

## Antibiotic-Resistant *Escherichia coli* Bacteria, Including Strains with Genes Encoding the Extended-Spectrum Beta-Lactamase and QnrS, in Waterbirds on the Baltic Sea Coast of Poland<sup>▽</sup>

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Individual cloacal swabs of mallards (*Anas platyrhynchos*) and of herring gulls (*Larus argentatus*), as well as samples of waterbird feces obtained in 2008 and 2009, were cultivated for *Escherichia coli*. Isolates of *E. coli* were tested for susceptibilities to 12 antimicrobial agents by the disk diffusion method. Moreover, the samples were subcultivated on MacConkey agar (MCA) containing cefotaxime (2 mg liter<sup>-1</sup>) to detect *E. coli* with extended-spectrum beta-lactamase (ESBL) and subsequently on MCA supplemented with ciprofloxacin (0.05 mg liter<sup>-1</sup>) and MCA with nalidixic acid (20 mg liter<sup>-1</sup>) to isolate fluoroquinolone-resistant *E. coli*. PCR was used to detect specific antibiotic resistance genes. We found 9 *E. coli* isolates producing ESBL with *bla* genes: *bla*<sub>CTX-M-1</sub> (6 isolates), *bla*<sub>CTX-M-9</sub> plus *bla*<sub>TEM-1b</sub> (1 isolate), *bla*<sub>CTX-M-15</sub> plus *bla*<sub>OXA-1</sub> (1 isolate), and *bla*<sub>SHV-12</sub> (1 isolate). In the isolate with *bla*<sub>CTX-M-15</sub>, the gene *aac(6)-Ib-cr* was also detected. The *bla* genes were harbored by transferable plasmids of the IncN and IncI1 groups. Nine quinolone-resistant *E. coli* isolates with *qnrS* genes were found and characterized. The gene *qnrS* was associated with a Tn3-like transposon on the IncX1 plasmid together with *bla*<sub>TEM-1</sub> in two isolates. The gene *qnrS* was also harbored by conjugative plasmids of the IncN and IncX2 groups. Even if populations of wild birds are not directly influenced by antibiotic practice, we have demonstrated that antibiotic-resistant *E. coli* strains, including strains with various ESBL and *qnrS* genes, are found in the feces of wild birds on the coast of the Baltic Sea in Poland.

There is considerable concern about antibiotic resistance in bacteria from humans and farm animals, but the spread of resistance into wider ecosystems has received much less attention (48). Usually, isolates of the common intestinal bacterium *Escherichia coli* are examined to detect antibiotic resistance in populations of wild animals. Wild birds are colonized with various strains of *E. coli*, including strains such as *E. coli* O157 that are pathogenic for humans (83). Fecal strains of *E. coli* resistant to antibiotics have been found at various prevalences in wild bird populations. In particular, bird populations sympatric to areas inhabited by people and areas with a high density of livestock were colonized with antibiotic-resistant *E. coli* strains possibly selected by the antibiotic practice in humans and domestic animals. Antibiotic-resistant *E. coli* isolates have been found in corvids (*Corvus corone*, *C. frugilegus*, *C. macrorhynchos*, *Pica pica*, and *Pyrrhocorax pyrrhocorax*) (3, 46, 48, 53, 74), house sparrows (*Passer domesticus*) (22, 61), house martins (*Delichon urbica*) (73), feral pigeons (*Columba livia*

forma domestica) (68), ducks, geese, and swans (*Anas platyrhynchos*, *Anas acuta*, *Branta canadensis*, and *Cygnus columbianus*) (17, 26, 51, 82), cormorants (*Phalacrocorax auritus* and *P. cristatus*) (20, 71), Egyptian vultures (*Neophron percnopterus*) (2), and, most frequently, in various gull species throughout the world (*Larus argentatus*, *L. atricilla*, *L. audouinii*, *L. cachinans*, *L. crassirostris*, *L. glaucoides*, *L. hyperboreus*, *L. marinus*, *L. ridibundus*, and *L. vagae*) (4, 7, 21, 23, 29, 66, 71, 76, 82). Corvids and gulls feeding on garbage dumps and in urbanized areas are frequently colonized with resistant strains of *E. coli*, and they are considered to be important reservoirs and vectors of these isolates in the environment (23, 46).

