antimicrobial resistance genes by PCR in *Pseudomonas* spp.

Extracted DNAs from 10 isolates of *Pseudomonas* spp. were amplified in PCR for detection of various genes of antimicrobial resistance. Different primers used for targeted antibiotic genes were *bla*CTX-M, *tet*A, *Mex*A, *Sul*1, *bla*SHV, *bla*TEM and *tet*B (Table 1). Amplification and detection of *bla*CTX-M gene was done using published primer as per Adesoji *et al.* (2015). An amplicon size of 538 bp was taken as positive for *bla*CTX-M gene. Out of 10, one (10%) isolate was positive for *bla*CTX-M gene (Plate 2). A desired product of 628 bp using primer as per Gundran *et al*. (2020) in PCR was produced by 2 (20%), strains thus showing the presence of *bla*SHV gene against beta lactam antibiotics (Plate 3). Amplification and detection of *bla*TEM gene was done using published primer as per Gundran *et al*. (2020). An amplicon size of 506 bp was taken as positive for *bla*TEM gene. Out of 10 isolates, 5 (50%) were positive for *bla*TEM gene (Plate 4). The detection of ESBL and beta-lactamase encoding genes: *blaTEM*, *blaSHV* and *blaCTX* in the ESBL-producing *Pseudomonas* species isolated from livestock samples were also reported by Falodun *et al*. (2020).

In this study the ESBL producing *Pseudomonas* isolates were dominated by encoding *bla*TEM gene, followed by *bla*SHV and *bla*CTX-Ms (Table 3). It has been reported that ESBL genes show variation depending on the geographical location. Dominance of genotype *bla*TEM was also reported by Chen (2015). On contrary, Jamali *et al*. (2017) reported the prevalent gene to be *bla*SHV.

Table 2: Thermocyclic condition for amplification of AMR genes of *Pseudomonas.*

Genes	Initial denaturation		Denaturation		Annealing		Extension		Final extension	
	Total no. of cycles (1)				Total no. of cycles (35)				Total no. cycles (1)	
	Temp (°C)	Duration	Temp (°C)	Duration	Temp (°C)	Duration	Temp (°C)	Duration	Temp (°C)	Duration
*bla*CTX-M	95	1 minute	96	30 seconds	52	30 seconds	72	30 seconds	72	10 minutes
*tet*A	95	1 minute	96	30 seconds	61	30 seconds	72	30 seconds	72	10 minutes
*Mex*A	95	15 minutes	95	30 seconds	69	30 seconds	72	1 minute	72	10 minutes
*Sul*1	94	5 minutes	94	30 seconds	54	30 seconds	72	90 seconds	72	10 minutes
*bla*SHV	95	3 minutes	94	1 minute	64	30 seconds	72	1 minute	72	7 minutes
*bla*TEM	95	3 minutes	94	1 minute	60	30 seconds	72	1 minute	72	10 minutes
*tet*B	94	5 minutes	94	30 seconds	58	30 seconds	72	1 minute	72	10 minutes

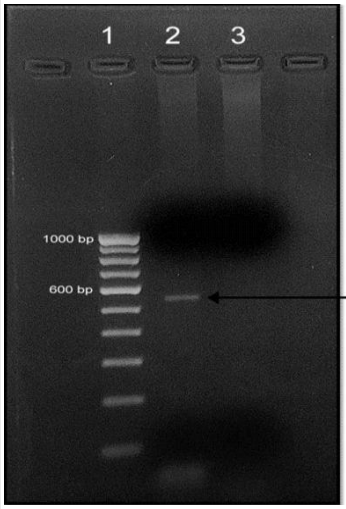


1. Lane 1 :Ladder (100 bp)
2. Lane 2-8 :Amplified product of oprL gene
3. Lane 9 :Negative sample

Plate 1: Agarose gel electrophoresis showing amplified product (504 bp) of *opr*L gene of *Pseudomonas aeruginosa.*

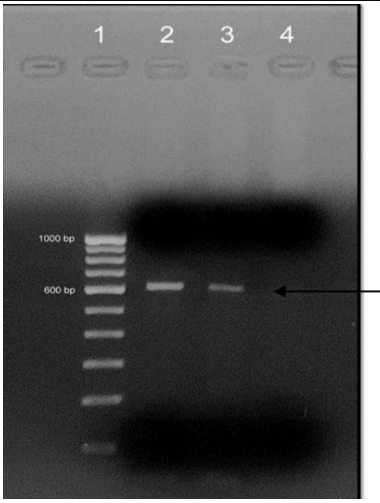
The least detected ESBL genotype from this study was *bla*CTX-M similar to records of Miranda (2015). In other studies Hassan *et al*. (2020) reported that resistance genes, *bla*CTX, was detected in 100% of the examined *P. aeruginosa* isolates although the lower prevalence was recorded by others (Peymani *et al*., 2017). The detection of *bla*CTX gene among all the tested isolates could explain the growing resistance pattern of *P. aeruginosa,* which reached to complete resistance against the third generation of cephalosporins represented by cefotaxime.

