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Phylogenetic characterisation, virulence factors, and multi-drug resistance of *Escherichia coli* strains isolated from faeces of feral pigeons (*Columba Livia* forma *urbana*)

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Abstract

Background Feral pigeons are a synanthropic species commonly found in cities worldwide. They are known to carry zoonotic pathogens, including *Escherichia coli*, and have long raised concerns about environmental contamination and public health risks.

Objectives The aim of the study was to phylogenetically classify, identify selected virulence genes and determine the phenotypic antimicrobial susceptibility profiles of *E. coli* isolated from pigeon faeces in urban agglomeration.

Methodology A total of 120 fresh faecal samples were collected from feral pigeons in urban areas. Groups of 4 samples from each location were tested in a total of 30 pools. A total of 97 faecal *E. coli* isolates were screened for enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) strain genes and thirteen selected virulence factors associated with pathogenic function and activity. Resistance patterns were determined by the Kirby-Bauer disk diffusion method for twenty antibiotics.

Result The most common phylogenetic group was group D (70/97, 72.2%), followed by group A (15/97, 15.5%), B1 (7/97, 7.2%) and B2 (3/97, 3.1%). EPEC and STEC were found in 5.2% and 22.7% isolates, respectively. The obtained results showed *katP*, $lpfA_{O157/OI-141}$, *tir*, *iha* and $lpfA_{O157/OI-154}$ genes in *eaeA*-positive and *stx*-positive isolates, mainly from phylogroups D and B2. The isolated *E. coli* strains were resistant to at least one antibiotic in 16.5%, and 2.1% were recognised as multidrug-resistant (MDR).

Conclusions The results of this study confirm that pigeons in the urban environment are carriers of potentially pathogenic strains of *E. coli*, including MDR strains. Twelve patterns of virulence genes were identified among *E. coli* strains, with a great predominance of the single gene stx_1 encoding Shiga toxin 1. The highest resistance was observed for imipenem (IMP), tetracycline (TE) and doxycycline (DO), respectively, and these antibiotics were also involved in most of the observed resistance patterns. The obtained results justify the implementation of preventive measures in cities and the introduction of surveillance programs for synanthropic pigeon populations to protect both the urban environment and public health.

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Table 3 Patterns of virulence genes identified in phylogroups of *E. coli* strains isolated from pigeon faeces

Virulence genes pattern	Phylogenetic group (No.*)					Total No. (%)
	A	B1	B2	D	X**	
eaeA	–	–	–	2	–	2 (2.06)
stx_1	9	7	1	48	–	65 (67.01)
stx_2	–	–	–	3	–	3 (3.09)
stx_1 + *katP*	–	–	–	1	1	2 (2.06)
eaeA + stx_1	6	–	–	4	–	10 (10.31)
eaeA + stx_1 + *katP*	–	–	–	3	1	4 (4.12)
eaeA + stx_1 + *katP* + $lpfA_{O157/OI-154}$	–	–	–	1	–	1 (1.03)
eaeA + stx_1 + $lpfA_{O157/OI-141}$	–	–	–	4	–	4 (4.12)
eaeA + stx_2 + *tir*	–	–	–	1	–	1 (1.03)
eaeA + stx_2 + *tir* + *iha*	–	–	1	–	–	1 (1.03)
eaeA + stx_2 + *tir* + *iha* + *katP*	–	–	1	–	–	1 (1.03)
eaeA + *katP*	–	–	–	3	–	3 (3.10)
Total of non-virulence isolates (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total no. (%)	15 (15.46)	7 (7.22)	3 (3.09)	70 (72.17)	2 (2.06)	97 (100.00)

*No. – number of isolates; **X – undefined phylogenetic group

At least one of the virulence genes was found in all samples tested. More than one gene was present in 29.9% (29/97) of the *E. coli* isolates (Table 3).

Discussion

Most epidemiological studies on wild birds focus on migration as a risk factor for the transmission of zoonotic pathogens to farms and the environment. Much data is available on the prevalence of diarrhoeal and MDR *E. coli* strains in migratory ducks, geese, quails, swans, cormo-rants, and other wild bird species [40–43]. The source of pathogenic *E. coli* may also be the faeces of synanthropic bird species, e.g., feral pigeons, which commonly and numerously inhabit urban agglomerations, however, data on the prevalence of different *E. coli* pathotypes are lim-ited. Some studies have analysed samples from a wide range of host birds, including pigeons [20, 44, 45]. Most of them focus on detecting the *eaeA*, stx_1 and stx_2 genes as the genetic determinants of common EPEC and STEC strains, which are one of the main causes of diarrhoeal diseases in children and adults around the world. The prevalence of potentially diarrhoea-causing human *E. coli* strains in urban pigeon faeces was estimated at 9.4% in Iran [46], 10.7% in Rome [47], and 12.1% in Brazil [48]. In Brazil, Silva et al. [48] identified 7.1% enterotoxigenic and 0.5% enteroinvasive strains among all diarrheagenic *E. coli* detected. Atypical EPEC was also found in four sam-ples from urban pigeons in Brazil [20] and three samples from pigeons in Hungary [49].