The occurrence of antibiotic resistance in *E. coli* from surface water and fecal pollution sources (wastewater treatment plants and bird feces) was studied in the inland Hamilton Harbor on Lake Ontario, Canada (24). Authors of the study suggested that at times, feces of wild geese, ducks, and gulls may be a more prominent contributor of resistant *E. coli* to a beach near Hamilton than are municipal wastewater sources.

Thousands of great cormorants (*Phalacrocorax carbo*), gulls (*Larus argentatus*, *L. ridibundus*, *L. canus*, and *L. marinus*) and mallards (*Anas platyrhynchos*) winter in Puck Bay on the Polish coast of the Baltic Sea (50). Many of them stay near the two biggest cities of this region, Gdynia and Gdansk, in harbors,

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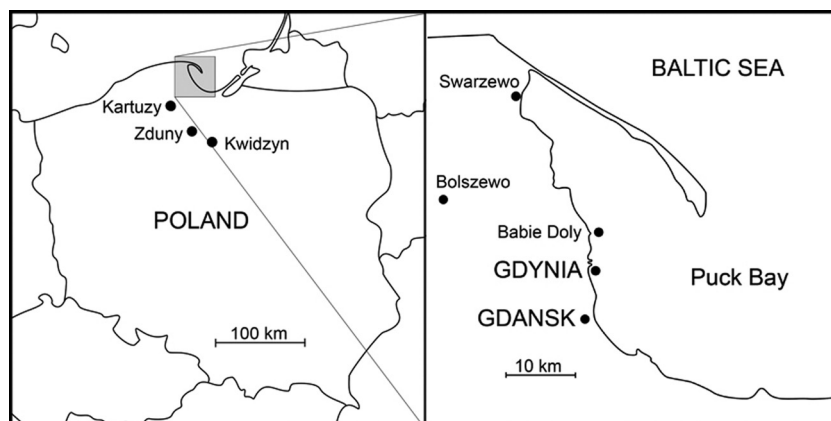


FIG. 1. Map of the Polish coast of the Baltic Sea, where the samples were collected. Mallards (*Anas platyrhynchos*) originated at Gdansk, Bolszewo, Kwidzyn, Zduny, and Kartuzy, herring gulls (*Larus argentatus*) from Gdynia, and samples of waterbird feces from Gdynia, Babie Doly, and Swarzewo.

municipal beaches, build-up areas, and city parks situated near the seacoast. We were concerned about antimicrobial-resistant *E. coli*, including strains producing extended-spectrum beta-lactamase (ESBL) and carrying class 1 and 2 integrons with resistant gene cassettes in these birds. Resistant *E. coli* isolates producing ESBL have been found in black-headed gulls (*L. ridibundus*) in the Czech Republic, yellow-legged gulls (*Larus michahellis*) in France, and seagulls of diverse species (not determined) in Portugal (4, 23, 66). Resistant isolates carrying class 1 and 2 integrons have been found in Canada geese (*Branta canadensis*) in the United States, black-headed gulls and rooks (*Corvus frugilegus*) in the Czech Republic, Mediterranean herring gulls (*Larus cachinans*) in Italy, and seagulls in Portugal (4, 17, 21, 23, 29, 46, 66). We also were concerned about strains resistant to fluoroquinolones, since such strains have emerged recently in gulls (*L. audouinii*, *L. cachinans*, *L. glaucooides*/*L. hyperboreus*, and *L. ridibundus*) in Italy, Portugal, Greenland, and the Czech Republic (7, 23, 29, 76).

