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Phenotypic and Genotypic Characterisation of ESBL Producing and Carbapenem-resistant *Escherichia coli* and *Salmonella* spp. in Japanese Quail Farms of Wayanad District

Hema Persis Andrews[2*], K. Asha[2], Prejit[1, 2], Jess Vergis[1, 2], R. Rajasekhar[3], Hamna Hakim[2], N. Suma[2] and K. L. Akarsh

[1]Centre for One Health Education, Advocacy, Research and Training, India
[2]Department of Veterinary Public Health, CV&AS, Pookode, India
[3]Department of Veterinary Microbiology, CV&AS, Pookode, India
Kerala Veterinary and Animal Sciences University, Pookode, Wayanad-673576, India

**Corresponding author*

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A B S T R A C T

The long-term indiscriminate usage of antimicrobials in poultry for growth promotion and therapeutic purposes has resulted in the emergence of multi-drug resistant (MDR) bacteria like *E. coli* and *Salmonella* spp. The food-producing birds harbouring MDR genes of ESBL and Carbapenemases may possess health risks to the human population and is a major pressing issue affecting the market for broiler meat. The present study was conducted to find the occurrence of *E. coli* and *Salmonella* spp. in broiler Japanese quails of the Wayanad district and to detect the presence of ESBL and carbapenem resistance. A total of 22 cloacal swabs, two feed and two water samples were analysed for *E. coli* and *Salmonella* spp. by conventional microbiological, biochemical and molecular methods and the occurrence was observed as 100 and 9.09 per cent, respectively. The phenotypic characterization of isolates for ESBL production and carbapenem resistance was tested through disc diffusion method as per CLSI 2019 guidelines. The isolates were screened for *bla*CTX-M, *bla*SHV and *bla*TEM genes for ESBL genotypic characterization and 90.90 per cent of *E. coli* isolates were found to harbour at least one ESBL gene of interest whereas, negative results were obtained for *Salmonella* spp. isolates in both phenotypic and genotypic assay. None of the isolates was found positive for the presence of carbapenem-resistant S genes *bla*NDM, *bla*OXA-48, *bla*IMP and *bla*VIM. Thus, a diverse phenotypic and genotypic pattern of ESBL and carbapenem resistance was observed among isolates.

Introduction

The emergence and dissemination of antimicrobial resistance by means of ESBL and Carbapenemase production is a global public health threat. Animals and birds reared intensively for food purposes act as a reservoir host for multidrug resistant bacteria mainly belonging to the family *Enterobacteriaceae*. The zoonotic transmission of these bacteria to humans occur either directly from broilers and farm

environments or indirectly as a foodborne disease (Agyare *et al.,* 2018). Japanese quail (*Coturnix japonica*) is one of the important broiler birds in Kerala and also the smallest bird farmed for meat and egg.

However, the research on antimicrobial resistance organisms in quail are meagre in India, especially in Kerala. Hence, the present study envisaged detecting the *Escherichia coli* and *Salmonella* spp. in poultry and its farm environment along with ESBL and carbapenem resistance shown by the recovered isolates.

Materials and Methods

Sample collection

Cloacal swabs of apparently healthy broiler quail birds and environment samples (feed, water samples) were collected from two organised quail farms in Wayanad District.

A total of 22 cloacal swabs were collected in Cary-Blair transport medium and feed and water samples from the farms were collected in sterile containers. All the collected samples were transported to the laboratory under insulated chilled conditions.

Microbiological isolation

The samples were enriched in BPW for *E. coli* and *Salmonella* spp. The enriched samples were streaked onto MacConkey agar for the isolation of *E. coli* and the typical pink coloured lactose- fermenting colonies were presumptively identified as *E. coli.* Further, selective plating of *E. coli* was done on EMB agar.

The selective enrichment of the inoculum from BPW in RV broth, followed by selective plating on XLD agar was performed for the isolation of *Salmonella* spp. The microbiological and biochemical tests were performed as detailed by Barrow and Feltham (2003) and Agarwal *et al.,* (2003).

