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Plasmid-Mediated Quinolone Resistance Genes in Fecal Bacteria from Rooks Commonly Wintering Throughout Europe

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This study concerned the occurrence of fecal bacteria with plasmid-mediated quinolone resistance (PMQR) genes in rooks (*Corvus frugilegus*, medium-sized corvid birds) wintering in continental Europe during winter 2010/2011. Samples of fresh rook feces were taken by cotton swabs at nine roosting places in eight European countries. Samples were transported to one laboratory and placed in buffered peptone water (BPW). The samples from BPW were enriched and subcultivated onto MacConkey agar (MCA) supplemented with ciprofloxacin (0.06 mg/L) to isolate fluoroquinolone-resistant bacteria. DNA was isolated from smears of bacterial colonies growing on MCA and tested by PCR for PMQR genes *aac(6')-Ib*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *oqxAB*. All the PCR products were further analyzed by sequencing. Ciprofloxacin-resistant bacteria were isolated from 37% (392 positive/1,073 examined) of samples. Frequencies of samples with ciprofloxacin-resistant isolates ranged significantly from 3% to 92% in different countries. The *qnrS1* gene was found in 154 samples and *qnrS2* in 2 samples. The gene *aac(6')-Ib-cr* was found in 16 samples. Thirteen samples were positive for *qnrB* genes in variants *qnrB6* (one sample), *qnrB18* (one), *qnrB19* (one), *qnrB29* (one), and *qnrB49* (new variant) (one). Both the *qnrD* and *oqxAB* genes were detected in six samples. The genes *qnrA*, *qnrC*, and *qepA* were not found. Wintering omnivorous rooks in Europe were commonly colonized by bacteria supposedly Enterobacteriaceae with PMQR genes. Rooks may disseminate these epidemiologically important bacteria over long distances and pose a risk for environmental contamination.

Introduction

The second generation of quinolones (fluoroquinolones) are commonly used in antimicrobial treatment in both human and veterinary medicine worldwide, including Europe. With the continuing development of new quinolone congeners with expanded clinical indications reflecting an expanding antibacterial spectrum for some members of the fluoroquinolones, understanding as to limitations posed by the occurrence of bacterial resistance to fluoroquinolones is of increasing importance.[17] Plasmid-mediated quinolone resistance (PMQR) was identified in Enterobacteriaceae bacteria for the first time in 1998.[31]

Wild bird populations sympatric to areas inhabited by people and areas with high density of livestock have been colonized with antibiotic-resistant strains that probably have

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been selected by antibiotic practice in humans and domestic animals. Antibiotic-resistant *Escherichia coli* isolates have been found in various corvids, including rooks (*Corvus corone*, *C. frugilegus*, *C. macrorhynchos*, *Pica pica*, and *Pyrrhocorax pyrrhocorax*).[3,27,28,32,41] Corvids and gulls feeding on garbage dumps in urbanized areas are frequently colonized with antibiotic-resistant strains of *E. coli* and they are considered to be important reservoirs and vectors of these isolates in the environment.

The rook (*Corvus frugilegus*) is a migrating omnivorous corvid with Palearctic distribution.[12] Large numbers of these birds regularly winter in central and western Europe. Rooks winter mostly in lowlands and they traditionally keep gregarious roosting places. Rooks leave their roosting places during the day to search for food, usually within 10–25 km around their roosting places. In the past, rooks migrated for the winter so far as to such south European countries as Italy and Spain.[4,39] Today, wintering rooks are rare in Italy and only a small sedentary population of rooks winters in Spain. The origin of those rooks wintering still in huge numbers in both central and western parts of continental Europe is mostly in eastern Europe—Russia, Belorussia, and Ukraine. Wintering rooks in central Europe can serve as reservoirs and vectors of *E. coli* and *Salmonella* isolates resistant to old-generation antibiotics and potentially can transmit these isolates over long distances during their migrations.[27]

This study concerned bacterial strains resistant to fluoroquinolones, as such strains have emerged recently in gulls in Italy, Portugal, Greenland, and the Czech Republic.[5,10,15,42] PMQR in *E. coli* isolates from wild birds has been reported very recently for mallards (*Anas platyrhynchos*), herring gulls (*Larus argentatus*), and great cormorants (*Phalacrocorax carbo*) in Poland and Czech Republic.[26,45] Given the scavenging diet of rooks in winter and the long distances traveled, it may be postulated that rooks could be important vectors for disseminating bacteria with PMQR throughout Europe.