The average total number of *E. coli* obtained in the current study (8.78×10^7 CFU/g) was lower than the results obtained by Karim et al. [50] in oral and cloacal swabs samples from individual birds 8.78×10^7 CFU/g and 6.8×10^9 CFU/ml, respectively. Karim et al. [50] and Sacristán et al. [51] found the presence of *E. coli in 50.0% and 78.6% of cloacal swabs, respectively.*

While Silva et al. [48] and Ghanbarpour and Daneshdoost [46] found higher recoveries for fresh urban pigeon faeces, 78.6% and 89.6%, respectively. As shown by the above authors, the recovery of *E. coli* strains from individual bird sam-ples is within the range estimated in this study for pooled faecal samples (25–100%). Differences in the total num-ber of bacteria obtained by microbiological culture meth-ods may vary depending on the location of the pigeon population under study, the source of biological material (e.g., cloacal swabs, faeces), or the type of medium used.

In the current study, most of the isolated strains belonged to phylogenetic groups related to ExPEC and diarrheagenic *E. coli*, including 72.2% to group D and 3.1% to group B2. The *eaeA* gene, which determines the production of intimin (adhesin) necessary for the attach-ment of bacteria to epithelial cells and typical of EPEC, as well as genes responsible for the production of Shiga toxins and other virulence factors, were identified in 18 and 15 strains, respectively (Table 3). Although the prevalence of the specific *E. coli* phylogenetic groups iso-lated in urban pigeons may vary [21, 46, 52], the *eaeA*, stx_1 and stx_2 genes appear to be common in these bird populations. In Europe, there are only a few reports on the occurrence and characteristics of the pathogenic *E. coli* (STEC and EPEC) originating from feral pigeons in cities. The frequency of these genes obtained in the cur-rent study (37.1% *eaeA*-positive, 88.7% stx_1-positive and 6.2% stx_2-positive) is higher than that reported in other studies. In Spain, 6% (2/33) *E. coli* isolated from urban pigeons were positive for *eaeA* gene and negative for stx_1 and stx_2. However, only *eaeA*-positive strains were ana-lysed for the toxin [51]. The presented results showed that these genes can be detected independently of each other (Table 3). In research conducted in Finland, the *eaeA* gene was confirmed in two (6.9%) pigeons out of

29 tested [53]. Gargiulo et al. [54] showed the prevalence of *E. coli* O157 in 7.8% of cloacal swab samples collected from a total of 1,800 urban pigeons in Italy. The genes responsible for the production of Shiga toxin and encoding intimin in STEC and EPEC *E. coli* strains were isolated from faeces of urban pigeons in Germany [55], Portugal [56], Iran [46], Brazil [20].

In the current study, the virulence genes of translocated intimin receptor (*tir*) on the locus of enterocyte effacement pathogenicity island, gene encoding IrgA homologue adhesin (*iha*), two markers of long polar fimbriae ($lpfA_{O157/OI-154}$, $lpfA_{O157/OI-141}$) and catalase-peroxidase gene (*katP*) were detected in STEC strains and only in phylogenetic group D and B2. According to Kobayashi et al. [57], these genetic features include: those associated with high virulence of various STEC seropathotypes (A, B, C, D/E) and the course of the disease in humans. Bujňáková et al. [58] showed the prevalence of other virulence genes in a group of carrier and domestic pigeons in Slovakia, but similarly to this study, the largest number of them was obtained for *E. coli* strains from groups B2 and D. To the best of our knowledge, the results of the current work are among the first in Europe focusing on the occurrence of virulence factors other than commonly researched *eaeA*, stx_1 and stx_2 gene in feral pigeons from urban agglomeration. So far, only Borges et al. [20] investigated the presence of the *iha*, *saa*, $lpfA_{O113}$, *espA*, *nlB* and *nlE* genes among *E. coli* isolates from free-living urban pigeons in Brazil. The genes mentioned were previously detected in clinical samples from humans symptomatically infected with *E. coli*.