#### MATERIALS AND METHODS

**Locations, birds, and feces samples.** The samples were collected on the Polish coast of the Baltic Sea within or near Gdynia and Gdansk (Fig. 1). Mallards (*Anas platyrhynchos*) were caught in the city parks of Gdansk ( $n = 46$ ) and also in Bolszewo ( $n = 32$ ), Kwidzyn ( $n = 5$ ), Zduny ( $n = 2$ ), and Kartuzy ( $n = 1$ ) during winter 2008/2009 and in November and December 2009. The latter four places are located in a region of lakes around Gdansk within a distance of up to 50 km from that city. Cloacal smears were obtained from 86 mallards in total. Herring gulls (*Larus argentatus*) were caught in Gdynia during winter 2008/2009 (adults) and during the nesting period (pulli) in 2009. Cloacal smears were obtained from 27 herring gulls. There is a large wave breaker within Gdynia's harbor that has no connection with the coast. On the shore at a distance of about 6 km from the harbor, there is also an old and abandoned army building standing on water near (300 m) a coast, near one of the Gdynia districts called Babie Doly. Especially inasmuch as public entry is prohibited to both of these, they are frequently used by waterbirds (gulls, cormorants, and sometimes also ducks) for resting, and many of their feces occur there. We collected samples of bird feces from both of these structures in January 2009. We obtained in total 244 samples (119 and 125 from the wave breaker and army building, respectively). A large roosting place for wintering great cormorants lies along the coast in Swarzewo, about 30 km from Gdynia. Here we collected 125 samples of cormorant feces in January 2009. Cormorants were daily moving from this roosting place to various places near the seacoast, including the sea close to Gdynia and Gdansk.

**Nonselective antibiotic-resistant *E. coli* isolation and characterization.** Individual cloacal swabs and samples of feces were transported to the laboratory and placed overnight in buffered peptone water (BPW) at 37°C and then cultivated

for *E. coli* on selective chromogenic medium for *E. coli* and coliform bacteria (CM0956; Oxoid, United Kingdom). One colony of each plate was tested for susceptibilities to antimicrobial agents by the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (16). Susceptibilities to the following antibacterial substances were tested: amoxicillin-clavulanic acid (30 µg), ampicillin (10 µg), cephalothin (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (25 µg), sulfonamide compounds (300 µg), and tetracycline (30 µg) (Oxoid). In *E. coli* isolates found to be resistant to one or more of the antibiotics listed above, PCR was used to detect specific antibiotic resistance genes, integrase genes *int1* and *int2*, and gene cassettes within class 1 and 2 integrons (47). The presence of the genes *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *bla<sub>OXA-1</sub>* like, *cat*, *cmlA*, *floR*, *sul1*, *sul2*, *sul3*, *strA*, *int1*, *int2*, variable regions of class 1 and class 2 integrons, *dhfr12*, *dhfr17*, *aadA1*, *aadA2*, *aadA5*, *estX*, and *sat1* and *sat2* were tested with primers and under conditions as described elsewhere (47). All resistant *E. coli* strains were identified by the API 10S test (bioMérieux, France).

**Selective isolation of ESBL and quinolone-resistant isolates.** All samples from BPW were subsequently enriched in MacConkey broth and subcultivated on MacConkey agar (MCA) containing cefotaxime (2 mg liter<sup>-1</sup>) to detect *E. coli* strains with ESBL (84) and subsequently on MCA supplemented with ciprofloxacin (0.05 mg liter<sup>-1</sup>) to isolate fluoroquinolone-resistant *E. coli*. Samples of waterbird feces from BPW were enriched in MacConkey broth and also subcultivated on MCA containing nalidixic acid (20 mg liter<sup>-1</sup>) to detect *E. coli* strains resistant to nalidixic acid and possibly other quinolones.

