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Quinolone resistance determinants in environmental *Escherichia coli* isolates from Portugal, Spain and Sweden

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Summary

Antibiotic resistance in the environment is a major concern for public health and the spread of antibiotic resistant microbes in natural environments is increasing at an alarming rate over time. Since the first implementation of antibiotics for clinical purposes, the treatment effectiveness of many antibiotics has declined dramatically as microbes have adapted to cope. Resistance to ciprofloxacin, a second-generation quinolone that is widely used in Europe, has also been observed. To investigate the extent of ciprofloxacin resistance in the environment, we isolated *Escherichia coli* from various species of wild birds from Portugal, Spain and Sweden. Wild birds are considered important reservoirs and dissemination vectors for antibiotic resistance traits, as they live in close contact with humans and travel great distances. Once they acquire resistant microbes, for example via feeding on human waste, they can spread these resistant microbes into even quite remote natural environments. We also investigated ciprofloxacin resistance prevalence in urban water bodies, hypothesizing that aquatic environments play an important role in the dissemination of antibiotic resistance. As different countries apply different antibiotic usage, the levels of selective pressures imposed by antibiotics are also considered to differ regionally. We therefore compared the phylogenetic relationships among quinolone resistant *E. coli* from Portugal, Spain and Sweden using multilocus sequence typing (MLST). Subsequently, we interpreted the diversity of the antibiotic resistance patterns and quinolone resistance determinants in relation to the genetic lineage. We analyzed the phenotypic resistance using the Disc Diffusion method and genotypic resistance was investigated using PCR analysis for the resistance genes *gyrA, parC, qnrA, qnrB, qnrS, qepA* and *aac(6')-Ib-cr.*

Results showed considerable resistance patterns in all three countries, and especially isolates from Portugal mirrored the extensive antibiotic usage in Southern Europe. Especially isolates from Portugal mirrored the extensive antibiotic usage in Southern Europe. However, also Swedish isolates featured striking antibiotic resistance prevalence, which lies in contrast to the country's conservative antibiotic usage. Spanish isolates were also highly resistant to the tested antibiotics. MLST analysis illustrated the diversity of *E. coli* in the combined dataset. Based on this phylogenetic analysis, isolates typed together from the different countries and host species, thus, the phylogenetic diversity was high. Especially seagull and wastewater isolates from Portugal showed genetic diversity by typing together with isolates from other origins. In contrast, wild bird isolates from Portugal were comparatively related to each other. Resistance gene analysis of all isolates shed some light onto the resistance mechanisms of isolates from various origins. Only the plasmid-mediated quinolone resistance genes *qnrA, qnrS* and *aac(6')-Ib-cr* could be detected in the combined set of isolates, and there was no obvious pattern in their habitat distribution. Moreover, we conclude that the chromosomal resistance genes *gyrA* and *parC* appear to play a central role for ciprofloxacin resistance in the analyzed E. coli isolates, as the resistance-conferring mutations in these genes were widespread in the isolates.

Contents

Abbreviations

ATCC	American Type Culture Collection
BR	Wild Birds Portugal
CLSI	Clinical and Laboratory Standards Institute
ECDC	European Centre of Disease Control
HGT	Horizontal Gene Transfer
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
PCR	Polymerase Chain Reaction
SG	Seagulls Portugal
SP	Wild Birds Spain
ST	Sequence Type
SV	Wild Birds Sweden
SVu	Wild Birds Uppsala
UP	Water Sample from Uppsala Pond
UR	Water Sample from Uppsala River
WWZ	Wastewater Portugal

Antibiotics

CIP	Ciprofloxacin
CAZ	Ceftazidime
MEM	Meropenem
SXT	Trimethoprim- Sulfamethoxazole
TIC	Ticarcillin
CN	Gentamicin
NA	Nalidixic Acid

1. Introduction

When antibiotics became available for clinical use in the 1940's, modern healthcare had found a powerful weapon that would soon revolutionize the treatment strategy of infectious diseases. However, as a consequence of the wide exposure to antibiotics, bacteria would soon adapt and develop resistance to those drugs. It was Sir Alexander Fleming himself, the discoverer of the antibiotic Penicillin in 1928, who warned of the potential impact of antibiotic resistant microbes: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body… and by exposing his microbes to non-lethal quantities of the drug makes them resistant" (Fleming, Nobel Lecture 1945). And inevitably, the first antibiotic resistance cases would appear only a few years later.

1.1 Antibiotic resistance and reservoirs

Ever since, antibiotic resistance is a steadily growing public health concern, and there is great interest from the scientific community as well as the public to learn more about the mechanisms, pathways and potential reservoirs of antibiotic resistance. Interestingly, aquatic environments were repeatedly reported among the most important reservoirs for antibiotic resistance that could potentially spread as a result of as human and animal discharges into the surface waters, thus enabling microbes to spread and create an environment conducible for genetic exchange of resistance traits (Banquero et al., 2008; Kuemmerer et al., 2009; Taylor et al., 2011 and Figueira et al., 2011). Wild birds have been widely studied and considered to be important reservoirs and vectors of resistance dissemination to the environment (Simões et al., 2010). Not only do they live in vicinity to humans, where they can take up resistant microbes through feeding, for example on livestock waste, (Poeta et al., 2008; Sjölund et al., 2008; Cole et al., 2005 and Simões et al., 2010) but their high motility across great geographic distances also facilitates the low-range spread of resistant microbial strains. The further spread and existence of multiresistant bacteria across multiple continents and even the most remote locations such as the polar regions (Sjölund et al., 2008), suggest that migratory and free-living birds could mirror the global occurrence of antibiotic resistance in humans as well as in nature.

1.2 Target organism E. coli

The first bacterium, for which population genetic techniques were introduced, was *Escherichia coli*, a universal and commensal organisms found in the intestines of mammals and birds (Wirth et al., 2006; Brzuszkiewicz et al., 2011). While most *E. coli* are harmless, some of them have pathogenic potential, such as the recently emerged Entero-Aggregative-Haemorrhagic Escherichia coli (EAHEC) that caused broad publicity

during an outbreak in Germany in 2011 (Brzuszkiewicz et al., 2011). Pathogenic *E. coli* can also cause various other diseases, such as pneumonia, diarrhea, cholangitis, neonatal meningitis or endemic dysentery, all of which can be potentially fatal (Wirth et al., 2006). Additionally, it has been shown that *E. coli* can potentially develop and spread antibiotic resistance into the environment, making them a primary target for antibiotic resistance studies (Figueira et al., 2011). It is therefore important to generate more information on environmental dissemination of antibiotic resistance as there is no easy solution to the problem.