Several researchers have reported about the concurrence of different β-lactamase genes found in the same strains (Bahrami *et al*., 2018). ESBL combinations in our study observed were *bla*TEM + *bla*SHV and *bla*TEM + *bla*SHV+ *bla*CTX-M. Chen (2015) reported the commonest combination of genes to be *bla*SHV + *bla*CTX-M. Prior to now, *bla*TEM used to be the most prevalent gene but recent reports suggest that the CTX-M-type group of ESBLs may now be the most predominant type globally (Sahoo *et al*., 2019). These discrepancies may be due in part to varied geographic location, different levels of healthcare facilities involved, varied levels of exposure to healthcare settings, antibiotic use and antibiotic stewardship practices.



Lane 1 : Ladder (100 bp),
Lane 2 : Amplified product of *bla*CTX-M gene,
Lane 3: Negative sample

Plate 2: Agarose gel electrophoresis showing amplified product (538bp) of *bla*CTX-M gene.

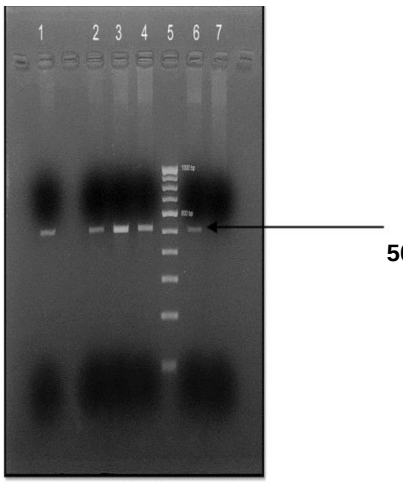


Lane 2,3: Amplified product of *bla*SHV;
Lane 1 : Ladder (100 bp)
Lane 4 : Negative sample

Plate 3: Agarose gel eleectrophoresis showing amplified product (628 bp) of blaSHV gene.

ESBLs are undergoing continuous mutations, causing the progression of new enzymes, showing expanded substrate profiles. So far, there have been more than 300 different ESBL variants which have been clustered into nine different structural and evolutionary families based on their amino acid sequences (Bokaeian *et al*., 2015).

A desired product of 316 bp using published primer as per Farghaly *et al*. (2017) in PCR was produced by 9 (90%), strains thus showing the presence of *Mex*A gene against beta lactam antibiotics (Plate 5). Detection of some efflux pumps system genes, such as *oprJ, mexA, etc* in a*vi*an *P. aeruginosa* isolates was conducted by Farghaly *et al*. (2017) and the results revealed the presence of these genes with percentages of 100% and 85.7%, respectively.



Lane 1,2,3,4,6 : Amplified product of *bla*TEM gene
Lane 5: Ladder (100 bp)
Lane 7: Negative sample

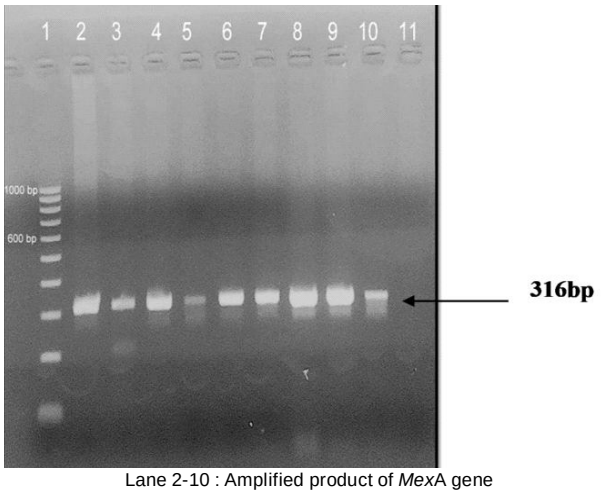
Plate 4: Agarose gel electrophoresis showing amplified product (506 bp) of *bla*TEM gene.

The DNA extracted from 10 isolates of *Pseudomonas* was subjected to PCR for detection of *Sul*1 gene using published primer pair (Shehata *et al*., 2016). A desired amplicon size of 822 bp was found in only 3 (30%) out of 10 *Pseudomonas* isolates (Plate 6). Adekanmbi *et al*. (2020) also reported *Sul*1 gene in *P. aeruginosa*. Resistance to sulfonamides occurs principally through the acquisition of the alternative dihydropteroate synthase (DHPS) gene *sul*, the product of which has a low affinity for sulfonamides (Changkaew *et al*., 2014).

