Prevalence of ESBL and/or carbapenemase-producing *Escherichia coli* isolated from yellow–legged gulls from Barcelona, Spain

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Running Head: ESBL-producing *E.coli* from yellow–legged gulls

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Abstract

Seventy two (54.5%) out of 132 fecal samples were positive for either extended spectrum beta-lactamases (ESBL) (51.5%), carbapenemase (1.5%) or cephamycinase (1.5%) producing *Escherichia coli* from a group of yellow–legged gulls in Barcelona, Spain. The isolation of two carbapenemase-producing *E. coli* strains is a matter of concern.

In the last decade, the number of bacterial pathogens presenting multidrug resistance to antibacterial agents has increased dramatically, becoming an emergent global concern and a major public health problem (*1*). The main cause behind the increasing rates of resistance can ultimately be found in the abuse and misuse of antibacterial agents, whether used in patients and livestock or released into the environment. Once antimicrobial resistant bacteria emerge, they can spread locally or globally. The main factors contributing to their spread at a global level comprise migrant birds, globalization of commercial food and international travelling.

There have been several studies about the presence of resistant bacteria in gulls (*2*,*3*), to the extent of being considered as an indicator of environmental antibiotic resistance occurrence, as they are distributed almost all around the world (*4*). Meerburg *et al* (*5*) showed that gulls feces contain a greater average concentration of *E. coli* than other wild animals and according to Stedt *et al* (*4*), Spain is the country in Europe with the highest levels of gull *E. coli* isolates resistant to ≥1 antibiotic.

The objective of this study was to investigate the prevalence of extended spectrum beta-lactamases (ESBL) and/or carbapenemase-producing *Enterobacteriaceae* from fecal swabs obtained from a group of yellow–legged gulls (*Larus michahellis*) in Barcelona, Spain.

The study was conducted from the beginning of May to late July 2014 in the city of Barcelona, including the breeding period of the yellow-legged gull in the city. The sampling program was part of the sanitary and epidemiological surveillance that is carried out by the Public Health Agency, Barcelona, institution responsible for the supervision and surveillance of the species. The sampling sites were chosen according to citizens' reports regarding the species nesting on their terraces or high roofs of the

city. Every gull chick from each nest found (Figure) was sampled, which amounts to 132 samples in total. All samples were obtained from young specimens born in that same year, and all nests were independent from each other, since the urban structure of cities promotes isolated instead of colonial nesting. Fecal material was obtained by sampling the cloaca of gull chicks with sterile swabs. Each swab was individually preserved in Cary-Blair medium, at 2-8 ºC and analyzed within 24 h in the Laboratory of the Public Health Agency, Barcelona.

The samples were plated on ESBL chromogenic agar (Biomérieux, France) and burgundy red colonies were selected, according to the manufacturer's instructions. Colonies were further identified by mass spectrometry (MALDI-TOF) (Bruker Daltonics Inc., Bremen, Germany). Susceptibility to ampicillin, amoxicillin/clavulanic acid, cefuroxime, ceftriaxone, cefotaxime, meropenem, gentamicin, amikacin, nalidixic acid, and ciprofloxacin was determined by the disk diffusion method following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and breakpoints (version 5.0, 2015; http://www.eucast.org). The double-disk diffusion technique was performed for phenotypic ESBL detection. The presence of carbapenemases was assessed with the modified Hodge test according to phenotypic susceptibility results. Characterization of ESBL and carbapenemases genes was performed by PCR followed by DNA sequencing (*6*) (ESBL: bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ genes; carbapenemases: bla_{KPC}, $bla_{\text{OXA-48}}$, bla_{VIM}, bla_{IMP} and bla_{NDM} genes; cephamycinases: bla_{CMY}, bla_{DHA}, bla_{FOX}, bla_{ACC}, bla_{EBC}, bla_{MOX} genes). Seventy two (54.5%) out of 132 fecal samples were positive for either ESBL (68/132, 51.5%), carbapenemase (2/132, 1.5%) or cephamycinase (2/132, 1.5%) producing *Escherichia coli* (Table 1), with SHV being the most prevalent the group (38/132, 28.8%). Forty-five strains (62.5%) were resistant to quinolones, 22 (30.6%) to gentamicin and 9

(12.5%) to amikacin. Rep-PCR showed a high genetic heterogeneity among the strains with up to 57 different clones, 15 of them containing two different isolates each (data not shown). Agglutination with antiserum O:25 was used to identify CTXM-15 producing isolates belonging to the high risk clone O:25b-ST131, but all isolates were negative.