1.3 Antibiotic resistance in Portugal, Spain and Sweden

As reported by the European Surveillance of Antimicrobial Consumption (ESAC), the use of quinolones is generally highest in Southern Europe and lowest in Northern Europe. Portugal has one of the highest outpatient antibiotic consumption rates in Europe, and the use of especially ciprofloxacin is stable over time (Adriaenssens et al., 2011). As a logical consequence, high resistance rates have been observed in the country, thus making it an insightful environment to study. Sweden, in contrast, reports low and decreasing ciprofloxacin use over time, which is why it could be considered as a fairly unstressed environment in terms of antibiotic resistance prevalence. Ciprofloxacin is overall the most widely used quinolone in Europe for human therapy due to its high efficiency in treating urinary tract and respiratory tract infections. It is also the most frequently prescribed quinolone in Spain.

1.4 Resistance mechanisms

Chromosomal genes

During microbial DNA replication, a number of cellular proteins are needed, enabling the successful separation, recombination and replication of the chromosomal double helices. Topoisomerases (which also include gyrase) are involved in relaxing and unwinding the supercoiling of the DNA strand during replication. Ciprofloxacin binds to complexes that form between DNA and topoisomerases and thus inhibits the supercoiling removal of DNA, causing transcription errors (Hawkey et al., 2003; Périchon et al., 2007). It is therefore the benchmark quinolone with the highest potency against Gram-negative bacteria (Adriaenssens et al., 2011; Tortora et al., 2007).

Through extensive exposure to antibiotics, point mutations can evolve at the target site; this is the most common type of mutation where a single base at one point is substituted with another base. For *gyrA*, a gyrase subunit gene, mutations are mostly associated with Serine at position 83 and Aspartic Acid at position 87, (Weigel et al., 1998). Similarly, quinolone resistance can be developed in the *parC* gene, a topoisomerase subunit, if point mutations in Serine at position 80 and Glutamic Acid at position 84 are present. Those mutations in the quinolone resistance-determining regions (QRDR) of the drug targets are the most common mechanism of high-level resistance to quinolones.

While low-level ciprofloxacin resistance is often associated with a resistance at a breakpoint concentration of 4 µg/ml, bacteria are high-level resistant when their growth is not inhibited at a concentration of 64 µg/ml and higher. (Hawkey et al., 2003; Périchon et al., 2007 and Leavis et al., 2006).

1.5 Plasmid mediated antibiotic resistance

As microbes are capable of not only exchanging their genetic material from generation to generation, but also between individuals of the same generation, resistance genes may be acquired through horizontal gene transfer (HGT). Among the most frequent plasmid mediated resistance genes are the *q*ui*n*olone *r*esistance *(qnr)* genes, which confer low-level resistance by protecting type II topoisomerases (gyrases) from quinolone inhibition (Tortora et al., 2007). Other mechanisms of quinolone resistance were described by Périchon et al., 2007, highlighting the recently discovered plasmid-borne *q*uinolone *e*fflux *p*ump (*qepA*) that enables the organism to actively reduce their susceptibility to hydrophilic quinolones by pumping the antibiotic out of the cell (Cavaco et al., 2009; Robicsek et al., 2006) and the *aac(6')-Ib-cr*, a variant aminoglycoside acetyltransferase which modifies the ciprofloxacin molecule has also been found to significantly hamper quinolone effectiveness.

1.6 Objective

To carry out an integrated study of antibiotic resistance, dissemination pathways and resistance reservoirs, quinolone resistance was assessed in environmental *E. coli* isolates from three different regions in Northern and Southern Europe. We studied the genetic relatedness of ciprofloxacin resistant *E. coli* from gulls, wild birds and wastewaters in Portugal, Spain and Sweden to compare related isolates and their resistance phenotypes and genotypes.

It was hypothesized that specific antibiotic resistant lineages may have an advantage in certain habitats with strong selection pressure and that this would be reflected in different geographic dispersal patterns. Therefore the lineages of quinolone resistant *E. coli* isolated from sites with different levels of antibiotic pressure were identified and their phylogenetic relationship was described with multilocus sequence typing (MLST). Phylogenies based on partial sequencing of seven housekeeping genes were then compared to the diversity of the antibiotic resistance patterns. Phenotypic resistance was analyzed using the Disc Diffusion method and genotypic resistance was investigated using PCR analysis for the resistance genes *gyrA, parC, qnrA, qnrB, qnrS, qepA* and *aac(6')-Ib-cr.* The major aim of this study was to gain more knowledge about quinolone resistant *E. coli* in Europe as this could potentially be a hazard to public and animal health.

2. Materials and Methods

2.1 Sample collection

For Portugal, *E. coli* isolates resistant to ciprofloxacin had previously been isolated from the feces of seagulls (Isolate ID: SG) (*Larus fuscus, Larus cachinnans*), wild birds (Isolate ID: BR) (*Buteo buteo*, *Hieraaetus pennatus, Milvus migrans, Aegypius monachus, Strix aluco, Accipiter nisus, Hieraaetus fasciatus,* and *Bubo bubo)* and urban wastewater (Isolate ID: WWZ). Fecal droppings were collected during a period from December 2007 to April 2008, using sterile spatulas (Simões et al., 2010) from Matosinhos and Leça da Palmeira beaches in Porto, Portugal. Wild bird feces were collected from the National Park Serra da Estrela, during the period of March to May, 2008. The wastewater was collected from a local wastewater treatment plant during 2004 in the region of greater Porto in the north of Portugal. This wastewater treatment plant serves approximately 100.000 inhabitants and uses activated sludge treatment (Ferreira da Silva et al., 2006).

We also received *E. coli* isolated from gulls and wild birds in Sweden (Isolate ID: SV) and Spain (Isolate ID: SP) that had been collected during 2009. All these isolates were resistant to the quinolone nalidixic acid but their resistance phenotypes to ciprofloxacin were not known and therefore tested. Since it was difficult to obtain ciprofloxacin resistant isolates from Sweden, we chose to also include Spanish isolates for this study. Isolates were provided by the University of Kalmar.

For Sweden, 40 fecal samples were collected from wild birds and gulls residing in a pond close to the Uppsala University hospital, Sweden (Isolate ID: SVu) during the period of June and July 2012. At the same time, water samples were collected from that same pond (Isolate ID: UP) and the nearest river (Fyrisån) (Isolate ID: UR). Close to this river, there was a wastewater treatment plant serving the city of Uppsala. For each sample, a cotton swab swirled in bird droppings, was submerged in bacterial freeze media and handled as described previously (Bonnedahl et al., 2010). One liter of water sample was taken from four different locations of the river and the pond (two in each).