Molecular detection

The PCR mixture was prepared for the respective genes separately in a 0.2 mL PCR tube as mentioned in Table 7 and vortexed thoroughly to mix the reagents properly. PCR reaction was performed in an automated thermal cycler with a preheated lid.

The PCR targeting the *uid*Agene (Bej *et al.,* 1991) for *E. coli* and the *inv*A gene (Rahn *et al.,* 1992) for *Salmonella* spp. were used for the genus level confirmation.

Antibiotic susceptibility testing

The antibiotic susceptibility testing in the recovered isolates was performed as per CLSI guidelines (2019) by following the standard double disc synergy test using the commercial antibiotic disc of Ceftazidime, Cefotaxime and the corresponding clavulanate combinations and a ≥5mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanate versus the zone of diameter when tested alone was considered positive for ESBL production.

The antibiotic discs of Ertapenem (ETP 10 µg), Doripenem (DOR 10 µg), Meropenem (MRP 10 µg) and Imipenem (IPM 10 µg) were used for the genotypic characterization of carbapenem resistance and the obtained zone diameters of the isolates were compared with interpretative categories as per CLSI 2019 guidelines to grade the test isolates as sensitive (S), intermediate (I) and resistant (R) for the respective antibiotics.

Genotypic detection of ESBL and carbapenem resistance

The genotypic detection of ESBL production was performed using PCR targeting the selected ESBL genes viz, *bla*CTX-M, *bla*SHV and *bla*TEM and for carbapenem resistance, genes like *bla*NDM-1, *bla*IMP, *bla*VIM, *bla*OXA-48 were targeted. The primer details and cycling conditions for various genes used in the study are detailed in Table 1.

Results and Discussion

Isolation of *E. coli* and *Salmonella* spp. by Culture Method

The lactose fermenting pink coloured colonies on MacConkey agar plates were selected for further selective isolation of *E. coli* on EMB agar and purplish-black colonies with greenish metallic sheen were identified as *E. coli* (Fig. 1).

For *Salmonella* spp., after inoculation and incubation in BPW, pre-enrichment in Rappaport Vassiliadis (RV) broth was done followed by selective plating on XLD agar where the organism showed red coloured colonies with a black centre (Fig. 2).

PCR confirmation of *E. coli* and *Salmonella* spp. isolates

The presumptive colonies of *E. coli* from EMB agar and *Salmonella* spp. were confirmed by PCR targeting *uid*A (162bp) and *inv*A(264bp), respectively (Fig. 3 &4). All the 22 cloacal swabs were confirmed positive for *E. coli* and two were positive for *Salmonella* spp. whereas none of the feed and water samples was found positive for *E. coli* or *Salmonella* spp. (Table.2).

Occurrence of *E. coli* and *Salmonella* spp. in Japanese quail

The occurrence of *E. coli* was obtained as 100 per cent in the present study whereas 76.66 per cent of samples were reported as positive for the presence of *E. coli* by Sekhar *et al.,* (2017) and a much lower isolation rate of 6 per cent was documented by Farghaly *et al.,* (2017). The higher rate of occurrence of *E. coli* in the present study may be due to a lack of implementation of a proper biosecurity plan on farms or due to the variation in the factors such as geographical location, sample size, season and techniques of sampling and isolation. Out of the 22 quail cloacal samples analysed, two isolates (9.09 per cent) were positive for *Salmonella* spp. and the findings were similar to the nine per cent,11.11 per cent and 13.3 per cent reported by Awadallah *et al.,* (2013); Palanisamy and Bamaiyi (2015) and Jahan *et al.,* (2018), respectively in the earlier studies.

A higher occurrence of 65 per cent was reported by Khoshbakht *et al.,* (2017). However, Omoshaba *et al.,* (2017) documented a lower rate of 3.5 per cent from 400 quail cloacal samples which may be due to the large sample size in comparison with the present research work. No *Salmonella* spp. isolate was recovered by Dipineto *et al.,* (2014) from quail cloacal swabs sampled.