Antimicrobial resistance phenotypes of cultured *E. coli* strains from pigeon faeces were confirmed, with a low percentage of resistant strains of 16.5%, of which over 7.0% were classified as MDR. Karim et al. [50] obtained much higher results, where 95.2% of isolated *E. coli* from 20 pigeons raised under household conditions were resistant to at least one to six antibiotics, and 23.8% were found to be MDR, with the highest resistance found to ampicillin, amoxicillin, erythromycin, and tetracycline (over 50% of isolates each). The obtained results may be related to treatment with different groups of antibiotics in domestic pigeons, the development of resistance to another antibiotic due to cross-resistance or co-selection. Although domestic animals seem to be more predisposed as a source of resistant bacteria, a relatively high prevalence of MDR *E. coli* isolated from wild mammals (wild boars (*Sus scrofa*), hedgehogs (*Erinaceus europaeus*), red fox (*Vulpes vulpes*)) and wild birds (e.g., common kestrels (*Falco tinnunculus*), white storks (*Ciconia Ciconia*), red kites (*Milvus milvus*), booted eagles (*Hieraaetus pennatus*), tawny owls (*Strix aluco*) has been found (71.9%), with mainly resistant to ampicillin (84.4%), trimethoprim–sulfamethoxazole (43.8%), tetracycline (39.1%), nalidixic acid (39.1%), and amoxicillin–clavulanic acid (33.6%) [59]. Among wild birds rescued in North-Western Italy, including birds of prey and synanthropic species, MDR *E. coli* was demonstrated in 13.2% of isolates, with the highest resistance rate observed for cephalexin (84.6%), amoxicillin-clavulanic acid (39.6%), and tetracycline (31.5%) [60]. Bueno et al. [21] obtained resistance to at least one antibiotic in all isolates from healthy free-living pigeons, and MDR was much higher and amounted to 63.0%. The greatest resistance was found in the aminoglycoside class, with the highest percentage of resistance found to streptomycin (88.0%). In turn, of the 203 *E. coli* isolates obtained from wild pigeons tested in the Czech Republic, drug resistance was detected in only 3 isolates (1.5%) [61].

The resistance results of *E. coli* strains obtained in this study differ from those reported by other authors. Unexpectedly, the highest number of imipenem-resistant strains was detected (9.3%). Importantly, the imipenem-resistant isolates originated from collection points located near hospitals (Fig. 2). Imipenem, a member of the carbapenem class, is a bactericidal beta-lactam antibiotic used in hospitals to treat severe infections in humans. Furthermore, it is particularly relevant in the context of the global problem of bacterial resistance to carbapenems and has been classified as "critically important for human medicine." [62]. Sabença et al. [59] showed that all *E. coli* isolates from faecal samples from different wild birds and mammals in Portugal were susceptible to imipenem. Other studies conducted in wild animals showed varied resistance profiles of bacteria, with the most common resistance to ampicillin (71.5%) and tetracycline (63.6%), but susceptibility to imipenem was not tested [63]. Imipenem resistance of almost 7.0%, exhibiting five different MDR phenotypes, was found in a population of wild Canarian Egyptian vultures (*Neophron percnopterus majorensis*) endemic to the Canary Islands, Spain [64, 65]. Bueno et al. [21] showed high imipenem resistance in pigeon strains (almost 40%) and lower resistance to tetracyclines (approximately 25%), including three strains resistant to both antibiotics simultaneously. Ghanbarpour and Daneshdoost [46], although they showed an overall high level of resistance in *E. coli* isolated from pigeons, did not include imipenem in their study.

The available literature data on antibiotic resistance in wild animals, especially synanthropic species, are very limited, and the results obtained are variable. Differences in the results of antibiotic sensitivity tests in wildlife may be caused by factors such as the type region or habitat (e.g., agricultural, urban areas), source and level of environmental contamination, wild animal species' feeding strategies, movement and migration, and the degree of synanthropization of the animal species tested [29, 66].

It is also possible that there are previously unknown acquired mechanisms of antibiotic resistance among commensal *E. coli* strains of wild animals [67].