2.2 Bacterial isolation and identification

Each fecal sample collected from Uppsala was plated on CLED plates (BD, Sweden) and incubated overnight at 37°C. Each fecal sample was also enriched in LB broth supplemented with 2, 5 µg/ml ciprofloxacin to screen selectively for ciprofloxacin resistant isolates. Putative *E. coli* were identified by conventional biochemical testing (Oxidative-fermentative analysis, o-nitrophenyl-beta-D-galactopyranoside, Urea, Voges Proskauer and Sulfur- Indole- Motility). Isolated *E. coli* were stored at -80°C for further investigations.

Each water sample was filtered through cellulose nitrate membranes (0, 5µm pore size, 47mm diameter, Millipore, USA) and the filters were subsequently placed into LB broth supplemented with 2, 5 µg/ml ciprofloxacin and incubated at 37°C for 24 hours. Putative *E. coli* were isolated and identified as described previously.

2.3 Antibiotic susceptibility testing

All isolates were tested against six different antibiotics, belonging to four different classes of antibiotics, using the Disc Diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) 2012. Six different antibiotics were used (Table 1), all of which were provided by Oxoid, UK. Briefly, a single *E. coli* colony from Plate Count Agar (Liofilchem, Italy for Portuguese isolates) and BLOOD agar plates (Oxoid, UK for Swedish isolates) cultures was dissolved in saline solution (0.85%), and adjusted to a turbidity of 0.22-0.24 OD at a wavelength of 610nm. Then the solution was spread over Mueller Hinton agar plates (Oxoid, UK), using sterile cotton swabs. Antibiotic discs were dispensed on the growth media using an antibiotic dispenser (Oxoid, UK). MIC (Minimum Inhibitory Concentration) was determined following overnight incubation at 37°C. Isolates were regarded either as Susceptible (S), Intermediate (I) or Resistant (R), based on their phenotypic expression against antibiotics (Table 1). All isolates that expressed intermediate resistance to antibiotics were considered to have reduced susceptibility and categorized as resistant. *Escherichia coli* ATCC 25992 was used as a quality control strain.

Table 1 – *Antibiotics used in the antibiotic susceptibility testing, concentrations and breakpoints as given by the Clinical and Laboratory Standards Institute (CLSI, 2012).*

Name	Group	Concentration	Susceptible (S)	Intermediary (I)	Resistant (R)
ciprofloxacin	quinolones	5 µg	≥ 21 mm	16 – 20 mm	≤ 15 mm
ceftazidime	cephalosporins	30 µg	≥ 21 mm	18 – 20 mm	≤ 17 mm
meropenem	carbapenems	10 µg	≥ 23 mm	20 – 22 mm	≤ 19 mm
ticarcillin	penicillins	75 µg	≥ 20 mm	15 – 19 mm	≤ 14 mm
trimethoprim-sulfamethoxazole	sulfonamides	25 µg	≥ 16 mm	11 – 15 mm	≤ 10 mm
gentamicin	aminoglycosides	120 µg	≥ 15 mm	13 – 14 mm	≤ 12 mm

2.4 DNA isolation

Isolates that were found resistant to quinolones (ciprofloxacin) were plated on blood agar plates and incubated overnight. Two to three colonies were picked and suspended in 200 µl deionized water and heated at 99°C for 10 minutes. In Portugal, a water bath was used for this step while a heating block was used in Sweden. The cell suspension was then cooled on ice for 5 minutes and subsequently centrifuged at 14000 rpm for 2.5 minutes. The supernatant was collected and used for further analysis.

2.5 Genetic characterization of resistance determinants

All ciprofloxacin resistant isolates were screened for mutations in the quinolone resistance-determining regions (QRDR) of the chromosomal genes *gyrA* and *parC* (Weigel et al., 1998) applying PCR and comparative sequence analysis. Further, plasmid-borne resistance genes *qnrA, qnrB, qnrS, qepA* and *aac(6')-Ib-cr* were screened using previously described primers (Table 2) and PCR conditions (Table 3).

For the chromosomal genes *gyrA* and *parC*, PCR assays were performed in a final reaction volume of 50 µl. Each reaction contained 10 µl of dNTP (1mM), 5 µl of 10x buffer (KCl), 3 µl of $MgCl_2$ (25mM), 1 µl of each primer (Table 2), 1.5 µl of Taq Polymerase (1U), (Fermentas, Germany), 26 µl of sterile water and 2.5 µl of previously extracted DNA template. Cyclic conditions were as follows: 5 min at 95°C, 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, 15 min at 72°C for 35 cycles (McDonald et al., 2001).

Point mutations in the sequences of the genes *gyrA* and *parC* were detected by comparison with homologous nucleotide sequences of quinolone susceptible strains available in GenBank (Goñi-Urriza et al., 2002; Figueira et al., 2011): *Aeromonas punctata* CIP 7616T (AYO27899 and AF435418) and *Aeromonas hydrophila* subsp. *hydrophila* CIP 7614T (AYO27901 and AF435419).

Positive controls for the plasmid-mediated resistance genes were used (Table 3). Positive PCR products for the antibiotic resistant variant *aac(6')-Ib-cr* were purified and sequenced. Sequences were identified using BLAST, (National Center for Biotechnology Information website).

Table 2 – *Primers used for detection of resistance genes*.

Gene	Primers	Sequence	Fragment length	Reference
gyrA	gyrA6 gyrA631R	CGACCTTGCGAGAGAAAT GTTCCATCAGCCCTTCAA	583 bp	Yáñez et al., 2003
parC	HJL3 HJL4	AATGAGCGATATGGCAGAGC CTGGTCGATTAATGCGATTG	806 bp	Goñi-Urriza et al., 2002
aac(6')-Ib-cr	aac(6)-F aac(6)-R	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	911 bp	Park et al, .2006
qnrA	qnrAmF qnrAmR	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	878 bp	Cattoir et al., 2007
qnrB	qnrBmF qnrBmR	GGCATCGAAATTCGCCACTG TTTGCTGTTCGCCAGTCGAA	932 bp	Cattoir et al., 2007
qnrS	qnrSmF qnrSmR	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	816 bp	Cattoir et al., 2007
qepA	qepA-F qepA-R	TGGTCTACGCCATGGACCTCA TGAATTCGGACACCGTCTCCG	780 bp	Périchon et al., 2007

Table 3 - *PCR conditions and positive controls used for the detection of resistance genes.*