Double disc synergy test for the detection of ESBL production

A total of six out of 22 (27.27 per cent) cloacal swabs sampled were phenotypically positive for ESBL production when tested as per CLSI 2019 recommendations (Fig.5) and a similar result of 25 per cent was reported by Carissa *et al.,* (2013) in poultry birds. The two *Salmonella* spp. isolates were tested negative for ESBL production on DDST.

ESBL Genotypic characterisation

Out of 22 *E. coli* isolates recovered, 20 isolates (90.9 per cent) carried at least one ESBL gene of interest (Fig.7, 8 & 9). Out of this, 45.45 per cent (10 numbers) and 68.18 per cent (15 numbers) were found to carry *bla*CTX-M and *bla*TEM genes, respectively and five isolates were co-harboured *bla*CTX-M and *bla*TEM. Only one (4.45 per cent) *E. coli* isolate was found positive for the *bla*SHV gene which co-harboured *bla*CTX-M and *bla*TEM genes. No positive isolates for *bla*SHV were obtained in the study by Effendi *et al.,* (2021) in broiler chicken. The two *Salmonella* spp. positive isolates recovered found negative for the presence of *bla*CTX-M, *bla*SHV and *bla*TEM genes.

Antibiotic susceptibility testing for carbapenem resistance

Among the 22 *E. coli* positive isolates, 13 (59.09 per cent) were found as Imipenem- intermediate and

five were Imipenem resistant (22.72 per cent), seven (31.81 per cent) were Meropenem-intermediate and three (13. 63 per cent) were Meropenem resistant on Antibiotic susceptibility testing (ABST). None of the *E. coli* isolates showed phenotypic resistance against Doripenem and Ertapenem (Fig.6).

Buyukunal *et al.*, (2019) reported 97.2, 1.4 and 1.4 per cent of the isolates as Ertapenem sensitive, intermediate and resistant, respectively and also a similar antibiotic susceptibility pattern of the isolates were obtained in the study for both Imipenem and Meropenem i.e., 95.8, 2.8 and 1.4 per cent of the isolates were found as sensitive, intermediate and resistant, respectively for both the antibiotics. All the *Salmonella* spp. Isolates were found to be sensitive to the tested carbapenem antibiotics on ABST

Table.1 Details of oligonucleotides used in the study

Target organism	Gene	Gene Primer sequence	Amplicon size	Reference
Escherichia coli	*uid*A	F: TGGTAATTACCGACGAAAACGGC R: ACGCGTGGTTACAGTCTTGCG	162bp	(Bej *et al.*, 1991)
Salmonella spp.	*inv*A	F:GTGAAATTATCGCCACGTTCGGGCA R: TCATCGCACCGTCAAAGGAAC	284bp	(Rahn *et al.*, 1992)
ESBL producing *Enterobacteriaceae*	*bla*CTX-M	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	550bp	(Ahmed, 2004)
	*bla*SHV	F:5'- GATGAACGCTTTCCCCATGATG-3' R: 5'-CGCTGTTATCGCTCATGGTAA-3'	214bp	(Yazdi*et al.*,2012)
	*bla*TEM	F:5'- ATGAGTATTCAACATTTCCG-3' R: 5'-GTCACAGTTACCAATGCTTA-3'	847bp	
Carbapenemase producing *Enterobacteriaceae*	*bla*NDM-1	F: 5'-GCAGCTTGTCGGCCATGCGGGC-3' R: 5'-GGTCGCGAAGCTGAGCACCGCAT-3'	782bp	(Doyle *et al.*, 2012)
	*bla*IMP	F: 5'-GAAGGCGTTTATGTTCATAC-3' R: 5'-GTACGTTTCAAGAGTGATGC-3'	587bp	
	*bla*OXA-48	F: 5'-GCGTGGTTAAGGATGAACAC-3' R: 5'-CATCAAGTTCAACCCAACCG-3'	438bp	
	*bla*VIM	F: 5'-GTTTGGTCGCATATCGCAAC-3' R: 5'-AATGCGCAGCACCAGGATAG-3'	389bp	