Three (30%) out of 10 *Pseudomonas* strains showed the presence of *tet*A gene against tetracycline group of antibiotics, as they produced a desired product of 494 bp in PCR as per Adesoji *et al.* (2015) (Plate 7). Two (30%) out of 10 *Pseudomonas* strains showed the presence of *tet*B gene against tetracycline group of antibiotics, as they produced a desired product of 773 bp in PCR as per Van *et al*. (2008) (Plate 8). One of the most common resistance mechanisms in Gram-negative bacteria is the energy-dependent efflux pump system which is encoded by the genes *tet*A, *tet*B, *tet*C, *tet*D and *tet*G, with *tet*A and *tet*B genes being the most frequently described (Lewis *et al*., 2002).

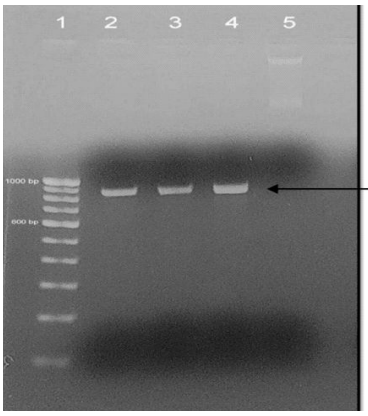
Prevalence of different antimicrobial genes in *Pseudomonas* isolates

Seven antimicrobial resistant genes (ARGs), belonging to three antimicrobial families, were detected in varying percentage from all the *Pseudomonas* isolates of different samples as shown in Table 3. Analysis revealed that the most prevalent gene found in *Pseudomonas* isolates was *Mex*A detected in 9 (90%) isolates followed by *bla*TEM (50%), *tet*A and *Sul*1 (30%), *tet*B and *bla*SHV (20%) and *bla*CTX-M (10%) genes (Fig 1). Maximum 7 genes detected in one sample followed by 5, 4, 2 and 1 in other samples (Fig 2). MexA gene was detected in maximum 9 isolates and *bla*CTX-M in minimum one isolate (Table 3).



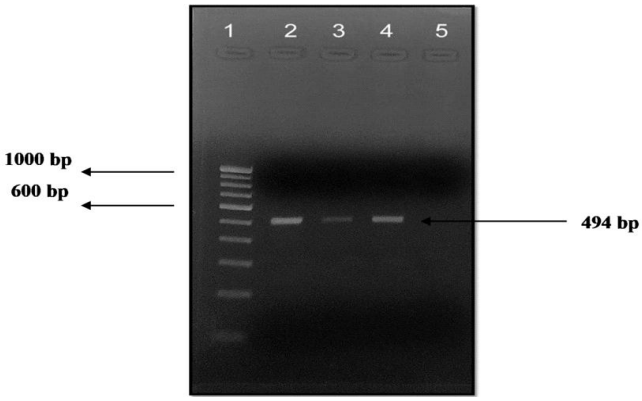
Lane 2-10 : Amplified product of *Mex*A gene
Lane 1 : Ladder (100 bp)
Lane 11: Negative sample

Plate 5: Agarose gel electrophoresis showing amplified product (316 bp) of *Mex*A gene.



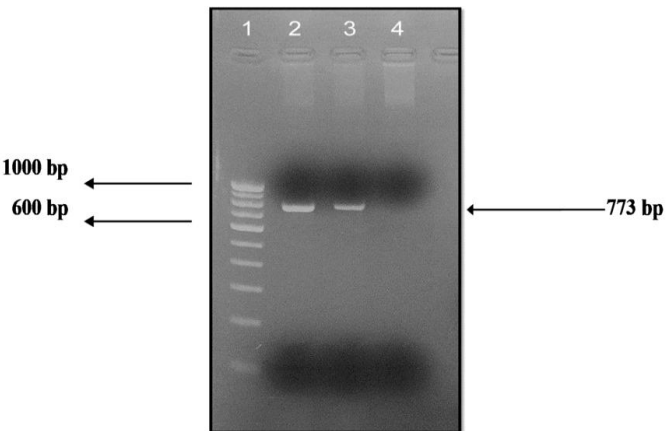
Lane 2-4: Amplified product of *Sul*1 gene
Lane 1: Ladder (100 bp)
Lane 5: Negative sample

Plate 6: Agarose gel electrophoresis showing amplified product (822bp) of *Sul*1 gene.



Lane 2-4: Amplified product of *tet*A gene
Lane 1: Ladder (100bp)
Lane 5: Negative sample

Plate 7: Agarose gel electrophoresis showing amplified product (494bp) of *tet*A gene.



Lane 2-3 :Amplified product of *tet*B gene
Lane 1: Ladder (100 bp)
Lane 4: Negative sample

Plate 8: Agarose gel electrophoresis showing amplified product (773bp) of *tet*B gene.

Table 3: Prevalence of different ARGs in *Pseudomonas* isolates.