Materials and Methods

Rooks examined

Samples of rook feces were taken in roosting places throughout Europe during winter 2010/2011, except for France, where samples were collected on 8 April 2011 (Fig. 1). The roosts were used by hundreds or thousands of rooks together with various numbers of other corvids, such as jackdaws (*C. monedula*) or crows (*C. corone* and *C. cornix*). Fecal samples were collected at each location only once. They were picked up individually in the morning from a large plastic film exposed overnight on the ground beneath the roosting place. Rooks were dropping on the film during the evening when they arrived to the roosting place and in early morning when leaving the location. Samples were collected in the Czech Republic (Prerov, 49°28′ N, 17°27′ E, 12 December 2010, 150 samples), France (Pire sur Seiche, 48°00′ N, 1°25′ W, 8 April 2011, 31 samples), Germany (Wilhelmshaven, 53°32′ N, 8°04′ E, 5 February 2011, 100 samples), Italy (San Benedetto Po, 45°2′ N, 10°55′ E, 27 February 2011, 150 samples), Poland (Gdynia and near vicinity, 54°31′ N,

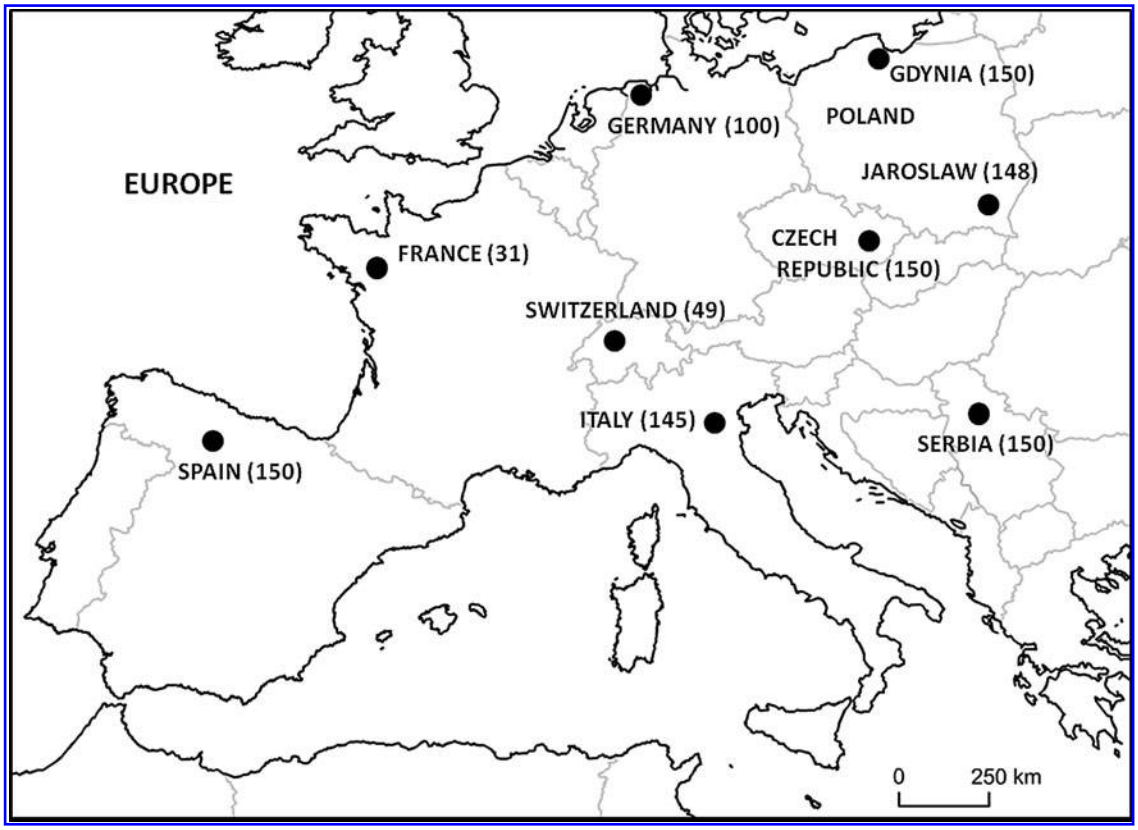

FIG. 1. Map of Europe indicating where fecal samples from rooks (*Corvus frugilegus*) were collected during winter 2010/2011. Full circles indicate locations of sampling. At each location, the number of samples collected is presented in parentheses.