Gene	mix	Final volume	cycles	Controls and References
aac(6')-Ib-cr	11.75 µl of sterile water 5.0 µl dNTP (1 mM) 2.5 µl Buffer (KCl) 1.5 µl $MgCl_2$ (25 mM) 1.0 µl primer Forward 1.0 µl primer Reverse 1.25 µl Taq Polymerase (1U)	25µl	94°C – 5 min 94°C – 45 sec 55°C – 45 sec 72°C – 45 sec 72°C – 10 min (35 cycles)	*Salmonella enteria* serovar *typhimurium* GSS-HN-2007-03 Cavaco et al., 2009
qnrA, qnrB, qnrS	12.35 µl of sterile water 5.0 µl dNTP (1 mM) 2.5 µl Buffer (KCl) 1.5 µl $MgCl_2$ (25 mM) 1.0 µl primer Forward 1.0 µl primer Reverse 1.25 µl Taq Polymerase (1U)	25µl	95°C – 10 min 95°C – 1 min 54°C – 1 min 72°C – 1 min 72°C – 10 min (35 cycles)	*Escherichia coli* L0 (qnrA1+) *Klebsiella pneumoniae* B1 (qnrB1+) *Enterobacter cloacae* S1 (qnrS1+) Cattoir et al., 2007
qepA	8.5 µl of sterile water 5.0 µl dNTP (1 mM) 2.5 µl Buffer (KCl) 3.0 µl $MgCl_2$ (25 mM) 2.5 µl DMSO 1.0 µl primer Forward 1.0 µl primer Reverse 0.5 µl Taq Polymerase (1U)	25µl	94°C – 4 min 94°C – 1 min 56°C – 1 min 72°C – 1.30 min 72°C – 7 min (30 cycles)	*Escherichia coli* TOP10+ paT851 Périchon et al., 2007

2.6 MLST of quinolone resistant isolates

Every isolate was genotyped using Multilocus Sequence Typing (MLST) technique as described in the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli), using specific primers for seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA)* (Table 4). This database also provided references for sequence types of all seven genes. Sequences were therefore aligned with the respective reference allowing a sequence quality analysis and editing. Alignments were performed using MEGA 5.05 and compared with the raw sequence version in Bioedit. The partial sequences of the housekeeping genes obtained in this study and the respective reference sequences were aligned with ClustalW in MEGA 5.05. In addition to analysis of each housekeeping gene separately, a single analysis based on the seven concatenated sequences was also performed. The phylogenetic analysis was carried out with Maximum Likelihood (Tamura et al., 2007; Figueira et al., 2011) (Figure 1). PCR products in Portugal and Sweden were purified using the EzWayTM PCRClean-up Kit (Komabiotech) and sent for sequencing.

Table 4 – *Primers used for MLST analysis*.

Gene	Primers	Sequence	Fragment length	Reference
16S rRNA	27F 1492R	GAGTTTGATCCTGGCTCAG TACCTTGTTACGACTT	1465 bp	Lane, 1991
adk	adk Fw adk Rv	ATTCTGCTTGGCGCTCCGGG CCGTCAACTTTCGCGTATTT	583 bp	Wirth et al. 2006
fumC	fumC Fw fumC Rv	TCACAGGTCGCCAGCGCTTC GTACGCAGCGAAAAAGATTC	806 bp	Wirth et al. 2006
gyrB	gyrB Fw gyrB Rv	TCGGCGACACGGATGACGGC ATCAGGCCTTCACGCGCATC	911 bp	Wirth et al. 2006
icd	icd Fw icd Rv	ATGGAAAGTAAAGTAGTTGTTCCGGCACA GGACGCAGCAGGATCTGTT	878 bp	Wirth et al. 2006
mdh	mdh Fw mdh Rv	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	932 bp	Wirth et al. 2006
purA	purA Fw purA Rv	CGCGCTGATGAAAGAGATGA CATACGGTAAGCCACGCAGA	816 bp	Wirth et al. 2006
recA	recA Fw recA Rv	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGGA	780 bp	Wirth et al. 2006

3. Results

3.1 Isolates

In total, eighty-six resistant isolates (Portugal, 49; Spain, 33; Sweden, 4) were collected from the Universidade Católica Portuguesa and the University of Kalmar. From enrichment of the fecal and water samples that were sampled in Uppsala and cultured with LB broth, five ciprofloxacin resistant isolates (2 from feces and 3 from water samples) were recovered. Additionally, 26 isolates were recovered following culturing on CLED plates without selective antibiotic pressure.

3.2 Antimicrobial susceptibility testing

A final set of 117 *E. coli* isolates from the various origins of Portugal, Spain and Sweden were selected for susceptibility testing. Of these, sixty-six isolates confirmed resistance to ciprofloxacin and were selected for further investigation. All Portuguese isolates (BR, SG and WWZ) were known to be resistant to ciprofloxacin and this was also confirmed with duplicate testing. One isolate originating from the wastewater (WWZ10) showed an intermediate resistance phenotype; however, as the intermediate resistance is considered to represent reduced susceptibility, the isolate was included in the final set of isolates. Out of all 33 Spanish isolates (SP) that were tested for their quinolone (nalidixic acid) resistance by the University of Kalmar, 11 were found resistant to also ciprofloxacin. In addition, ciprofloxacin resistance was observed in all of the four Swedish isolates (SV) that were sent by the University of Kalmar (Table 5).

All 26 *E. coli* isolates from birds (SVu) in Uppsala were susceptible to ciprofloxacin. Following enrichment with supplementation of ciprofloxacin, two isolates from birds confirmed ciprofloxacin resistance. Also the three isolates from water samples from Uppsala were resistant to ciprofloxacin.

3.3 Resistance to beta-lactams, sulfonamides and aminoglycosides

Isolates originating from Portugal are largely resistant to ticarcillin (45/47), but also trimethoprim -sulfamethoxazole (38/47). Ceftazidime and gentamicin resistance were less considerable. However, all Portuguese isolates were susceptible to meropenem (Table 6). For Spanish isolates, antibiotic resistance profiles followed a similar distribution, with most frequent resistances to ticarcillin and trimethoprim – sulfamethoxazole, while ceftazidime resistance was observed only once. All of the Spanish isolates were completely susceptible to meropenem and gentamicin (Table 6). Swedish isolates were all resistant to ticarcillin. Also, there were considerable resistant phenotypes to ceftazidime (5/9), gentamicin (4/9) and trimethoprim –sulfamethoxazole (3/9) (Table 5) Also all Swedish isolates were susceptible to meropenem. Multidrug resistance (MDR, defined here as resistance to 3 or more antimicrobial classes) was common in Sweden (7 out of 9), Portugal (42 out of 46) and Spain (5 out of 11).