Genes	Group	Isolate No.									
		1	2	3	4	5	6	7	8	9	10
*bla*CTX-M	Beta lactam								+		
*tet*A	Tetracycline								+	+	+
*Mex*A	Beta lactam		+	+	+	+	+	+	+	+	+
*Sul*1	Sulfa								+	+	+
*bla*SHV	Beta lactam								+		+
*bla*TEM	Beta lactam	+					+	+	+	+	
*tet*B	Tetracycline								+		+
Total no. of genes in each isolates		1	1	1	1	1	2	2	7	4	5

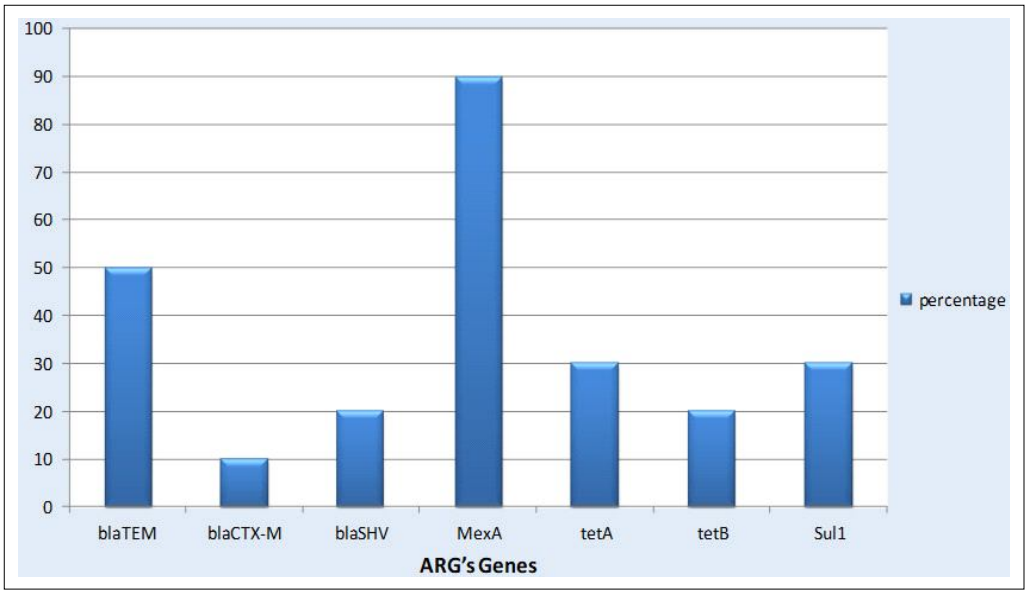


Fig 1: Prevalence of antimicrobial resistance genes.

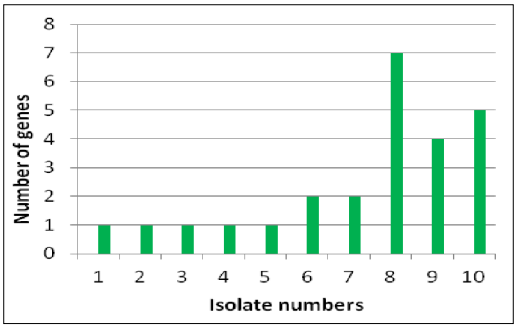


Fig 2: Total number of genes in each isolate of *Pseudomonas.*

Analysis revealed that the most prevalent gene found in *Pseudomonas* isolates was *Mex*A detected in 9 (90%) isolates followed by *bla*TEM (50%), *tet*A and *Sul*1 (30%), *tet*B and *bla*SHV (20%) and *bla*CTX-M (10%) genes (Fig1). Maximum 7 genes detected in one sample followed by 5, 4, 2 and 1 in other samples (Fig 1). MexA gene was detected in maximum 9 isolates and *bla*CTX-M in minimum one isolate (Table 3).

Gene sequencing

For the sequence analysis, the PCR product of two genes (*opr*L and *Mex*A) were got sequenced from Eurofins. Nucleotide base analysis of all sequence in GeneBank database revealed 98-100% homology with *Pseudomonas aeruginosa.*

CONCLUSION

The study concludes that the presence of ARGs such as *bla TEM, bla CTX, bla SHV, mex A, tetA, tet B* and *sul 1* in *Pseudomonas* strains may be responsible for coding resistance to different antibiotics. Antibiotic resistant bacteria in poultry could be a major threat to public health as there is the distinct possibility that genes encoding antibiotic resistance determinants that are carried on mobile genetic elements may be transferred to other bacteria of human clinical significance.

ACKNOWLEDGEMENT

The present study was supported by Dean, Veterinary College, Mhow and NDVSU university officials.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

The research methodology was approved by the Institutional Animal Ethics Committee (IAEC).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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