18°33′ E, 2–7 February 2011, 150 samples), Poland (Jaroslaw, 50°00′ N, 22°41′ E, 9 February 2011, 150 samples), Spain (La Baneza, 42°18′ N, 5°54′ W, 4 February 2011, 150 samples, sedentary population), Serbia (Novi Sad, 45°14′ N, 19°51′ E, 17 January 2011, 150 samples), and Switzerland (Bern, 46°56′ N, 7°26′ E, 8 February 2011, 49 samples).

Bacteriological examinations and DNA analyses for detecting PMQR

Samples from fresh feces were taken by cotton swabs that were then transported in Amies transport medium to the laboratory and pre-enriched overnight in buffered peptone water (BPW) at 37°C. The samples from BPW were selectively enriched in MacConkey broth and subcultivated onto MacConkey agar (MCA) supplemented with ciprofloxacin (0.06 mg/L) to isolate fluoroquinolone-resistant enterobacteria.

DNA was isolated from smears of bacterial colonies growing on MCA with ciprofloxacin (one smear from one rook sample). The DNA from these colonies was tested by PCR for PMQR genes *aac*(6′)-*Ib*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *oqxAB* (Table 1).[7,9,23,24,35,36,38,47] All the PCR products were further analyzed by sequencing (ABI 310 Genetic Analyzer, Applied Biosystems).

In each case of a sample where new variants of PMQR genes might possibly appear, a smear of unidentified bacteria from each sample was frozen at −80°C for later bacterial species determination. In such case, the bacteria were cultivated on MCA supplemented with ciprofloxacin (0.06 mg/L) and subsequently identified using the API 20E system (bioMerieux).

Results

In total, 1,073 samples of rook feces were collected. Ciprofloxacin-resistant bacteria supposedly Enterobacteriaceae were isolated from 392 (37%) samples (Table 2). Proportions of samples with bacteria resistant to ciprofloxacin ranged greatly—from 3% to 92%—in the different countries. The highest frequencies of ciprofloxacin-resistant Enterobacteriaceae bacteria of 92%, 69%, and 41% were found in the Czech Republic and the two locations in Poland, respectively. Numbers of samples with PMQR genes ranged from 0% to 60% in different countries and the highest frequencies of 60%, 27%, and 14% were again in the Czech Republic and the two locations in Poland, respectively.

The most numerous gene *qnrS* was found in 156 samples originating mainly from the Czech Republic and Poland. Sequence analysis of the *qnrS* genes showed the presence of two variants, *qnrS1* and *qnrS2*. The gene *qnrS1* occurred most frequently and was found in 154 samples, while *qnrS2* was identified in two samples.

The fluoroquinolone-aminoglycoside resistance gene *aac*(6′)-*Ib-cr* was the gene second most frequently detected in rook feces. It was found in 16 (1.5%) samples. The *aac*(6′)-*Ib-*

Table 1. Primers for Plasmid-Mediated Quinolone Resistance Genes Used in This Study

