

Original Research Article

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Colistin Resistance and Molecular Determinants in *Salmonella enterica* Isolates from Poultry Processing Line of Central Kerala, India

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ABSTRACT

Salmonellosis is an important foodborne cause of deaths in humans. Contaminated poultry meat is the major vehicle for *Salmonella* infections. Colistin is a critically important antibiotic used as last resort drug in the treatment of intestinal infections in humans. Widespread use of colistin as growth promoter in poultry industry has led to the emergence of drug resistance. The present study was conducted to detect colistin resistant *Salmonella enterica* from poultry processing lines. A total of 450 samples were collected which include 75 samples each of cloacal swabs, caecal contents and carcass rinsates from two poultry processing plants viz., Plant A, Thrissur and Plant B, Ernakulam districts of Kerala. The samples were subjected to isolation of *Salmonella* by conventional culture technique and molecular confirmation of the positive isolates was done by targeting *invA* gene for *Salmonella* genus and *iroB* gene for *Salmonella enterica* species. Further, the *Salmonella enterica* isolates were subjected to antibiotic susceptibility testing against colistin by colistin agar test. Molecular confirmation of colistin resistance was assessed by targeting mutations in *pmrA* and *pmrB* genes and by detection of plasmid mediated *mcr-1* gene. *Salmonella* was detected in 14.22 per cent of the samples. There was significant difference in the occurrence of *Salmonella* in cloacal swabs and carcass rinsates of both the processing plants. Among the positive isolates, 67.19 and 81.4 per cent of isolates harboured *invA* and *iroB* gene, respectively. Out of three colistin resistant isolates, two isolates had mutations in *pmrA* gene at 345th and 346th position of the sequence, respectively and two *pmrB* isolates showed mutations at 79th, 244th, 319th, 388th and 577th position of the sequence. None of the isolates harboured *mcr-1* gene. The results of the study revealed occurrence of colistin resistance in *Salmonella enterica* isolates from poultry processing line. In order to prevent colistin resistant *Salmonella* contamination in the poultry processing line elucidating risk factors and implementation of one health approach in meat production is necessary to ensure food safety to consumers.

Keywords

Salmonella enterica, colistin resistance, poultry processing line, *mcr-1* gene, Central kerala

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Introduction

Salmonella enterica is one of the most significant foodborne pathogen across the world forming the important cause of gastroenteritis. Each year, non-typhoidal *Salmonella* causes 1,55,000 deaths and 93 million instances of gastroenteritis worldwide (Castro-Vargas *et al.*, 2020). Frequent cause of illness include the ingestion of food with animal origin, primarily poultry and poultry products (WHO, 2017). Colistin belongs to the class of polymyxins, produced by *Paenibacillus polymyxa*. It is a polypeptide antibiotic and one of the primary classes of antibiotics with activity against most Gram-negative bacteria (Poirel *et al.*, 2017).

Colistin has been extensively used in the veterinary practice for prophylaxis, metaphylaxis, treatment of bacterial infections and growth promotion at low doses. It is also considered treatment of last resort against multidrug-resistant strains frequently found among nosocomial pathogens such as *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Enterobacter* and *Escherichia coli*. (Lima *et al.*, 2019).

Antibiotic resistance is a major public health problem and a lot of the antibiotic-resistant organisms have been found in contaminated foods of animal origin, putting human health at risk and driving up healthcare costs (Fierer *et al.*, 2001). In *Salmonella* spp., colistin resistance is mediated by decreased binding of polymyxin to LPS due to mutations in polymyxin resistance A and B (*pmrA* and *pmrB*) genes forming two-component system in the cell membrane. Recently, plasmid-mediated colistin resistance gene called as mobile colistin resistance (*mcr-1*) gene has been found in organisms of public health significance. Until now, a total of nine variants (*mcr-1* to *mcr-9*) genes has been found.