Table.2 Occurrence of *E. coli* and *Salmonella* spp. among Japanese quail

Type of sample	No. of samples collected	*E. coli* isolates obtained	Occurrence of *E. coli* (per cent)	*Salmonella* spp. isolates obtained	Occurrence of *Salmonella* spp.(per cent)
Cloacal swab	22	22	100	2	9.09
Feed	2	0	0	0	0
Water	2	0	0	0	0

Fig.1 *E. coli* on EMB agar: Blue-black colonies with greenish metallic sheen

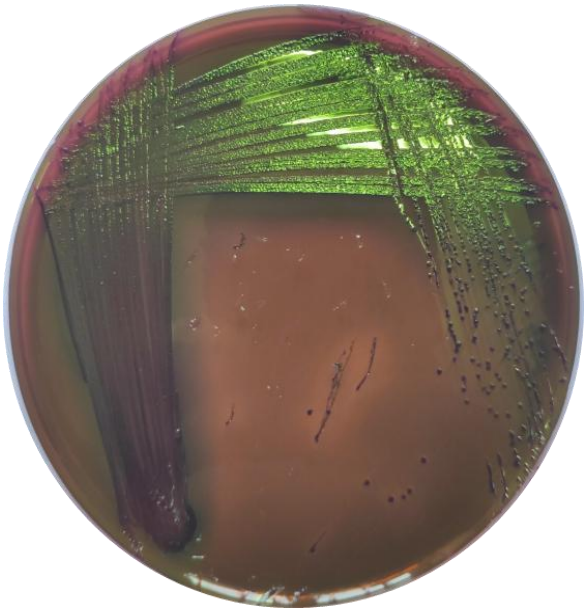


Fig.2 *Salmonella* spp. on XLD agar: Red colonies with black centre

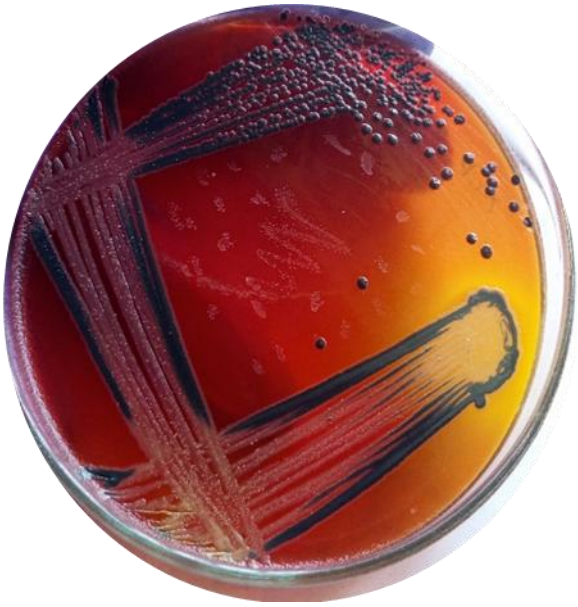


Fig.3 PCR Standardisation of *inv*A
Lane M-Marker, Lane N-Negative control, Lane P-Positive control (amplicon size 284bp), Lane 1&2: *inv*Apositive isolates