The drug resistance patterns in *E. coli* strains isolated from pigeons may differ depending on the studied population. Ghanbarpour and Daneshdoost [46] found twenty different resistance testing patterns for only eight selected antimicrobials. In this study, the level of resistance obtained in *E. coli* strains isolated from the faeces of feral pigeons living near hospitals (58.3%) may indicate a relationship between the environment and the occurrence of antibiotic resistance in bacterial populations. The low number of resistant *E. coli* strains in the other locations suggests that birds may have had limited exposure to resistant bacteria strains or anthropogenic antimicrobials. Since feral pigeons are not treated with antibiotics, it is suggested that acquired resistance is related to their urban environment and feeding behaviour [21, 61]. However, studies confirm that the prevalence of MDR strains of *E. coli* from these birds is quite high and may be an important factor in the spread of antibiotic resistance in urban environment. In addition, the presence of the complete set of AcrAB-TolC complex genes in almost all isolated strains found in the current study indicates the effectiveness of removing many antimicrobial agents from bacterial cells, including antibiotics. The AcrAB – TolC efflux pump contributes to multidrug resistance in gram-negative bacteria, especially in *Enterobacteriaceae* [68]. It appears that the role of synanthropic pigeons in acquiring, maintaining, and spreading antibiotic-resistant bacteria in urban ecosystems is still not fully understood, and further research is necessary.

Limitations of the study

Despite careful observations of the locations where wild pigeons occur, it cannot be ruled out that the collective sample included a faecal sample from more than one individual. Pooling samples may have reduced the specificity of the research methods used and thus influenced the interpretation of the results. Therefore, tests for antibiotic resistance and the presence of virulence genes included only *E. coli* strains whose isolated DNA was confirmed by genetic methods. Although biochemical methods allow for preliminary identification of isolated bacteria, they may have some limitations. In this study, three strains (3/100, 3.0%) initially identified as *E. coli* were excluded from further research procedures due to the lack of confirmation by genetic methods.

Conclusion

The faeces of feral urban pigeons are a source of both "*pathogenic*" and "*non-pathogenic*" *E. coli* strains, with mixed strains predominating. Most strains were classified into phylogenetic groups D and A, related to human sources. Twelve patterns of virulence genes were identified among *E. coli* strains, with a great predominance of the single gene stx_1 encoding Shiga toxin 1. For the first time in Europe, the presence of the virulence genes: *tir*, *iha*, $lpfA_{O157/OI-154}$, $lpfA_{O157/OI-141}$, *katP* was confirmed in STEC strains from phylogenetic groups D and B2 of *E. coli* isolated from pigeon faecal samples. The highest resistance was observed for imipenem (IMP), tetracycline (TE), and doxycycline (DO), and these antibiotics were also involved in most of the observed resistance patterns, including MDR. The obtained results justify the implementation of preventive measures in cities and the introduction of surveillance programs for synanthropic pigeon populations. This may help to protect both the urban environment and public health.

Abbreviations

µg	Microgram
16S rRNA	16 Svedberg ribosomal ribonucleic acid
AM	Ampicillin
AMC	Amoxicillin-clavulanic acid
Ami	Aminoglycosides
ATCC	American Type Culture Collection
ATM	Aztreonam
AZM	Azithromycin
bp	Base pair
C	Chloramphenicol
Car	Carbapenems
CAZ	Ceftazidime
CC	ChromID Coli
Cep	Cephalosporins
CFU/g	Colony-forming unit per gram
CXM	Cefuroxime
DNA	Deoxyribonucleic acid
DO	Doxycycline
E. coli	Escherichia coli
EHEC	Enterohemorrhagic
ENT	Ears, Nose, and Throat
EPEC	Enteropathogenic
ExPEC	Extraintestinal pathogenic Escherichia coli
Flu	Fluoroquinolones
I	Intermediate sensitive
IMP	Imipenem
InPEC/IPEC	Intestinal pathogenic Escherichia coli
K	Kanamycin
MDR	Multidrug-resistant
MEM	Meropenem
MH	Mueller-Hinton
Mon	Monobactams
NA	Nalidixic acid
No.	Number of strains/isolates
OFX	Ofloxacin
Pen	Penicillins
PRL	Piperacillin
R	Resistant
R + EB	Rebecca agar with EB Supplement
S	Streptomycin
S	Susceptible
SAM	Ampicillin-sulbactam
STEC	Shiga toxin-producing
T3SS	The type III secretion system
TE	Tetracycline
Tet	Tetracyclines
TPZ	Piperacillin-tazobactam
TS	Trimethoprim-sulfamethoxazole
TTC	Ticarcillin-clavulanic acid

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
KK: project administration, planning and organization of the study, methodology, collection of samples and investigation, writing - original draft. AWF: project and investigations supervision, methodology, writing – review and editing. All authors read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this published article. The partial sequences are deposited in GenBank under the following accession numbers: PQ632963.1, PQ632964.1, PQ632965.1, PQ632966.1, PQ632967.1, PQ632968.1, PQ632969.1, PQ632970.1, PQ632971.1, PQ632972.1.

Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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