**PCR for ESBL/quinolone resistance genes.** The colonies grown on MCA with cefotaxime were examined using the double-disk synergy test (DDST) for the production of ESBL (16, 80). The genes responsible for the ESBL phenotype (*bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>CTX-M</sub>*) were identified by PCR as described elsewhere (5, 45, 64). All the PCR products were further analyzed using sequencing (ABI 310 genetic analyzer; Applied Biosystems). The colonies grown on MCA with ciprofloxacin or nalidixic acid were tested for the plasmid-encoded quinolone resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, and *aac(6')-Ib* by PCR as described elsewhere (9, 11, 25, 41, 57, 63, 70, 78, 85), and MICs for nalidixic acid and ciprofloxacin were determined by the agar dilution method in accordance with Clinical and Laboratory Standards Institute standards (16). In all the isolates positive for plasmid-harbored quinolone resistance genes with high MIC levels for nalidixic acid and ciprofloxacin, the *gyrA* and *parC* genes were detected by PCR and analyzed by sequencing. All isolates resistant to ampicillin and/or amoxicillin-clavulanic acid, as determined during nonselective isolations, were tested also by DDST for the production of ESBL. All isolates resistant to nalidixic acid and/or ciprofloxacin, as determined during nonselective isolation, were also tested for the presence of the *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, and *aac(6')-Ib* genes.

**Molecular characterization of ESBL/quinolone-resistant isolates.** Antimicrobial susceptibilities of the ESBL-positive and quinolone-resistant *E. coli* isolates were examined by the disk diffusion method, and the isolates were tested for additional antibiotic resistance genes (see above). Identification of *E. coli* phylogenetic groups and determination of the presence of the *cib* gene for

TABLE 1. Antibiotic-resistant *Escherichia coli* obtained by nonselective isolation and numbers of strains with some resistance patterns from mallards, herring gulls, and waterbird feces

| Antibiotic resistance phenotype <sup>a</sup> | Antibiotic resistance gene(s) <sup>b</sup>                                                                                                        | No. of isolates from: |               |                 |
|----------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|---------------|-----------------|
|                                              |                                                                                                                                                   | Mallards              | Herring gulls | Waterbird feces |
| A                                            | <i>bla</i> <sub>TEM</sub>                                                                                                                         | 3                     |               |                 |
| Gn                                           | NT                                                                                                                                                | 1                     |               |                 |
| Na                                           | ND                                                                                                                                                | 1                     | 1             |                 |
| S                                            | <i>aadA1</i>                                                                                                                                      |                       |               | 1               |
| S                                            | ND                                                                                                                                                | 6                     |               | 1               |
| ANa                                          | <i>bla</i> <sub>TEM</sub>                                                                                                                         |                       |               | 1               |
| GnS                                          | <i>aadA1</i>                                                                                                                                      | 1                     |               |                 |
| GnS                                          | ND                                                                                                                                                | 1                     |               |                 |
| SSu                                          | <i>strA</i> , <i>sul2</i>                                                                                                                         | 1                     |               | 1               |
| ANaS                                         | <i>bla</i> <sub>TEM</sub> , <i>strA</i>                                                                                                           |                       |               | 1               |
| ANaT                                         | <i>bla</i> <sub>TEM</sub> , <i>tetA</i>                                                                                                           |                       | 1             |                 |
| AST                                          | <i>bla</i> <sub>TEM</sub> , <i>strA</i> , <i>tetB</i>                                                                                             | 1                     |               |                 |
| ANaST                                        | <i>bla</i> <sub>TEM</sub> , <i>strA</i> , <i>tetB</i>                                                                                             | 2                     |               |                 |
| AAcCfCipNa                                   | <i>bla</i> <sub>CTX-M-1</sub>                                                                                                                     | 1                     |               |                 |
| ACipNaSxtT                                   | <i>bla</i> <sub>TEM</sub> , <i>tetA</i> , <i>tetB</i> , <i>int1</i> ; I1, 1.5 kb: <i>dhfr1-aadA1</i>                                              |                       | 1             |                 |
| NaSSuSxtT                                    | <i>sul1</i> , <i>tetA</i> , <i>int1</i> ; I1, 2.2 kb: <i>aadA1</i>                                                                                | 1                     |               |                 |
| AAcCfCipNaS                                  | <i>bla</i> <sub>TEM</sub> , <i>aadA1</i>                                                                                                          | 2                     |               |                 |
| ACfSSuSxtT                                   | <i>bla</i> <sub>CTX-M-1</sub> , <i>strA</i> , <i>sul2</i> , <i>aadA1</i> , <i>tetB</i>                                                            | 1                     |               |                 |
| ACCfSSuSxtT                                  | <i>bla</i> <sub>CTX-M-1</sub> , <i>cat</i> , <i>strA</i> , <i>sul1</i> , <i>sul2</i> , <i>tetB</i> , <i>int1</i> ; I1, 2.5 kb: <i>dhfr1-aadA1</i> | 1                     |               |                 |
| Total                                        |                                                                                                                                                   | 23                    | 3             | 5               |

