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Detection and Molecular Characterization of bla_{VEB} and $bla_{CTX-M-1}$ Genes Producing Imipenem-resistant *Klebsiella pneumonia* from Birds in Hillah City, Iraq

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ABSTRACT

The tool of the current investigation was to assess the frequency of *Enterobacteriaceae* from fecal samples of local birds in Hillah city during the period from February, 2022 to the end of April, 2022. Thirty-six per cent isolates were identified as *Enterobacteriaceae* when *K. pneumonia* demonstrated higher frequency 14 (14%) followed by *E. coli* 10 (10%), *Proteus* spp. 6 (6%), *K. oxytoca* 3 (3%) and *Enterobacter* spp. 3 (3%). Disk diffusion test was employed to check antimicrobial susceptibility profile of *Enterobacteriaceae*. All bacterial isolates displayed highest resistant level (100%) for ampicillin, cloxacillin, amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, cefepime and aztreonam. Lower resistance was observed to levofloxacin and imipenem. Genotypic detection of bla_{VEB} and $bla_{CTX-M-1}$ ESBLs was investigated by PCR technique among imipenem-resistant *K. pneumoniae* isolates. Six (100%) and two (33.33%) isolates had bla_{VEB} and $bla_{CTX-M-1}$ gene, respectively.

Key words: Antibiotics resistance, *Klebsiella pneumoniae*, ESBLs, bla_{VEB}, $bla_{CTX-M-1,}$ local birds, PCR

INTRODUCTION

Resistance to antimicrobial is an emerging global threat due to the increasing numbers of microorganisms resistant to commonly used antimicrobials (Oduyebo *et al.*, 2017). A significant concern is the resistance to antibiotics of β-lactammediated by β-lactamases and their encoding resistant genes are widely distributed among Gram-negative bacteria especially *Enterobacteriaceae* and *Pseudomonas* spp. (Shrestha *et al.*, 2022). The use of antibiotics of β-lactam in an extensive rate worldwide can lead to excessive proliferations of antibiotic resistant pathogens and extensive β-lactamases evolution (Bottery *et al.*, 2021). These enzymes include extended spectrum β-lactamases (ESBLs), AmpC and carbapenemases which have been recognized as the most important epidemiological resistant mechanisms to antimicrobial agents in *Enterobacterales* (Marti´nez-Marti´nez and Gonzalez-Lopez, 2014).

ESBLs are mobilized by conjugative plasmid and can be easily shifted between different bacterial species or strains (Gekenidis *et al*, 2020). ESBLs-carrying bacteria can cause infections linked with increased mortality, morbidity and costs (Ghafourian *et al.*, 2015; Ghenea *et al.*, 2022). *Enterobacteriaceae* harboring these enzymes have major implication for animals and humans through contamination of both water and food (Maciuca *et al.*, 2015). Several studies proved the presence of ESBL-carrying bacteria in wild and urban birds (Ngaiganam *et al.*, 2019; Athanasakopoulou *et al.*, 2022). The responsibility of birds from wild type in the globally circulation of β-lactamases among *Enterobacterales* has been demonstrated (Wang *et al.*, 2017).

This work was aimed at describing the prevalence of *Enterobacteriaceae* isolates from fecal samples of local birds in Hillah city, Iraq to determine their resistance pattern as well as detect ESBLs genes (bla_{VEB} and $bla_{CTX-M-1}$) in imipenem resistant *K. pneumonia* isolates using Polymerase Chain Reaction assay.

MATERIALS AND METHODS

One hundred fresh stool samples were collected from different local birds in Hillah city, between the period from February, 2022 to April, 2022. The swabs were brought to the laboratory directly in transport media for microbiological examination. Each swab sample was cultured on different enrichment and selective media.

Identification of isolated bacteria was achieved following standard microbiological tests as previously described (MacFaddin, 2000).

All *Enterobacteriaceae* strains collected from birds fecal specimens were checked with antimicrobial susceptibility testing (AST) by disc diffusion test in accordance with the criteria of Clinical and Laboratory Standards Institute (CLSI, 2016). A reference strain *Escherichia coli* ATCC 25922 (University of Kufa, College of Medicine) was used for susceptibility procedure. The agents selected were: levofloxacin (LE^{5}), cloxacillin (OX), ampicillin (AMP), ceftazidime (CAZ), aztreonam (ATM), ceftriaxone (CRO), cefepime (FEP), imipenem (IMP) and amoxicillin-clavulanic acid (AMC). The inhibition zone diameters were determined and the tested bacterial isolates were classified as sensitive, intermediate or resistant in line with the criteria of the CLSI (CLSI, 2016).