The possibility of horizontal transfer of these genes has raised worldwide concern (Carfora *et al.*, 2018). Due to the emergence of plasmid mediated *mcr-1* gene, several countries banned the use of colistin as growth promoter in animal production. In 2019, Ministry of Health, Government of India prohibited the use of colistin in food producing animals. Still most of middle income and low income countries use colistin as growth promoter. Hence, it is crucial to monitor resistance to this agent in isolates from food producing animals especially poultry where large amount of colistin is used as growth promoter (Olaiton *et al.*, 2021)

Materials and Methods

Sample Collection

The samples were collected from two poultry processing plants located at Thrissur (Plant A) and Ernakulam (Plant B) districts, respectively. Seventy five samples each of cloacal swabs, caecal contents and carcass rinsates were collected from both the processing plants. Cloacal swabs of birds at lairage were collected randomly using sterile cotton swabs dipped in sterile buffered peptone water (Kadry *et al.*, 2019). The caeca of selected birds were cut using sterile scalpel and five grams of caecal contents were collected (Ali *et al.*, 2020). The carcass rinsate of the birds were collected after final wash by dipping the carcasses in 400 ml of buffered peptone water (Rivera-Perez *et al.*, 2014).

Isolation and identification

The isolation and identification of *Salmonella* spp. was done by conventional culture techniques (Andrews *et al.*, 2001). Initially, the samples were pre-enriched in buffered peptone water (BPW). The cloacal swabs were pre-enriched by dipping the swabs in 10 ml of BPW and caecal contents were pre-enriched by adding five grams of caecal contents to 45 ml of buffered peptone water. The carcass rinsates were pre-enriched by adding 30ml of carcass rinsates to equal volume of BPW and incubated for 18-20 h at 37°C. For enrichment, a 0.1ml of pre-enriched samples were transferred to 10 ml each of modified Rappaport-Vassiliadis broth and incubated at 42.5°C for 24h. The enriched samples were streaked on Xylose-lysine deoxycholate agar. The characteristic pink colonies with /without black centre of *Salmonella* spp. were selected as presumptive colonies and subjected to biochemical tests.

DNA extraction and polymerase chain reaction

The DNA of the *Salmonella* spp. positive isolates was extracted by boiling and snap chilling technique followed by Ram *et al.*, (2019). The molecular confirmation of *Salmonella* spp. was done by targeting *invA* gene. The primers used for identification are listed in table 1. The *iroB* gene was targeted by PCR to confirm *Salmonella enterica* isolates and the primers used for identification are listed in table 2. The *invA* gene and *iroB* genes were standardised at 244 and 606 base pairs respectively. For the PCR amplification of *invA* gene was carried out with

cyclic conditions as initial denaturation 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 35 sec. followed by final extension at 72°C for 5 min. The *iroB* gene was amplified with 35 cycles of denaturation at 94°C for 40 sec, annealing at 65°C for 40 sec and extension at 72°C for one min.

Antibiotic resistance profiling against colistin

The isolates were subjected to colistin agar test as described in CLSI, 2020. The isolates were cultured in nutrient agar plate and 3-5 colonies from the agar plates were transferred to 4-5mL of Normal Saline (NS). Turbidity of the suspended culture was adjusted to 0.5 McFarland standard. Later, the standardised inoculum was diluted in 1:10 NS. Muller-Hinton agar plates with increasing dilution of 0µg/mL (growth control), 1µg/mL, 2µg/mL and 4µg/mL of colistin was prepared in duplicates. These agar plates were divided with a marker to test upto 10 isolates per plate and each part was labelled with appropriate isolate number. Using a 10-µL loop 1:10 dilution of each standardised inoculum was streaked on respective marked part on agar plates. The plates were incubated at 33-35°C for 16-20h.