Table 5 – *Prevalence of Antibiotic resistance in Spain, Portugal and Sweden. SP, wild birds Spain; BR, wild birds Portugal; SG, seagulls Portugal; WWZ, wastewater Portugal; SV, wild birds Sweden (provided by the University of Kalmar); Uppsala, wild birds Uppsala (fecal and water samples)*

Antibiotic	Spain		Portugal		Sweden	
	SP n=11	BR (n=18)	SG (n=19)	WWZ (n=9)	SV (n=4)	Uppsala (n=5)
Ciprofloxacin	11	18	19	9	4	5
Ceftazidime	1	0	14	1	4	1
Meroperem	0	0	0	0	0	0
trimethoprim-sulfamethoxazole	6	18	14	6	0	3
Ticarcillin	9	18	19	8	4	5
Gentamicin	0	0	12	2	4	0

Table 6 - *Antibiotic resistance and characterization of chromosomal and acquired quinolone resistance in E. coli isolates. CAZ, ceftazidime; TIC, ticarcillin; SXT, trimethoprim-sulfamethoxazole; CN, gentamicin. Resistance phenotypes to ciprofloxacin and meropenem are not shown, all isolates are resistant to CIP and susceptible to MEM.*

Isolate ID	Resistance phenotype				Mutations		qnr genes
	CAZ	TIC	SXT	CN	gyrA	parC	
E. coli ATCC 25992	S	S	S	S	AGC (Ser)^{83}, GAC (Asp)^{87}	AGT/AGC (Ser)^{80}, GAA (Glu)^{84}	-
BR6	I	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR7	I	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR8	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR1	I	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
SP422	S	R	R	S	TTG (Leu)^{83}, -	ATC (Ile)^{80}	-
BR14	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
WWZ2	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SP281	S	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SP480	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SG5	I	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SG7	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SG3	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
SG16	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SG15	R	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SG20	R	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SP254	S	R	S	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	-
SP367	S	S	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
BR3	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
BR4	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR5	I	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
SG6	S	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR9	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
SG12	S	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SP444	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-

Isolate ID	Resistance phenotype				Mutations		qnr genes
	CAZ	TIC	SXT	CN	gyrA	parC	
SP238	S	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrS
SG4	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SG10	R	R	R	I	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SG11	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SG8	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SG9	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SP215	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SP217	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
WWZ10	S	R	S	S	TTG (Leu)^{83}, -	-	-
SP418	R	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
WWZ4	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
WWZ9	S	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}, GGA (Gly)^{84}	qnrA
SG17	S	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SV173	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}, GGA (Gly)^{84}	-
SG14a	R	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr, qnrA
UP2	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
WWZ11	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	-
SV32	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SV83	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}, GGA (Gly)^{84}	-
SV230	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}, GGA (Gly)^{84}	-
UP1	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	No Data	aac(6')-ib-cr
UR2	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
SVu22	S	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	No Data	-
SG19	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}, GTA (Val)^{84}	aac(6')-ib-cr
SG1	R	R	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	No Data	aac(6')-ib
WWZ3	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	No Data	
WWZ6	S	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	No Data	
WWZ5	R	S	S	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	
SVu32	R	R	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	
WWZ7	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	
SG2	S	R	R	S	TTG (Leu)^{83}, TAC (Tyr)^{87}	CGC (Arg)^{80}, GTA (Val)^{84}	
SG18	R	R	R	R	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	aac(6')-ib-cr
BR19	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	
BR16	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR17	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR12	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR18	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR10	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR11	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
BR13	S	R	R	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA
BR15	S	R	R	S	TTG (Leu)^{83}, GGC (Gly)^{87}	ATC (Ile)^{80}	qnrA
SP292	S	S	S	S	TTG (Leu)^{83}, AAC (Asn)^{87}	ATC (Ile)^{80}	qnrA

3.4 Strain diversity

Genotyping of all 66 ciprofloxacin resistant *E. coli* isolates from Portugal, Spain and Sweden was performed and the phylogenetic tree represents the diversity and relatedness of strains from the various origins (Figure 1). Ciprofloxacin resistant *E. coli* isolates were assigned to 27 different sequence types (STs) (Table 7). 15 of the isolates could not be assigned with the allelic profiles in the MLST database and are yet to be assigned with a novel ST. Interestingly we found some strains belonging to the ST that were previously reported in human hospitals: ST131, ST405, ST156, ST58, ST10 and ST354. Other sequence types have been reported previously as common in poultry (ST10 and ST155) or wild birds (ST648) (Gibreel et al., 2010; Madec et al., 2011).

Table 7 – MLST analysis of quinolone resistant E. coli isolates from wild birds and water samples

Isolate ID	Sequence type (ST)
BR1, BR6, BR7, BR8	ST1998
BR3, BR4, BR5	ST1800
BR9, SG12	ST359
BR10, BR13	ST2309
BR11, BR12, BR15, BR16, BR17, BR18, BR19	ST115
SG1, SG19, WWZ6, SVu22, UP1, UR2	ST131* (human)
SG2	ST405* (human)
SG3	ST224
SG4	ST1284
SG5, SG7	ST205
SG14a	ST617
SG15, SG16, SG20	ST156* (human)
SG17, SG18	ST3004
WWZ2	ST58* (human)
WWZ4, WWZ9, SV173	ST10* (human and poultry)
WWZ5	ST354* (human)
WWZ11, SV230, SV32, SV83	ST167
SP215	ST57
SP444	ST398
SP422	ST345
SP238	ST1626
SP480	ST448
SP254	ST533
SP281	ST155 (poultry)
SP292	ST770
SVu32	ST648 (pigeon, goose)
BR14, SG6, SG8, SG9, SG10, SG11, WWZ3, WWZ7, WWZ10, SP367, SP418, SP217, UP2	New sequence types

*= human pathogen

Interestingly, Portuguese seagull isolates were highly diverse and in some cases closely related to wastewater isolates. Portuguese wild bird isolates were closely related to each other but different isolates were quite distant from each other (Figure 1). Therefore, the cluster of wild birds was clearly divided into three unrelated branches. Isolates from wastewater in Portugal and wild birds in Spain were dispersed among the tree, indicating that they are similar to each other and to the rest. They did not form any distinct habitat clusters.

The isolates from wild birds in Sweden were closely related to each other. However, they grouped together with other isolates from Portugal. The same *E. coli* strain was detected in both, water sample isolates and fecal sample isolates. In one exceptional case, an isolate from pigeons was unrelated to gull and water sample isolates.

3.5 Genetic determinants of quinolone resistance

For *gyrA*, sequences of all isolates [n=66] showed identical mutations in codon 83, substituting Serine with Leucine. In codon 87, three different amino acid changes were detected (Asp to Asn [n=53] or Asp to Gly [n=9] or Asp to Tyr [n=2]). Two isolates displayed a single amino acid change, as for them, no mutation occurred in position 87 (Table 6).

In the gene sequence of *parC*, fifty-nine isolates carried a mutation from Ser to Ile at codon 80. The sequence of one isolate had an amino acid change from Ser to Arg and one sequence showed no mutation at this site. Further, the majority of isolates [n=55] did not harbor any mutations at codon 84. Four isolates had an amino acid change from Glu to Gly and two isolates converted from Gly to Val. Of all 66 isolates, the amplification of *parC* was not successful for five isolates.