<sup>a</sup> A, ampicillin; Ac, amoxicillin-clavulanic acid; C, chloramphenicol; Cf, cephalothin; Cip, ciprofloxacin; Gn, gentamicin; Na, nalidixic acid; S, streptomycin; Su, sulfonamides; Sxt, sulfamethoxazole-trimethoprim; T, tetracycline.

<sup>b</sup> ND, not determined; NT, not tested; I1, class 1 integron.

colicin production were performed using PCR (15). The isolates were typed by XbaI pulsed-field gel electrophoresis (PFGE) (12). Those samples with no band differences were designated indistinguishable and possibly epidemiologically linked.

**Transferability of antibiotic resistance genes.** Transferability of ESBL and plasmid-encoded quinolone resistance genes was tested by conjugation. Plate-mating experiments were done using plasmid-free, rifampin and nalidixic acid-resistant *E. coli* MT102RN and *Salmonella enterica* serovar Typhimurium SL5325 as recipients (in the case of a donor strain having resistance to quinolone, recipients resistant to rifampin and sodium azide were used) (1).

**Plasmid analysis.** For plasmid analysis, transformants with a single plasmid carrying ESBL or quinolone resistance genes were prepared. Primarily plasmid DNA was extracted using the Qiagen Plasmid Midi kit (Qiagen, Germany). Plasmid DNA was introduced into competent *E. coli* Top10 (Invitrogen) by chemical transformation, followed by selection of transformants on LB agar supplemented with cefotaxime (2 mg liter<sup>-1</sup>) or ciprofloxacin (0.05 mg liter<sup>-1</sup>). The presence of relevant *bla* and quinolone resistance genes in transformants was confirmed by PCR. The size of plasmids with ESBL or quinolone resistance genes from transformants was designated by S1-PFGE. Plasmids were characterized by replicon typing and restriction fragment length polymorphism (RFLP) analysis. Plasmid DNA from transformants was digested with EcoRV and HincII and then subjected to gel electrophoresis in a 1% agarose gel for 5 h at 100 V/cm. Plasmids were replicon typed as previously described (8). The presence of Tn3-like transposons in *qnrS* transformants was tested by PCR (1).

## RESULTS

**Antimicrobial-resistant *E. coli* in mallards, herring gulls, and waterbird feces.** In total, 65 suspected *E. coli* isolates were obtained from 86 samples from mallards. Antibiotic resistance was detected in 23 *E. coli* isolates. The prevalence of mallards with antimicrobial-resistant *E. coli* was 27% (23 resistant *E. coli* isolates/86 mallards tested). Three of the resistant isolates showed resistance to ciprofloxacin (but plasmid-encoded quinolone resistance genes were not detected). Isolates resistant to ampicillin and amoxicillin-clavulanic acid were

tested for ESBL production by DDST, and three of them were found to produce ESBL. Phenotypes and resistance genes, including integrons and gene cassettes, are summarized in Table 1. With selective cultivation on MCA with cefotaxime, one ESBL-producing *E. coli* isolate was detected (upon further analysis, however, this isolate was found to be identical to an isolate obtained by nonselective cultivation from the identical cloacal swab sample) (Table 2). Resistance to ciprofloxacin was found in 25 isolates by selective cultivation, and the *qnrS* gene was detected in four of these (Table 3).