Molecular confirmation of colistin resistant isolates was assessed by targeting mutations in genes *pmrA* and *pmrB*, the primers used for identification of the isolates are listed in table 3. Plasmid mediated colistin resistance mediated by *mcr-1* gene was also targeted by PCR and the primers used for identification are listed in table 4. The PCR amplification of *pmrA* and *pmrB* genes was carried out with cyclic conditions as initial denaturation 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 62.4°C for 40 sec and extension at 72°C for 40 sec, followed by final extension at 72°C for 5 min. For *pmrB* gene amplification, annealing temperature was set at 60°C and denaturation, annealing and extension time was set for 1 min, keeping all the other parameters same as *pmrA* gene.

Nucleotide sequence analysis

The amplicons of *Salmonella enterica* specific *iroB* gene and the amplicons obtained consequent to PCR for colistin resistant genes viz., *pmrA* and *pmrB* and genes were purified and were outsourced for sequencing at Sci Genome Labs Private Limited. Cochin, using Sanger's dideoxy nucleotide chain termination method. The obtained sequence of each gene product were aligned

using EMBOSS and NCBI BLAST tool and gene sequences were submitted to NCBI Genbank using Bankit tool with necessary details of the gene.

Statistical analysis

The results obtained were statistically analysed using SPSS version 24.0 software. The significant difference in occurrence of *Salmonella* spp. and *Salmonella enterica* in Plant A and Plant B was analysed using chi-square test.

Results and Discussion

Out of 450 samples analysed, the samples positive for *Salmonella* spp. from Plant A and Plant B were 45 and 19, respectively. There was significant difference in the occurrence of *Salmonella* spp. in cloacal swabs and caecal contents of Plant A and Plant B. The overall occurrence of *Salmonella* spp. in poultry processing lines of both the processing plants was 14.22 per cent (Table 5). All the *Salmonella* spp. positive isolates were subjected to molecular confirmation by targeting *invA* gene by PCR. Out of 64 positive isolates, 43 (67.18 per cent) were harbouring *invA* gene. For the confirmation of *Salmonella enterica iroB* gene was targeted (Table 6).

Out of 43 samples analysed, 35 samples (81.4 per cent) were positive for *iroB* gene (Table 7). There was no significant difference in the occurrence of *Salmonella enterica* in poultry processing line (p-value < 0.05). Out of 450 samples analysed the occurrence of *Salmonella enterica* was found to be 7.7 per cent. Mir *et al.*, (2015) from Rajasthan reported a similar occurrence of 6.33 per cent of *Salmonella enterica* species. Sohail *et al.*, (2021) from Karnataka reported 37.91 per cent occurrence *Salmonella enterica* from broiler supply chain which is higher than the present study. Amplification of *iroB* gene by PCR is the rapid and sensitive method of detection of *Salmonella enterica*. The present study revealed the occurrence of *Salmonella enterica* in the poultry processing line. *Salmonella enterica* species are the most pathogenic species with more than 2600 serovars. These organisms commonly causes food poisoning in humans and are capable of getting transmitted from farm to fork.

The antibiotic resistance profiling of all the 35 *Salmonella enterica* isolates was tested against colistin by colistin agar test following the procedure of CLSI (2020) and the isolates were classified as resistant or intermediate resistant based on Minimum Inhibitory Concentration (MIC).

The phenotypic colistin resistance of the *Salmonella enterica* isolates were tested using colistin agar test following the procedure of Clinical and laboratory standards institute (CLSI, 2020). The isolates of *S. enterica* were plated on the agar plates with concentration of 0µg/mL, 1µg/mL, 2µg/mL and 4µg/mL. After incubation the agar plates were carefully examined for colony or light film of growth. The MIC of the isolates was read as the lowest colistin agar plate that completely inhibits the growth of the test isolate. The isolates with $\leq 2\mu\text{g/mL}$ was considered as intermediate resistant and the isolates with $\geq 4\mu\text{g/mL}$ was considered resistant.