Interestingly, one isolate (SG2) deviated from the major pattern of amino acid changes in both, *gyrA* and *parC* (Ser to Leu in 83 and Asp to Tyr in 87 for *gyrA*; Ser to Arg and Glu to Val for *parC*) (Table 6). Another isolate differed from the overall mutation patterns by merely showing a single mutation in codon 83 of *gyrA*.

Plasmid mediated quinolone resistance was detected only in the form of *qnrA* [n=22], *qnrS* [n=1] and *aac(6')-Ib-cr* [n=12]. Of all 12 isolates that were positive for the *aac(6')-Ib* gene, eleven were present in the *–cr* form that is known to confer quinolone resistance. In two isolates, the presence of both, *qnrA* and *aac(6')-Ib-cr* was detected.

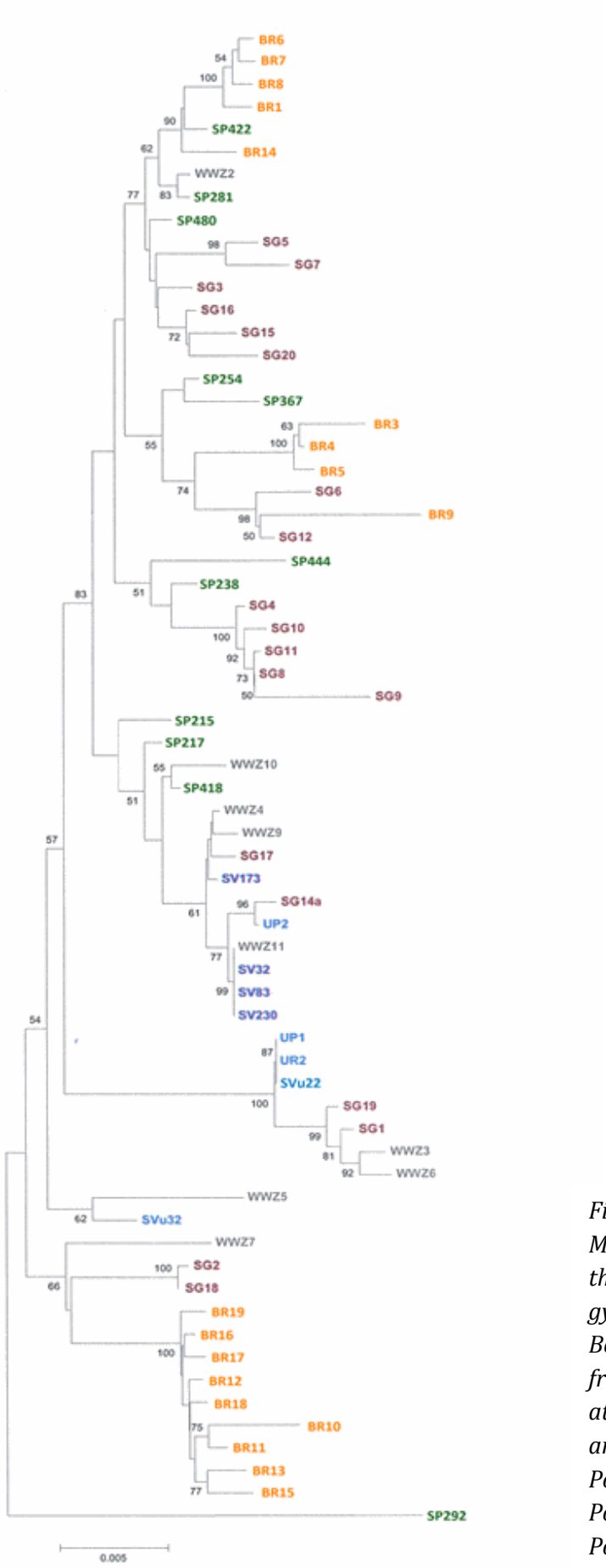

Figure 1 - Dendrogram based on MLST concatenated sequences of the housekeeping genes adk, fumC, gyrB, icd, mdh, purA and recA. Bootstrap values (≥ 50%) generated from 1000 replicates are indicated at branchpoints. Strains colour code and designation: red, Seagulls Portugal; orange, Wild Birds Portugal; grey, Wastewater Portugal; green, Wild Birds Spain; purple, Wild Birds Sweden; cyan, Wild Birds Uppsala.

4. Discussion

4.1 Overall resistance

The present study reports the antibiotic resistance profile of a small set of isolates from different avian hosts and wastewater, which is known to be an important interface between bird populations and human settings. Among the environmental isolates from the described countries, many isolates were noticed carrying resistance to many antibiotics. In this study, birds of prey and seagulls from Portugal were compared with ducks, pigeons and gulls from Sweden and Spain and hence, resistance profile diversity cannot clearly be designated to either geographic location or host species. More than 81% (54/ 66) of the ciprofloxacin resistant isolates were found to be resistant to three or more classes of antibiotics, indicating substantial multidrug resistance. Multidrug resistant microbes were detected on a frequent basis, especially in areas with high human density (Guenther et al., 2009; Cole et al., 2005; Simões et al., 2010), substantiating the threatening scenario of the emergence of pathogenic multidrug resistant strains.

Interestingly, the overall resistant phenotypes in Spanish isolates were less frequent when compared to Portuguese and Swedish isolates even though antibiotic usage is less controlled in Spain and Portugal than in Sweden (Adriaenssens et al., 2011). The high resistance in Swedish isolates was surprising. However, antibiotic resistance in Sweden is generally low, although there are reports suggesting that such traits have been increasing over time (Bonnedahl et al., 2010). Swedish samples were collected in a pond very close to the Uppsala university hospital featuring 1100 beds to cover health care in this region. The recovery rate of ciprofloxacin resistant isolates from these samples was very low, which is in agreement with the previously acknowledged low background resistance in Sweden (Bonnedahl et al., 2010). However, due to the low number of Swedish isolates (4), statistical analyses of resistance prevalence are not statistically robust.

Due to the low number of Swedish ciprofloxacin-resistant isolates that could be provided from other sources, resistance investigation was done on *E. coli* isolates from Spain. The inclusion of Spanish isolates allowed us to compare resistance profiles in isolates from two Southern European countries. Despite decreasing first- and third generation quinolone prescription rates in Spain, the prescription of second generation quinolone (i.e. ciprofloxacin) has been high and stable (Adriaenssens et al., 2011). However, the situation in Portugal was reported to be more uncontrolled than in other countries. The differences in the resistance profiles from Southern Europe (Portugal and Spain) and Northern Europe (Sweden), together with different antibiotic prescription regimes in the respective countries, give rise to the assumption that the extent of antibiotic usage may affect the resistance development (Adriaenssens et al., 2011) like hypothesized. Similarly, meropenem resistance was observed in none of the three countries, as carbapenems are widely reserved as the 'last resort' antibiotics in the treatment of infections caused by bacteria that are resistant to penicillins (e.g.

ticarcillin) and cephalosporins (e.g. ceftazidime) (Nukaga et al., 2008). The overall susceptibility of *E. coli* from Portugal, Spain and Sweden is therefore good news, showing that there are still antibiotics that can effectively treat bacterial infections when other antibiotic agents have failed.