Plasmid DNA template of pure imipenem-resistant *K. pneumonia* was extracted following modified method. The presence of genes encoding for (*bla*$_{VEB}$ and *bla*$_{CTX-M-1}$) ESBLs was investigated using conventional PCR assay with specific primers (Bioneer, Korea). For VEB/ F: (5^{-}-ACCAGATAGGAGTACAGAC ATATG3^{-}) and VEB/ R: (5^{-}TTCATCACCGCGA TAAAGCAC-3-) (727bp); CTX-M-1/F : (5^{-}-AAAGTGATGGCCGTGGCC -3^{-}) and CTX-M-1 R: (5'-GATATCGTTGGTGGTGCCA-3') (522bp) (Laudy *et al.*, 2017). The PCR conditions for each gene were illustrated (Table 1). The products of PCR were resolved on agarose (1.5%) by electrophoresis run for 2-3 h at 70 volts and the visualization of the gel was achieved using UV-Trans-illuminator.

RESULTS AND DISCUSSION

The results exposed that 36/100 (36%) bacterial isolates were found to be *Enterobacteriaceae*, the most commonest identified bacterium was *K. pneumoniae* 14 (14%) followed by *E. coli* 10 (10%), *Proteus* spp. 6 (6%), *K. oxytoca* 3 (3%) and *Enterobacter* spp. 3 (3 %; Table 2). A work conducted in Egypt documented the occurrence of *Pseudomona aeruginosa*, *E. coli*, *K. pneumonia* and *K. oxytoca* in wild birds fecal samples (Ahmed *et al.*, 2019). In Greece, Athanasakopoulou *et al.* (2022) detected *E. coli* from wild bird fecal samples. Mohamed *et al.* (2022) stated that the incidence of *E. coli* from birds of wild type was (47.4%) in Malaysia. Other study carried out in Ghana reported *E. coli* from farms fecal samples and chicken samples with a prevalence rates of 28 (56.2%) and 8 (32%), respectively (Mensah *et al.*, 2022). For *Proteus* spp. recovered from urban birds, low prevalence rate was recorded by Ngaiganam *et al.* (2019) in France.

Table 2. Distribution of *Enterobacteriaceae* isolates obtained from birds fecal samples (n=100)

Type of isolated bacteria	Numbers	Percentages
Klebsiella pneumoniae	14	14
Escherichia coli	10	10
Proteus spp.	6	6
Klebsiella oxytoca	3	3
Enterobacter spp.	3	3
Bacterial isolates other than Enterobacteriaceae	64	64
Total	100	100

However, some researchers suggested that the differences in feeding habits can affect the existence of various bacterial species in birds population (Vittecoq *et al.*, 2017; Sharma *et al.*, 2018). The possibility of birds to contact with human and other animals makes them an ideal model to investigate the exchange of pathogenic bacteria between human and the environment (Modupe *et al.*, 2021).

Concerning antibiotic susceptibility testing data obtained by standard disk diffusion test, results showed that all *Enterobacteriaceae* strains in the present research were realized to be completely resistance (100%) to ampicillin, cloxacillin, amoxicillin- clavulanic acid, ceftazidime, ceftriaxone, cefepime and aztreonam (Table 3). These results correlate with the findings of Rybak *et al.* (2022) who

Table 1. PCR cycling conditions for ESBL gene amplification

Target gene	Cycling conditions					Cycle number
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	
bla$_{VEB}$	93/3 min	93/min	55/1 min	72/1 min	72/7 min	40
bla$_{CTX-M-1}$	94/5 min	94/30 sec.	63/30 sec.	72/1 min	72/5 min	35

Table 3. Rate of resistance for all *Enterobacteriaceae* isolated from birds fecal samples (n = 36)

Agent tested	Type of resistant bacteria				
	Klebsiella pneumoniae No. (%)	*Escherichia coli* No. (%)	*Proteus* spp. No. (%)	*Klebsiella oxytoca* No. (%)	*Enterobacter* spp. No.(%)
Ampicillin	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Cloxacillin	14 (100)	7 (70)	6 (100)	3 (100)	3 (100)
Amoxicillin-clavulanic acid	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Ceftriaxone	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Ceftazidime	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Cefepime	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Aztreonam	14 (100)	10 (100)	6 (100)	3 (100)	3 (100)
Imipenem	6 (42.85)	2 (20)	2 (33.33)	0 (0)	1 (33.33)
Levofloxacin	6 (42.85)	4 (40)	2 (33.33)	1 (33.33)	1 (33.33)

detected *E. coli* strain recovered from living birds with higher degree of resistance (100%) to ampicillin and cefuroxime (97%), to ceftriaxone and cefotaxime (91%), to amoxicillin/clavulanic acid (88%) to ceftazidime (70%) to cefepime (61%), to piperacillin/tazobactam followed by (76%) resistance rate to sulphamethoxazole/ trimethoprim.

However, the lower level of antibiotics resistance was found for imipenem and levofloxacin antibiotics and zero resistance was recorded for imipenem by *Klebsiella oxytoca* (Table 3). Giacopello *et al.* (2016) detected multi-drug resistant (MDR) to antimicrobial agents (three or more classes) tested in his study when significant rates of resistance were observed to amoxicillin/clavulanic acid, ampicillin and streptomycin among *Enterobacteriaceae* strains isolated from European wild bird, imipenem displayed the low level of resistance.