Among the isolates 8.57 per cent of isolates were resistant to colistin and 91.43 per cent of the isolates showed intermediate resistance to colistin (Table 8). [Rau et al., \(2021\)](#) from Brazil reported that six per cent *Salmonella enterica* isolates were resistant to colistin. In a study conducted by [Sakdinun et al., \(2018\)](#) from Thailand, 5.1 per cent of *Salmonella enterica* isolates were found to be resistant to colistin which is in accordance with the present study. However, [Anju et al., \(2014\)](#) from Kerala and [Amer et al., \(2020\)](#) from Egypt reported that 40 per cent and 43.8 per cent of the isolates, respectively were resistant to colistin which is higher than the present study. Increased resistance to colistin has public health significance since the drug is used as a last resort antibiotic against carbapenemase- producing non-typhoidal *Salmonella* species ([Apostolakis and piccirillo, 2018](#)).

Molecular confirmation of colistin resistance in *Salmonella enterica* isolates

Molecular confirmation of colistin resistance was done by targeting two colistin resistant genes *pmrA* and *pmrB*. Mutations in *pmrA* and *pmrB* genes confers resistance to colistin antibiotic. Thus, the mutations were assessed by comparing the sequences of resistant isolates with that of susceptible isolates and also with the established gene sequence from NCBI. The *pmrA* and *pmrB* genes were standardised with amplicon size of 1067 and 1359 base pairs, respectively (Fig.1 and 2). Plasmid mediated *mcr-1* gene was standardised with amplicon size of 320bp (Fig. 3). All the three colistin resistant isolates were sequenced by Sanger's dideoxy method of sequencing and the sequences were submitted to the Genbank.

The resistant isolates harbouring *pmrA* gene viz., *pmrA1*, *pmrA2*, *pmrA3* and resistant isolates of *pmrB* gene viz.,

pmrB1, *pmrB2*, *pmrB3* were sequenced. Whereas, *pmrA4* and *pmrB4* are the sequences of colistin susceptible *Salmonella enterica* isolates. The *pmrA2* and *pmrA3* isolates showed change in nucleotide at 345th and 346th position of the 3' to 5' sequence where guanine is replaced by adenine (Table 9). In *pmrB2* and *pmrB3*, a total of 5 substitutions is noted at 79th, 244th, 319th, 388th and 577th position of the 3'-5' direction of the sequence (Table 10). The observed mutations did not cause any change in amino acid upon translation thus, these mutations are synonymous substitution. Similar results of mutations in 345th position of *pmrA* gene and 79th, 319th and 577th position of *pmrB* sequences was reported by [Quesada et al., \(2014\)](#).

[Sun et al., \(2009\)](#) reported five missense mutations in *pmrA* gene and 22 missense mutations in *pmrB* gene which are contrary to the present study. Whereas, [Jovčić et al., \(2020\)](#) reported that four colistin resistant isolates showed non synonymous mutations in *pmrB* gene such as V164G, V164M, R92P and V164M and no mutations was reported in *pmrA* gene of the resistant isolates. Whereas, none of the isolates had harboured *mcr-1* gene. [Brorwiak et al., \(2020\)](#) and [Proietti et al., \(2022\)](#) reported that 3 per cent of the isolates had harboured *mcr-1* gene. Zoonotic *Salmonella enterica* serovars may transfer the plasmid mediated colistin resistant *mcr-1* gene to human microbiota through food chain So, It is important to prevent the spread of colistin resistant isolates and also to implement infection control measures to prevent their spread.

In the present study *Salmonella enterica* isolates were prevalent in all the three points of processing line viz., cloacal swabs, caecal contents and carcass rinsates. This indicates the cross contamination of carcasses throughout the processing lines. The isolates showed resistance to colistin, considering the critical importance of the antibiotic, misuse and over the counter use of drug must be avoided. Colistin resistant isolates can contaminate the final products and resulting in transfer of resistance to gut microbiota in humans. Molecular analysis of all bacterial isolates for *mcr*-like genes along with phenotypic resistance mediating transfer of resistance to other organisms should be considered in future studies. There is need for strict implementation of risk management strategies and actions to reduce the misuse of drug in poultry industry. One health approach is necessary to minimise the use of colistin antibiotic and to avoid the transfer of resistant isolates to humans through food chain.