Primers	*Sequence (5′–3′)*	*Target gene*	*Annealing temperature (°C)*	*Amplicon size (bp)*	*Positive control*	*Reference*
qnrA-F	ATT TCT CAC GCC AGG ATT TG	*qnrA*	53	516	*Escherichia coli* J53 pMG252	38
qnrA-R	GAT CGG CAA AGG TTA GGT CA					
qnrB-F	GAT CGT GAA AGC CAG AAA GG	*qnrB*	53	476	*E. coli* J53 pMG298	23
qnrB-R	ATG AGC AAC GAT GCC TGG TA					
qnrB_VI	CTARCCAATMAYCGCGATGCCAAG	*qnrB*	53	645	*E. coli* J53 pMG298	Primers designed for this study
qnrB_VII	ATGRCTCTGGCRTTAGTTRGCGAAA					
qnrB19.seq1F	ATG ACT CTG GCA TTA GTT GG	*qnrB19* part 1	53	411	*E. coli* DH5T15	11
qnrB19.seq1R	CCA CAG CTC ACA CTT TTC CA					
qnrB19.seq2F	TGC CAT TTT CAA AAG CTG TG	*qnrB19* part 2	53	457	*E. coli* DH5T15	11
qnrB19.seq2R	GTA ACC AAT CAC AGC GAT GC					
qnrC-F	GGG TTG TAC ATT TAT TGA ATC G	*qnrC*	53	307	*E. coli* DH10B pHsII Tf1	23
qnrC-R	CAC CTA CCC ATT TAT TTT CA					
qnrS-F	GCA AGT TCA TTG AAC AGG GT	*qnrS*	53	428	*E. coli* J53 pMG306	6
qnrS-R	TCT AAA CCG TCG AGT TCG GCG					
qnrS1.seq1F	ATGGAAACCTACAATCATACATATCG	*qnrS1* part1	55	443	*E. coli* J53 pMG306	11
qnrS1.seq1R	TTCGTTCCTATCCAGCGATT					
qnrS1.seq2F	TTC GTG ATG CAA GTT TCC AA	*qnrS1* part2	55	467	*E. coli* J53 pMG306	11
qnrS1.seq2R	TTA GTC AGG ATA AAC AAC AAT AAC C					
qnrD-F	CGA GAT CAA TTT ACG GGG AAT A	*qnrD*	55	582	*E. coli* TG1 p2007057 Tf1	8
qnrD-R	AAC AAG CTG AAG CGC CTG					
aac(6′)Ib-F	TTG CGA TGC TCT ATG AGT GGC TA	*aac(6′)-Ib*	55	482	*Salmonella* Infantis 14	35
aac(6′)Ib-R	CTC GAA TGC CTG GCG TGT TT					
qepA-F	TGG TCT ACG CCA TGG ACC TCA	*qepA*	53	1136	*E. coli* 20III-09	36 47
qepA-R	TGA ATT CGG ACA CCG TCT CCG					
oqxBs	TTCTCCCCCGGCGGGAAGTAC	*oqxB*	64	512	*E. coli* CSH26 RifR/pOLA52	24
oqxBa2	CTCGGCCATTTTGGCGCGTA					
oqxA-F	CTCGGCGCGATGATGCT	*oqxA*	60	392	*E. coli* CSH26 RifR/pOLA52	24
oqxA-R	CCACTCTTCACGGGAGACGA					

Table 2. Plasmid-Mediated Quinolone Resistance Genes in Fecal Bacterial Samples from Rooks (*Corvus frugilegus*) Wintering in Europe During Winter 2010/2011

	Czech Republic	*France*	*Germany*	*Italy*	*Poland, Gdynia*	*Poland, Jaroslaw*	*Spain*	*Serbia*	*Switzerland*	*Total (%)*
No. of samples	150	31	100	145	150	148	150	150	49	1,073 (100)
No. of samples with bacteria resistant to ciprofloxacin (%)	139 (92)	1 (3)	20 (20)	9 (6)	104 (69)	61 (41)	26 (17)	30 (20)	2 (4)	392 (37)
No. of samples with plasmid-mediated quinolone resistance genes	89 (60)	0 (0)	9 (9)	1 (0.7)	40 (27)	20 (14)	9 (6)	7 (5)	0 (0)	175 (16)
No. of samples with *qnrA*	—	—	—	—	—	—	—	—	—	— (0)
No. of samples with *qnrB6*	—	—	—	—	—	1	—	—	—	1 (0.1)
No. of samples with *qnrB18*	—	—	—	—	—	1	—	—	—	1 (0.1)
No. of samples with *qnrB19*	3	—	—	—	6	—	—	—	—	9 (1)
No. of samples with *qnrB29*	1	—	—	—	—	—	—	—	—	1 (0.1)
No. of samples with *qnrB49*	—	—	—	1	—	—	—	—	—	1 (0.1)
No. of samples with *qnrC*	—	—	—	—	—	—	—	—	—	— (0)
No. of samples with *qnrD*	4	—	—	—	—	2	—	—	—	6 (0.6)
No. of samples with *qnrS1*	82	—	4	—	38	15	9	6	—	154 (14)
No. of samples with *qnrS2*	2	—	—	—	—	—	—	—	—	2 (0.2)
No. of samples with *aac(6′)-Ib-cr*	8	—	7	—	—	—	—	1	—	16 (1.5)
No. of samples with *oqxAB*	4	—	—	—	—	1	—	1	—	6 (0.6)
No. of samples with *qepA*	—	—	—	—	—	—	—	—	—	— (0)