It is also noteworthy that sampling dates for isolates from Portugal varied from 2004 (for wastewater) to 2008 (for wild bird and seagulls), which can substantially affect the image of ciprofloxacin resistance mirrored from the environment. The use and misuse of antibiotics can be mirrored in the different environmental reservoirs, as for example in avian scavengers (Bonnedahl et al., 2010; Hasan et al., 2012; Guenther et al., 2009). However, due to their migration over long distances, wild birds can displace the reflectance of antibiotic usage per country. In the case of Swedish isolates, the *E. coli* isolates that were found resistant to ciprofloxacin were substantially resistant to also other antibiotics, indicating that these resistant microbes might have been introduced to the Swedish environment from elsewhere, for example Portugal.

Comparing wild birds from Portugal and Spain demonstrated similar antibiotic resistance phenotypes, illustrating a common feeding behavior of birds in different ecological niches and habitats, to scavenge from human waste. Isolates from both countries were completely susceptible to gentamicin and meropenem, and in some cases resistance were noticed to ceftazidime (Portugal, 4/18; Spain, 1/11). Additionally, resistance to ticarcillin and trimethoprim- sulfamethoxazole were most frequent in Portuguese (TIC: 18/18, SXT: 18/18) and Spanish (TIC: 9/11, SXT: 6/11) wild bird isolates. Similar results were obtained in avian isolates from Germany, where high resistance rates to ticarcillin and low resistance rates to gentamicin were found (Guenther et al., 2010). Nevertheless, analyzed bird species were not the same in Portugal and Spain, and therefore it may be suggested that for further comparative studies about geographic distribution of antibiotic resistance, bacterial isolates from one species are used.

The implementation of two isolation methods (Culturing on CLED plate and Enrichment in LB broth with ciprofloxacin supplementation) on samples from Uppsala, Sweden indicated the importance of methodological differences in regard to quinolone resistant isolates. Enrichment in LB broth with ciprofloxacin supplementation (2.5 µg/ml) is due to its selectiveness the better method for isolation of quinolone resistant *E. coli*. Moreover, methodological differences in different labs in Sweden and Portugal may influence the prevalence of quinolone resistance in different samples.

4.2 Acquired resistance genes

None of the isolates harbored the horizontally acquired *qnrB* or *qepA* genes, whereas *qnrA* was detected. Also *aac(6')-Ib-cr* was found. However, in a universal study of the prevalence of *qnr* genes, the frequency of *any qnr* gene in *E. coli*, collected from 1999 to 2004 was merely 4% (Robicsek et al., 2006a). In contrast to the present results, the

prevalence of *qnr* genes is reported generally lower than the prevalence of *aac(6')-Ib-cr (*Park et al., 2006). Certainly, the time of sampling may be an important factor impacting the results obtained in previous studies and the frequency and distribution of *qnr* genes and *aac(6')-Ib-cr* should be verified with a complete set of newly sampled isolates from all countries. As the dissemination of the acquired resistance genes is known to be highly dynamic, results today might differ from a few years ago. The quinolone efflux pump function of the *qepA* gene has only been discovered recently and prevalence of this gene needs further investigation. Despite of the low-level resistance conferred by *qepA*, its genetic dissemination among human pathogens could be enhanced by co-selection with aminoglycosides and beta-lactams (Périchon et al., 2007). It is thus of particular importance to promote a responsible stewardship in prescription of these antibiotic classes to control further dissemination.

The gene *qnrS* was found in one single isolate originating from wild birds in Spain, which is consistent with previous studies, stating that *qnrS* could be more prevalent in *Serratia marcescens* and *Enterobacter cloacae* while *qnrA* is thought to be more prevalent in *E. coli* (Robicsek et al., 2006a and Poirel et al., 2006). However, determinants for both, *qnrA* and *qnrS* were reported to be low for nalidixic acid (first generation quinolone) resistant *Enterobacteriaceae*, which may suggest that patterns would be similar for the second generation quinolones. While *qnrA* is known to be distributed on a geographically wide scale, the distribution and temporal changes in prevalence of the more recently discovered genes *qnrB* and *qnrS* have not been studied extensively (Robicsek et al., 2006b). Consequently, *qnrA* is reported to be more prevalent in the environment than *qnrB* and *qnrS*. The distribution of *qnr* genes found in this study supports this. Even more recently, new *qnrA* variants, multiple *qnrB*, *qnrS* and *qepA* alleles were reported in the clinical variants of Enterobacteriacae which indicate the vast diversity and dynamics of new emerging plasmid mediated quinolone resistant determinants (Robicsek et al., 2006a). The use of specific primers for known *qnr* genes could thus be a limitation of this study, because other, yet unknown *qnr* variants will not be amplified in PCR assays.

Interestingly, *qnrA* genes were exclusively detected in isolates from Portugal and Spain while *aac(6')-Ib-cr* was found in isolates from seagulls in Portugal and in water samples in Sweden, reflecting the distribution of ciprofloxacin resistance determinant in a wide scale. Transboundary dispersal would be possible through the migration process of birds that can take up resistant bacteria from surface water. It has been described that there seems to be no relationship between the presence of *aac(6')-Ib-cr* and *qnrA, -B* or *–S* (Park et al., 2006), which was consistent with this study. The distribution of *qnr* and *aac(6')-Ib-cr* genes did not seem to follow a country-specific pattern, but the cross-country distribution of those genes in this study substantiates previously reported wide geographical distribution of especially the *qnr* genes (Robicsek et al., 2006b). However, distribution patterns according to host species were not observed and could not be found in the literature. The association of acquired resistance genes and host species forms a clear limitation of this study and should be taken into consideration for further investigations.

It has been highlighted that the combination of *qnrA* and *aac(6')-Ib-cr* genes may potentially enhance the level of quinolone resistance to a fourfold than conferred by *qnrA* alone (Park et al., 2006). This can be supported by the high MIC of two isolates towards all tested antibiotics (data not shown). Two isolates (SG10 and SG14a) that carried both, *qnrA* and *aac(6')-Ib-cr* were resistant to more than three classes of antibiotics including quinolones. Also, there is an association between the presence of *aac(6')-Ib-cr,* and aminoglycoside (gentamicin) resistance, stating that *aac(6')-Ib-cr* is significantly more frequent in *E. coli* that are resistant to gentamicin than that resistant to ciprofloxacin or trimethoprim-sulfamethoxazole (Park et al., 2006). Results showed that of all eleven isolates that carry *aac(6')-Ib-cr*, eight are resistant to gentamicin. At the same time, however, the presence of the *aac(6')-Ib-cr* gene is not considered to be the responsible factor for gentamicin resistance in *E. coli* indicating that other genes may play an important role in conferring resistance (Park et al., 2006).