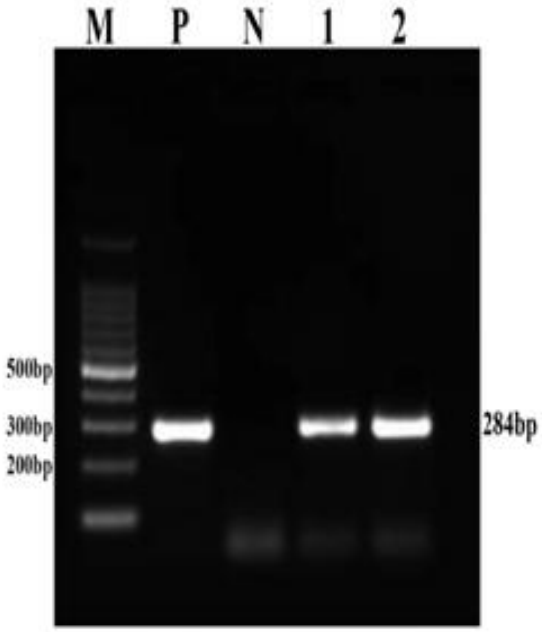


Fig.4 PCR Standardisation of *uid*A
Lane M-Marker, Lane N-Negative control, Lane P-Positive control (amplicon size 162bp), Lane1-3: *uid*Apositive isolates

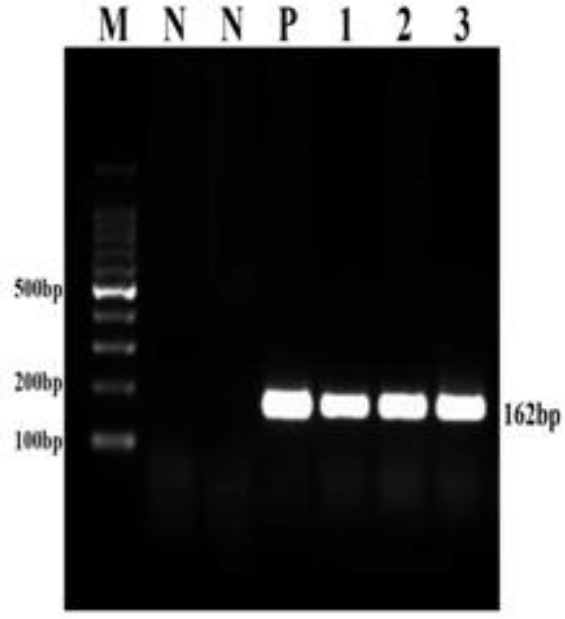


Fig.5 Antibiotic susceptibility pattern of isolates for ESBL production

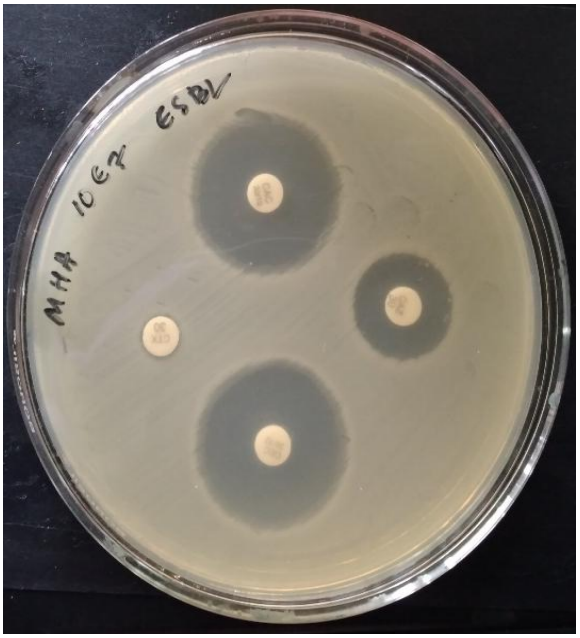
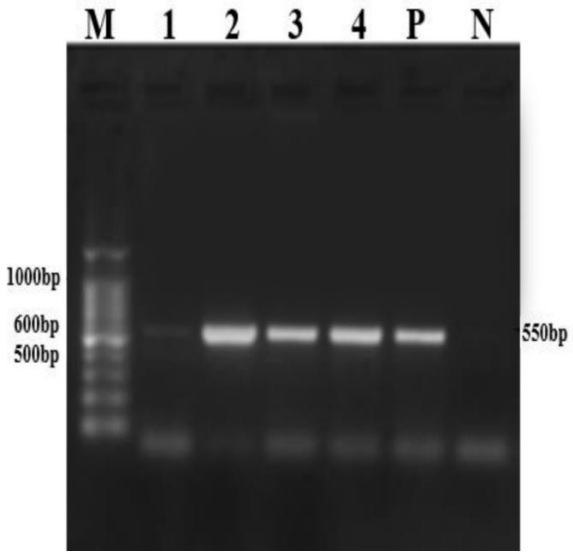