cr-positive samples originated mainly from the Czech Republic (eight samples) and Germany (seven). Thirteen samples were positive for the *qnrB* genes. These samples originated from the Czech Republic, Poland, and Italy. The *qnrB* genes were identified as the *qnrB6* (one sample), *qnrB18* (one), *qnrB19* (nine), and *qnrB29* (one) variants. A new variant of the *qnrB* gene was found in one sample. The nucleotide sequence of this gene was compared with other *qnrB* genes available in the GenBank database using BLAST sequence analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The new gene variant differed from the *qnrB20* in two nucleotides (GenBank Accession No. AB379831), meaning one amino acid substitution (Ala156Thr). The new variant was classified as *qnrB49*. The full sequence of this gene is included in GenBank under the accession number JQ582718. Retrospective analysis of the sample positive for the *qnrB* new variant showed this new variant to be present in two isolates of *Citrobacter freundii* isolated from a rook fecal sample collected in San Benedetto Po, Italy, on 27 February 2011.

Both the genes *qnrD* and *oqxAB* were detected in six (0.6%) different samples, four of which were collected in the Czech Republic. The genes *qnrA*, *qnrC*, and *qepA* were not detected in any rook fecal sample.

Discussion

In 2003, a single clone of *Shigella flexneri* was found to be resistant to ciprofloxacin and this strain was also harboring a unique conjugative plasmid that transferred quinolone resistance.[16] Cloning identified an open reading frame encoding a 218-amino-acid protein of the pentapeptide repeat family that was named QnrS (the gene is named *qnrS1*). A *qnrS* variant, *qnrS2*, was detected on a plasmid from *Salmonella enterica* serovar Anatum.[14] The *qnrS* gene has been identified as the most prevalent PMQR gene.[25] This gene has been detected in quinolone-resistant human, poultry, and swine *E. coli* and *Salmonella* isolates.[8,18,48] The *qnrS1* gene was predominant in the fecal bacteria from rooks throughout Europe. Rooks excreted feces with these bacteria commonly in the Czech Republic, both Polish locations, Spain, Serbia, and Germany. We consider that the sources of these bacteria could be food animals and their products and/or excrements because *Salmonella* and/or *E. coli* isolates with the *qnrS1* gene were found recently in food and turkeys in Germany; in cattle, pigs, chickens, and turkeys in Poland; in sheep in Italy; and in chickens in Spain.[46] The *qnrS1* gene was predominant among the *qnr* genes found in *S. enterica* and *E. coli* isolates from animals, humans, food, and the environment in 13 European countries,[46] and these findings accord with the result in rooks.

Two samples of rook feces obtained in the Czech Republic were positive for the *qnrS2* gene. This gene has been detected recently in *Aeromonas* spp. isolates recovered from diseased fish and water environments in different parts of Asia and in Switzerland, as well as from human clinical isolates in Spain.[1,6,30,37] The gene *qnrS2* has been found also in clinical isolates of *Salmonella* in Japan.[44] We report the *qnrS2* gene in bacteria from wild birds for the first time.

The spectrum of *qnrB* genes is broader than those of *qnrA* and *qnrS*.[43] While studying strains of *Klebsiella pneumoniae*, Jacoby *et al.*[22] found the PMQR gene *qnrB1*. A number of other variants (from *qnrB2* to *qnrB48*) were found subsequently among various Enterobacteriaceae (www.lahey.org/qnrstudies, accessed 13 February 2012). From all the *qnrB* genes, *qnrB19* was predominant in the fecal bacteria obtained from rook feces. This gene has very recently been demonstrated to be prevalent in Enterobacteriaceae from food-producing animals in different parts of Europe and in Nigeria, from humans in South America and the Netherlands, and from horses in the Czech Republic.[11,13,19,34,46] It seems that the frequency of the *qnrB19* gene in rooks reflects the general situation in domestic animals and humans in Europe.

Moreover, the genes *qnrB6*, *qnrB18*, and *qnrB29* were present in rook samples obtained from the Czech Republic

and Poland. The gene *qnrB6* has been detected mainly in clinical isolates of Enterobacteriaceae bacteria in China and Spain, in isolates obtained from dogs and ducks, and from the environment in China.[29,40,49,50] Until now, the genes *qnrB18* and *qnrB29* had been found only in *Citrobacter freundii* isolates in Spain and Korea (GenBank Accession No. AM919399; GenBank Accession No. HM439649). Our rare findings of these genes provide the first evidence of their occurrence in European wildlife.