Of note, two *E. coli* isolates co-carried both the bla_{KPC-2} and bla_{VIM} genes for carbapenem resistance. Multilocus sequence typing (MLST) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and PCR-based phylogroup analysis (*7*) identified them as belonging to the ST1011 phylogroup E (strain 40) and ST354 phylogroup F (strain 71), respectively, which have been previously reported in human strains (*8,9*). The genetic transference of carbapenemase genes was tested by biparental mating experiments using *E. coli* J53 AziR as the recipient strain. Transconjugants were selected in Mueller-Hinton agar plates containing 100µg/mL of sodium azide and 1µg/mL meropenem (Sigma Chemical Co., St Louis, MO). Successful conjugation was confirmed by specific PCR amplification. Table 2 shows the MICs determined by Etest of different antibiotics for the original and transconjugant strains with the corresponding carbapenemases harbored.

Plasmid analysis by S1 nuclease–pulsed-field gel electrophoresis (PFGE) (*6*) and replicon typing (*6*) were then performed on both the original strains and transconjugants to determine the size of these plasmids and classify them within the incompatibility groups. Digoxigenin-labeled probes for the bla_{VIM} and bla_{KPC} genes were hybridized against blotted nylon membranes from the S1-PFGE gels. The bla_{KPC-2} gene was located in a plasmid size ca. 60 kb in strain 40 and in a plasmid of <50kb in strain 71, both being non-typeable plasmids. The genetic environment of the bla_{KPC-2} genes was determined by inverse PCR, leading to the identification of a IS*Kpn27*-ΔTEM-bla_{KPC-2}-

115 IS*Kpn6-kor*C genetic arrangement, which was similar to those previously described
116 among different isolates of human origin from China and Taiwan (*10,11*). Further
117 analysis based on Next Generation Sequencing is needed to describe additional
118 elements of these plasmids for a more robust analysis.

119 The *bla*$_{\text{VIM-1}}$ gene was located in both strains in an In*3103* class I integron,
120 carried in a ca. 100kb plasmid belonging to the incompatibility group I1-Iγ. This
121 integron also cointained an aminoglycoside 6'-N-acetyltransferase (*aacA4*) gene and a
122 3'-(9)-O-adenylyltransferase (*aadA1*) gene. The presence of the *bla*$_{\text{VIM-1}}$ gene within an
123 In3103 class I integron was also described by at least one report in Spain, albeit in that
124 case it was located in a non-typeable plasmid of ca. 60 kb and recovered from a human
125 patient (*12*).

126 Our data showed a higher percentage of resistant *E. coli* in gulls fecal samples
127 compared with previous studies (*13–15*) but it also represents the first study reporting
128 the coexistence of two carbapenemase genes in *E.coli* recovered from yellow-legged
129 gulls. Although some OXA-48 producing *E.coli* could have been lost due to the
130 methodology followed that was specifically designed to search for ESBL. The fact that
131 carbapenem resistant isolates recovered from the fecal samples of gulls share the same
132 sequence types and resistance modules as those recovered from human samples in
133 different parts f the world highlights the potential role of migratory birds in the
134 dissemination and spread of antibiotic resistance genes.

135

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139

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208 Figure. Location of sites where positive (green) and negative (red) samples were

209 collected (Barcelona, Spain).

Table 1. Distribution of β-lactamases.

β-lactamase	Nº of strains	%
SHV Group	38	52.8
SHV-12	24	33.3
SHV-12 + TEM-1	13	18
SHV-2	1	1.4
CTX-M group	30	41.6
CTX-M-15	11	15.3
CTX-M-15 + TEM-1	2	2.8
CTX-M-1	1	1.4
CTX-M-1 + TEM-1	5	6.9
CTX-M-1 + TEM-84	1	1.4
CTX-M-14	4	5.5
CTX-M-14 + TEM-1	6	8.3
VIM-1 + KPC-2	2	2.8
CMY-2	2	2.8
TOTAL	72	100

212 Table 2. *In vitro* susceptibilities of original strains of *E. coli* and *E. coli* transconjugants expressing VIM-1 and/or KPC-2 (Etest, EUCAST).

	MIC (µg/ml)						
	Original strains			Transconjugants			
Antibiotics	*E. coli* 40[A] (VIM-1/KPC-2)	*E. coli* 71[B] (VIM-1/KPC-2)	*E. coli* J53 [C]	*E. coli* J53 40T3[D] (KPC-2)	*E. coli* J53 40T5[E] (VIM-1/KPC-2)	*E. coli* J53 71T1[F] (VIM-1)	*E. coli* J53 71T3[G] (VIM-1/KPC-2)
Cefoxitin	>256	>256	2	8	256	256	64
Cefotaxime	32	24	0.094	0.75	16	16	64
Ceftazidime	256	96	0.125	1	64	64	96
Imipenem	4	24	0.19	1.5	3	1	12
Meropenem	4	32	0.023	0.75	0.5	0.25	8
Ertapenem	6	12	0.008	0.38	0.38	0.64	4
Aztreonam	16	128	0.064	12	16	0.25	256
Ciprofloxacin	>32	>32	0.064	0.047	0.064	0.064	0.064
Gentamicin	>32	>32	0.25	0.25	1	2	2
Amikacin	3	3	1	1	1.5	1.5	1.5
Tobramycin	16	12	0.125	0.125	3	3	3
Colistin	0.25	0.125	0.125	0.25	0.125	0.19	0.25

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214 [A] *E. coli* strain 40 isolated from a yellow–legged gull.