Fig.6 Antibiotic susceptibility pattern of isolates for carbapenem resistance

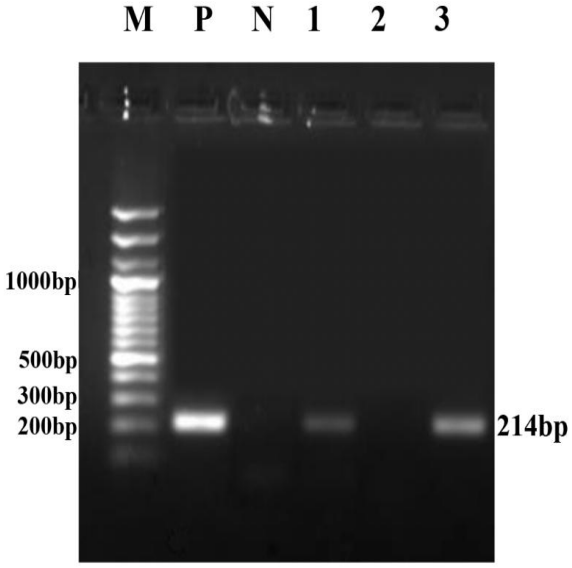
***bla*CTX-M**

Fig.7 PCR Standardisation of *bla*CTX-M Lane M-Marker, Lane N-Negative control, Lane P-Positive control (amplicon size 550bp), Lane1-4: *bla*CTX-Mpositive isolates



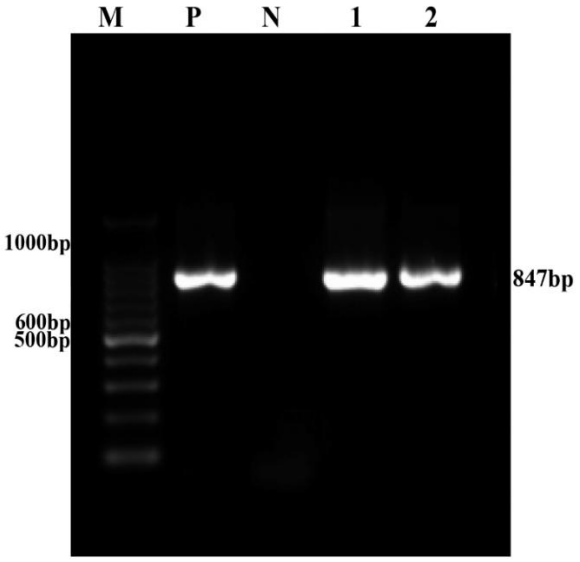
***bla*SHV**

Fig.8 PCR Standardisation of *bla*SHVLane M-Marker, Lane N-Negative control, Lane P-Positive control (amplicon size 214bp), Lane1&3- *bla*SHV positive isolates, Lane 2- *bla*SHV negative isolate



***bla*TEM**

Fig.9 PCR Standardisation of *bla*TEM
Lane M-Marker, Lane N-Negative control,
Lane P-Positive control (amplicon size: 847bp),
Lane1&3: *bla*TEM positive isolates



Genotypic characterization for carbapenem resistance

None of the *E. coli* and *Salmonella* spp. isolates was found to harbour the selected genes for forscarbapenem resistance in the present study and a similar result was documented by Roschanski *et al.*, (2018).

The molecular detection of ESBL genes and phenotypic resistance against carbapenem antibiotics obtained in spite of the small sample size in the present study advocates for further studies in the subject.

There are only a few reports of ESBL and Carbapenem resistant *Enterobacteriaceae* in broiler poultry and farm environment, especially in the case of Japanese quail in Kerala. Thus, the findings of the present study revealed the role of broiler farms as a reservoir of antimicrobial resistant bacteria and can be used as preliminary data for further research on the subject.

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