4.3 Chromosomal resistance genes

Leavis et al., 2006, reported *gyrA* to be the major target in mutation-mediated quinolone resistance in gram-negative bacteria, such as *E. coli*. Low-level ciprofloxacin resistance is often conferred by mutations in *gyrA* only, whereas higher levels of resistance were observed in isolates resistant to both, *gyrA* and *parC* (Leavis et al., 2006). Additionally, Weigel et al., 1998, reported that a single mutation in codon 83 of *gyrA* confers reduced susceptibility, while a double mutation in codon 83 and 87 seem to be the factor that determines high-level of resistance. In this study, mutations were ubiquitous in *gyrA* and accounted for at least one altered codon in almost all isolates in *parC*, suggesting high-level resistance in many isolates. The lower mutation rate in *parC* confirms *gyrA* as the primary target of antibiotic resistance development for *E. coli*. Considering the less frequently found and randomly distributed plasmid-mediated resistance genes, the extensive prevalence of mutations in isolates from all origins is even more striking. Our results demonstrate the major role of the chromosomal genes in ciprofloxacin resistance development.

4.4 Strain diversity

Even though Portuguese wild bird isolates were closely related to each other, bird species and their feeding behavior may influence the *E. coli* community. All isolates were recovered from the birds of prey such as the common buzzard (*Buteo buteo),* the booted eagle *(Hieraaetus pennatus)* and the cinereous vulture (*Aegypius monachus)* that do not live in groups. However, since they feed on prey (e.g. small mammals) that might be colonized with antibiotic resistant bacteria, antibiotic resistance dissemination into the pristine environment of a natural reserve is implied. Despite of the different feeding behavior of different species, all wild bird isolates are clearly related to each other, regardless the host species. However, taking into consideration that all wild bird species were predatory birds and that they resided in the same national park, it is not surprising

that they shared closely related *E. coli* isolates. Still, isolation location might play an important role and for further investigations.

The isolates derived from seagulls in Portugal also show a strong genetic relatedness, by clustering into phylogenetic branches, but they are more loosely aggregated and thus seem to be more diverse and mixed up with strains from other sources. Still, they mostly cluster together with Spanish and Portuguese wild bird isolates, and Portuguese wastewater isolates, substantiating a common genetic background of microbes in the Southern European region. Apart from clustering together with Spanish and other Portuguese isolates, *E. coli* isolates derived from wastewater in Portugal are randomly distributed among phylogenetic groups and subgroups and their appearance seems to follow no phylogenetic grouping at all. This and the unspecific resistance phenotypes showed by Portuguese wastewater isolates might be explained by the highly diverse bacterial environment in urban wastewaters. Especially wastewaters with combined effluents from various human origins are considered a major reservoir of antibiotic resistance and an ideal environment for bacteria to exchange genetic material.

The phylogeographic pattern of isolates from wild birds and gulls in Spain shows that they are more closely related to other Southern European isolates, than to Swedish isolates. However, one Spanish isolate proves to be an extreme outlier (SP292) at the unrelated bottom end of the tree, raising the question about the genetic determinants of this highly distinct resistance profile. Although the biochemical testing and the BLAST website confirmed this isolate as *E. coli*, it's clear deviation from the other isolates indicates that further investigations are needed to reveal the reasons for distinct it's distinct phylogenetic position. The isolates in Sweden also cluster together rather tightly, but not as tight and distinct as the wild birds from Portugal, possibly due to the various sampling locations. However, Swedish isolates provided by the University of Kalmar that were sampled in the city Hudiksvall typed together with isolates from Uppsala, thus revoking the possibility for Uppsala isolates to be diverse due to two different sampling locations within the city. Other factors such as host species, migratory behavior of the birds or precipitation may have a more substantially impact and more investigations on this are recommended. To conclude, there seem to be some distinct phylogeographic clusterings according to isolate origins. However, those results are based on a limited sample number. Still, clustering is distinct enough to distinguish the distribution according to large geographic distances, i.e. Swedish isolates are distinguished from isolates from Portugal and Spain. The presence of sequence types that were previously associated with strains of human pathogens and poultry substantiates the human footprint on microbes found in avian fecal flora.

5. Conclusion

This research shows that different avian species with different ecologies are important quinolone resistant *Escherichia coli* carriers. Therefore, wild birds could be an important indicator of environmental antibiotic resistance in Europe. Due to their scavenging feeding behavior, high mobility through migration and their close contact to human areas, wild birds and seagulls in particular could acquire quinolone resistant bacteria from human environments, and function as a reservoir of environmental antibiotic resistance. Through contact with natural water reserves, wild birds could pose a potential risk to the pristine environments and disseminate medically important pathogens. There was no clear difference in resistance phenotypes and genotypes in isolates from Portugal, Spain and Sweden, which might be due to the migratory behavior of the analyzed wild birds. Also, those isolates that typed together based on the multilocus sequence typing analysis, did not notably show analogy in resistance phenotypes or genotypes. Even so, the dissemination of resistant microbes may constitute a considerable hazard to public health and to control the alarming emergence of multi resistant bacteria, a responsible usage of antibiotics in human and animal health is of great importance.

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APPENDIX

Original code of Portuguese E. coli isolates

Wild birds

New Code	Original Code
BR1	1.102.AS
BR3	13.103.AS
BR4	14.101.AS
BR5	14.102.AS
BR6	15.101.AS
BR7	15.103.AS
BR8	20.101.AS
BR9	22.103.AS
BR10	40.101.AS
BR11	41.101.AS
BR12	42.101.AS
BR13	44.102.AS
BR14	44.51.AS
BR15	60.1.AS
BR16	72.100.AS
BR17	73.1.AS
BR18	73.100.AS
BR19	73.101.AS

Seagulls

New Code	Original Code
SG1	2.100.G
SG2	3.100.G
SG3	7.100.G
SG4	9.100.G
SG5	9.101.G
SG6	12.102.G
SG7	12.103.G
SG8	14.102.G
SG9	14.103.G
SG10	15.100.G
SG11	15.101.G
SG12	15.102.G
SG14a	20.101.G
SG15	21.101.G
SG16	21.103.G
SG17	21.104.G

Wastewater

New Code	Original Code
WWZ2	S3R22
WWZ3	S2R35
WWZ4	S1R62
WWZ5	S3R47
WWZ6	A5EL5
WWZ7	A4FC39
WWZ9	E3FC46
WWZ10	E4FC12
WWZ11	E4FC22