215 [B] *E. coli* strain 71 isolated from a yellow–legged gull.

216 [C] Sodium azide-resistant *E. coli* J53 strain used as a recipient in the conjugation experiment.

217 [D] *E. coli* transconjugant obtained from strains 40 and J53 that received only the $bla_{\text{KPC-2}}$.

218 [E] *E. coli* transconjugant obtained from strains 40 and J53 that received both the $bla_{\text{KPC-2}}$ and the $bla_{\text{VIM-1.}}$

219 [F] *E. coli* transconjugant obtained from strains 71 and J53 that received only the $bla_{\text{VIM-1.}}$

220 [G] *E. coli* transconjugant obtained from strains 71 and J53 that received both the $bla_{\text{KPC-2}}$ and the $bla_{\text{VIM-1.}}$

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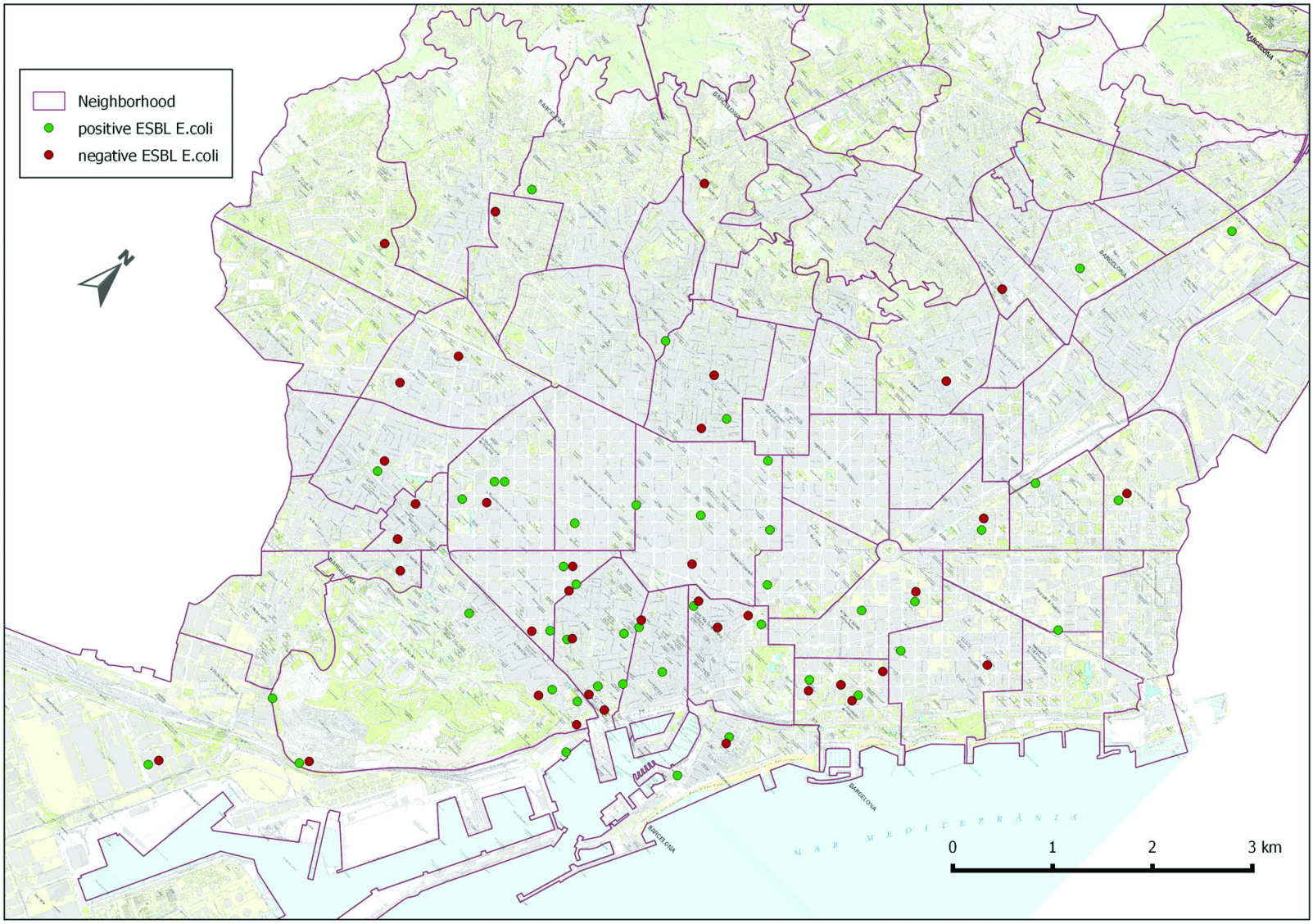
Neighborhood
positive ESBL E.coli
negative ESBL E.coli
0
1
2
3 km