Table.1 The *invA* gene primers used for identification of *Salmonella* spp.

Genes	Primer	Length (bases)	Primer sequence	Size (bp)	Ref.
<i>invA</i>	F	20	5'ACAGTGCTCGTTTACGACCTGAAT3'	244	Zadernowska and Chajęcka-Wierzchowska (2017)
	R	20	5'AGACGACTGGTACTGATCTAT3'		

Table.2 The *iroB* gene primers used for the identification of *Salmonella enterica* species

Genes	Primer	Length (bases)	Primer sequence	Size (bp)	Ref.
<i>iroB</i>	F	22	5'TGCGTATTCTGTTTGTCGGTCC3'	606	Bäumler <i>et al.</i> , (1997)
	R	21	5'TACGTTCCCACCATTCTTCCC3'		

Table.3 The primers used of identification of *pmrA* and *pmrB* genes in *Salmonella* spp.

Genes	Primer	Length (bases)	Primer sequence	Size (bp)	Ref.
<i>pmrA</i>	F	22	5'CGCGAATTTTCGTGCATGATATG3'	1067	Sun <i>et al.</i> , (2009)
	R	21	5'ATGTCCCGATGCTCATTGTC3'		
<i>pmrB</i>	F	19	5'AGGAAATTCTGGGCGAGCA3'	1359	
	R	19	5'CGTTTTTCAGCGAAGAGCGA3'		

Table.4 The primers used for identification of *mcr-1* gene in *Salmonella enterica* isolates

Genes	Primer	Length (bases)	Primer sequence	Size (bp)	Ref.
<i>mcr-1</i>	F	20	5'AGTCCGTTTGTCTTGTGGC3'	320	Rebelo <i>et al.</i> , (2018)
	R	20	5'AGATCCTTGGTCTCGGCTTG3'		

Table.5 Overall occurrence of *Salmonella* spp. by conventional culture technique

Sl. No.	Sample	Plant A	Plant B	Total		Chi- square value	p-value
				No.	Per cent		
1.	Cloacal swabs	19	8	27	18	5.46 ^s	0.02
2.	Caecal contents	11	5	16	10.66	2.51 ^{ns}	0.11
3.	Carcass rinsates	15	6	21	14	4.48 ^s	0.03
	Total	45	19	64	14.22		

Table.6 Occurrence of *invA* gene in *Salmonella* spp. Isolates

Sl. No.	Sample	Plant A	Plant B	Total		Chi- square value	p- value
				No.	Per cent		
1.	Cloacal swabs	13	4	17	39.53	5.37 ^s	0.04
2	Caecal contents	8	4	12	27.90	1.44 ^{ns}	0.37
3.	Carcass rinsates	10	4	14	32.56	2.83 ^{ns}	0.16
	Total	31	12	43			

Table.7 Occurrence of *iroB* gene in *Salmonella* spp. isolate

Sl. No.	Sample	Plant A	Plant B			Chi – square value	p-value
				No.	Per cent		
1.	Cloacal swabs	9	4	13	37.14	2.10 ^{ns}	0.24
2.	Caecal contents	7	4	11	31.42	0.88 ^{ns}	0.53
3.	Carcass rinsates	9	2	11	31.42	4.80 ^{ns}	0.05
	Total	25	10	35			

Table.8 Overall occurrence of colistin resistance in *Salmonella enterica* isolates

Sl. no.	Sample	Intermediate resistant ($\leq 2\mu\text{g/mL}$)		Resistant ($\geq 4\mu\text{g/mL}$)	
		Number	Per cent	Number	Per cent
1.	Cloacal swabs	11	31.42	2	5.71
2.	Caecal contents	10	28.57	1	2.86
3.	Carcass rinsate	11	31.42	0	0
	Total	32	91.43	3	8.57

Table.9 Mutations in the colistin resistant *Salmonella enterica* isolates of *pmrA* gene