In the present study, *Enterobacteriaceae* with multi-resistant phenotype were isolated from birds fecal samples, as transmission of these bacterial isolates to the surrounding environmental sources may happen via birds fecal deposits, so birds can be considered as a potential hazard to public health. Some researchers proposed that livestock constitute a big reservoir for MDR bacteria, dissemination of these isolates to humans and other animals, like wildlife can occur through manure, sewage, direct contact or via food chain (Bennani *et al.*, 2020; Hong *et al.*, 2020; Homeier-Bachmann *et al.*, 2021).

According to PCR assay, bla_{VEB} gene was observed in fecal samples of 6 (100%) imipenem –resistant *K. pneumoniae* isolates (Fig. 1). In a previous study, resistance gene encoding bla_{VEB} was not detected among bacteria of Gram-negative obtained from fecal specimens of urban birds, France (Ngaiganam *et al.*, 2019). Recently, bla_{VEB} gene was reported in *E.coli* recovered from farms of chicken in Egypt (Badr *et al.*, 2022). In this study, ESBLs of CTX-M-1 type were detected in 2 (33.33%) imipenem–resistant *K. pneumoniae* isolates (Fig. 2). In an investigation by Brendecke *et al.* (2022) characterized the presence of *K. pneumoniae* carrying $bla_{CTX-M-15}$ with low prevalence obtained from black-headed gulls in Germany. The detection of $bla_{CTX-M-1}$ was previously proved in *Escherchia coli* collected from urban birds in Marseille city, France (Ngaiganam *et al.*, 2019). Another research achieved in Greece identified CTX-M-1 alone or combined with TEM among the bacterium *Escherichia coli* obtained from different wild birds fecal samples (Athanasakopoulou *et al.*, 2022). However, ESBLs of VEB and CTX-M-1 harboring *K. pneumoniae* have also been demonstrated in clinical settings (Fazeli *et al.*, 2015; Abbas, 2017; Mirkalantari and Moghadas, 2018).

CONCLUSION

The finding of this research highlighted the incidence of *K. pneumonia* carrying VEB and CTX-M-1 ESBL genes from local birds fecal samples in Hillah city. These results reinforced the idea that birds were important vehicles for ESBLs transmission and dissemination between animals, environment and human that posed a greater risk to public health. Further epidemiological research and genome sequencing of multi-drug resistant and pan- drug resistant bacteria from birds of various geographical regions is required to investigate the role of these agents in circulating of such highly resistant pathogens.

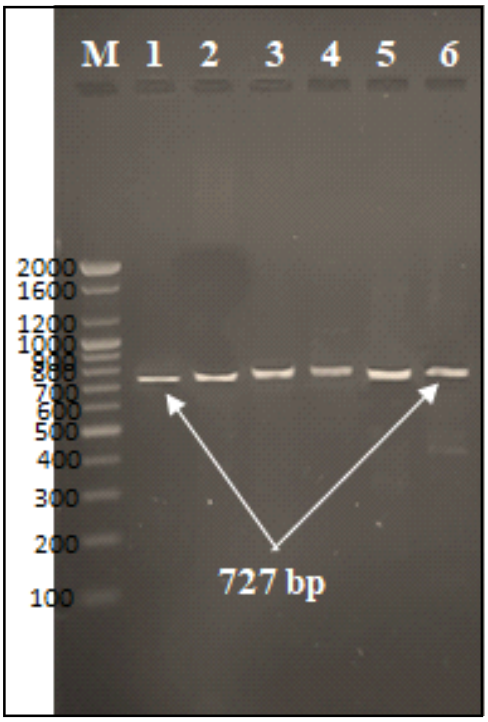

Fig. 1. Results of conventional PCR for VEB type in imipenem resist *K. pneumoniae*. Lane (M): DNA marker (100-bp) and Lanes (1, 2, 3, 4, 5, 6): Positive samples with VEB (727 bp) gene.

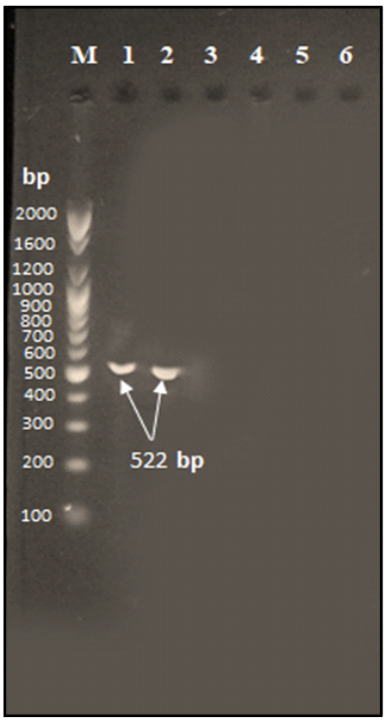

Fig. 2. Results of conventional PCR for VEB type in imipenem resist *K. pneumoniae*. Lane (M): DNA marker (100-bp) and Lane (1, 2): Positive samples with CTX-M-1 (522 bp) gene.

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