Six variants of *qnrB* (*qnrB2*, *qnrB4*, *qnrB6*, *qnrB7*, *qnrB12*, and *qnrB19*) were recently identified among PMQR *Salmonella* and/or *E. coli* isolates from Germany, Poland, and Spain.[46] Most of the *qnrB*-positive isolates in that study originated from turkeys. Hence, turkeys should be considered an important source for rooks of fecal bacteria carrying *qnrB* genes.

The new variant of the *qnrB* gene was found in *Citrobacter freundii* from a rook in Italy. Most known variants of the *qnrB* gene have been discovered from *Citrobacter freundii*, a potential reservoir for new variants of this gene.[21]

Another gene, designated *qnrD*, was found in *S. enterica* isolates, and it was transferable on small plasmids of about 4.3 kb.[9] This gene encodes a 214-amino-acid pentapeptide repeat protein designated QnrD. Six samples of rook feces obtained in the Czech Republic and Poland were positive for *qnrD*. This gene was recently identified in PMQR *Salmonella* isolates from Italy and Spain.[46] Most of the *qnrD*-positive isolates in that study originated from laying hens in Spain, while some isolates with the *qnrD* gene originated from chickens, turkeys, and food in Italy. Hence, chickens and turkeys should be considered as sources of fecal bacteria carrying *qnrD* genes found in rooks. In addition, *qnrD* has been detected in *E. coli* isolates obtained from pigs in China and in clinical isolates of *Proteus* and *Pseudomonas* in Nigeria.[33,49]

An additional important PMQR gene is *aac(6′)-Ib-cr*. It encodes a specific aminoglycoside acetyltransferase, AAC(6′)-Ib-cr, that confers increase of minimum inhibitory concentration selectively for norfloxacin and ciprofloxacin.[38] The gene *aac(6′)-Ib-cr*, like its parent *aac(6′)-Ib*, is an integron cassette with an associated *attC* site. It is hence found in various integrons, but especially on IncFII plasmids expressing CTX-M-15 that have spread rapidly such that CTX-M-15 has become the predominant extended-spectrum beta-lactamase in many countries throughout the world. The gene *aac(6′)-Ib-cr* has been associated with other PMQR genes and with other beta-lactamases. In our study, *aac(6′)-Ib-cr* was the gene second most frequently detected in rook feces and mainly among those obtained in the Czech Republic and Germany. In a contrast to our study, the *aac(6′)-Ib-cr* gene has only exceptionally been identified in PMQR Enterobacteriaceae bacteria in Europe.[46]

The plasmid-mediated quinolone transporter OqxAB was found in several samples. OqxAB is a multidrug efflux pump that confers resistance to quinolone, quinoxaline, and other agents, including chloramphenicol. Plasmid-mediated OqxAB has been detected in human clinical *E. coli* and the *oqxAB* gene was also found on the chromosome of *K. pneumoniae*.[24] Moreover, the *oqxAB* gene has been found in *E. coli* strains from pigs, chicken, farm workers, and a farm environment.[47] In our study, we report the first isolation of *oqxAB* gene in bacteria originating from wild animals.

Migratory birds in close contact with humans and domestic animals, such as rooks, can play an important role in the dispersion of *E. coli* and *Salmonella* isolates resistant to old-generation antimicrobials.[27] The sources colonizing rooks with these isolates could be food and/or drinking water. Direct observation in the field has shown rooks to have an omnivorous feeding pattern in agricultural, rural, and urban areas during winter.[20] Outside of the breeding season, huge flocks of this species forage in communal refuse dumps, which still exist in central and eastern Europe.[2] Thus, rooks could be colonized with antibiotic-resistant bacteria, including PMQR bacteria, from both animal and human sources even if their populations are not directly influenced by antibiotic practice. Consequently, once infected, rooks may disseminate these bacteria over long distances throughout Europe and pose a risk for environmental contamination.

Being congregative medium-sized birds, rooks excrete locally in various European countries large quantities of fecal coliforms. They are capable through their feces to contaminate environments inhabited by humans and domestic animals. It is important to prevent these birds from feeding on garbage dumps, the main suspected source of resistant bacteria and including bacteria with PMQR. Not to supply food for these birds is a simple way of limiting potential problems. The common occurrence of PMQR bacteria supposedly Enterobacteriaceae in populations of wild rooks, where there is no selective pressure, can imply that such resistance will be difficult to displace.

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Disclosure Statement

No competing financial interests exist.

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