Sl no.	Gene sequences	Accession number	Mutations
1.	<i>pmrA</i> 1	OM977020	c.345G>A
2.	<i>pmrA</i> 2	OM930732	c.346G>A
3.	<i>pmrA</i> 3	OM930733	No mutations
4.	<i>pmrA</i> 4 (Susceptible isolate)	OM977021	-

Table.10 Mutations in the colistin resistant *Salmonella enterica* isolates of *pmrB* gene

Sl no.	Gene sequences	Accession number	Mutations
1.	<i>pmrB</i> 1	OM977017	No mutations
2.	<i>pmrB</i> 2	OM977018	c.79T> C, c. 244G> A, c. 319A> G, c.388G> A, c. 577G> A
3.	<i>pmrB</i> 3	OM977019	c.79T> C, c. 244G> A, c. 319A> G, c.388G> A, c.577G> A
4.	<i>pmrB</i> 4 (Susceptible isolate)	OM977022	-

Fig.1 Detection of *pmrA* gene by PCR

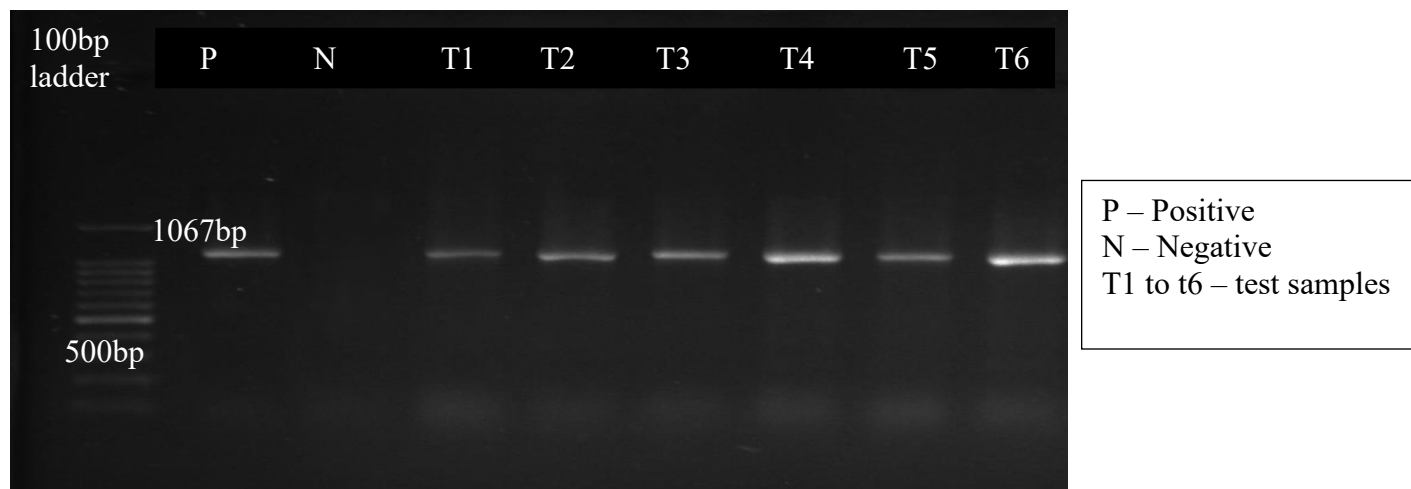


Fig.2 Detection of *pmrB* gene by PCR

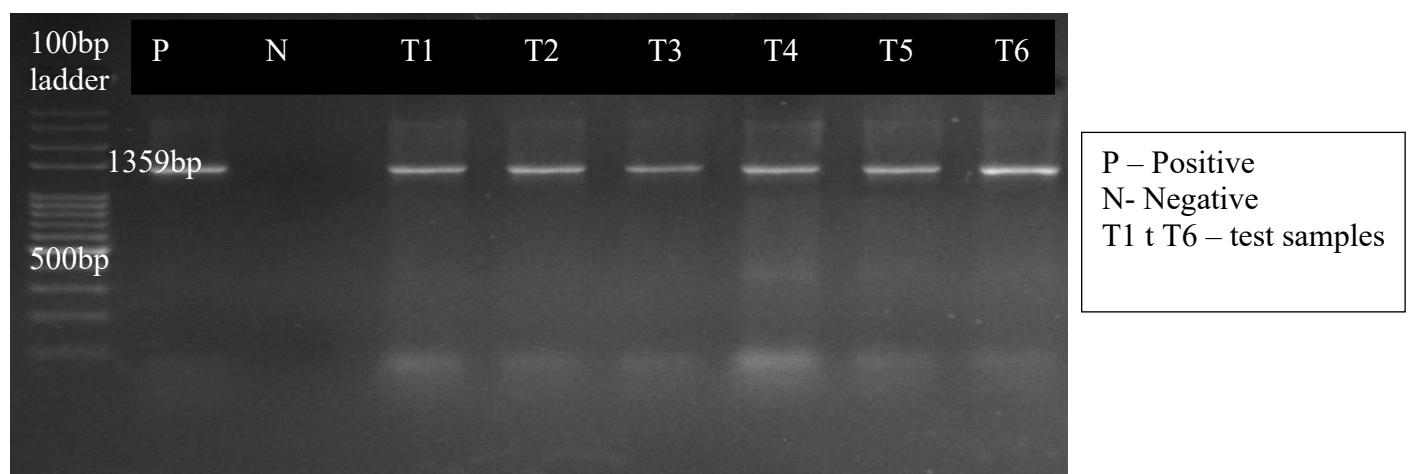
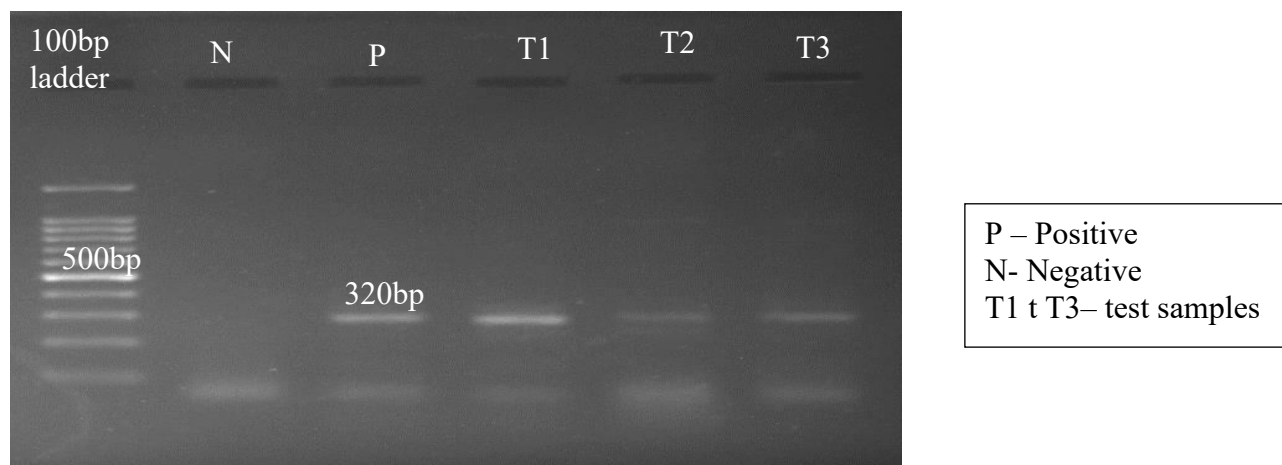


Fig.2 Detection of *mcr-1* gene by PCR



Author Contributions

A. N. Radhika: Investigation, formal analysis, writing—original draft. Binsy Mathew: Validation, methodology, writing—reviewing. C. Latha:—Formal analysis, writing—review and editing. K. Vrinda Menon: Investigation, writing—reviewing. T. Sathu: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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