In total, 18 suspected *E. coli* isolates were detected in 27 samples from herring gulls. Three *E. coli* isolates were antibiotic resistant (Table 1). The prevalence of herring gulls with antimicrobial-resistant *E. coli* was 11% (3/27). While one isolate was resistant to ciprofloxacin, plasmid-encoded quinolone resistance genes were not detected. Three ESBL-producing *E. coli* isolates were found by selective isolation with cefotaxime (Table 2). Using selective isolation with ciprofloxacin, 20 *E. coli* isolates were detected; the *qnrS* gene was detected in three of these (Table 3).

From 365 waterbird feces, 58 suspected *E. coli* isolates were obtained by nonselective cultivation. Antibiotic resistance was detected in five *E. coli* isolates (Table 1). The prevalence of waterbird feces with antimicrobial-resistant *E. coli* was 1.4% (5/365). By selective cultivation with cefotaxime, three ESBL-producing *E. coli* isolates were detected (Table 2). Using selective cultivation with ciprofloxacin, 23 *E. coli* isolates were found, and 2 of these were positive for *qnrS*. Another 5 isolates were obtained by selective cultivation on MCA with nalidixic acid; 1 of these isolates was positive for a mutant variant *aac(6')-Ib-cr*, and moreover, ESBL production was detected in this isolate (Table 2).

TABLE 2. Characterization of ESBL-producing *Escherichia coli* isolates from wild birds on the Baltic Sea coast of Poland

| Isolate no.        | Origin          | Antibiotic resistance phenotype <sup>a</sup> | ESBL <i>bla</i> genes          | Antibiotic resistance genes                                                                                                      | XbaI PFGE <sup>c</sup> | Presence of colicin (the gene <i>ctxB</i> ) | Phylogenetic group | Conjugation to <i>E. coli</i> | Conjugation to <i>Salmonella</i> | Plasmid incompatibility group <sup>d</sup> | RFLP pattern (EcoRV) | Plasmid size (kb) | Additional genes on ESBL-carrying plasmid                |
|--------------------|-----------------|----------------------------------------------|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------|------------------------|---------------------------------------------|--------------------|-------------------------------|----------------------------------|--------------------------------------------|----------------------|-------------------|----------------------------------------------------------|
| 1 PL               | Mallard         | AAC(2)IbNa                                   | <i>bla</i> <sub>CTX-M-1</sub>  | <i>strA</i> , <i>aadA1</i> , <i>su12</i> , <i>tetB</i>                                                                           | a                      | —                                           | A                  | +                             | +                                | —                                          | I                    | 35                |                                                          |
| 5 PL               | Mallard         | ACC(2)SSuXT                                  | <i>bla</i> <sub>CTX-M-1</sub>  | <i>cat</i> , <i>strA</i> , <i>su11</i> , <i>su12</i> , <i>tetB</i> , <i>int1</i> , 11, 4, 2.5 kb, <i>dhfr17-aadA1</i>            | b                      | —                                           | B1                 | +                             | +                                | —                                          | II                   | 90                | <i>cat</i> , <i>su12</i>                                 |
| 78 PL <sup>e</sup> | Mallard         | ACC(2)SSuXT                                  | <i>bla</i> <sub>CTX-M-1</sub>  | <i>cat</i> , <i>strA</i> , <i>su11</i> , <i>su12</i> , <i>tetB</i> , <i>int1</i> , 11, 4, 2.5 kb, <i>dhfr17-aadA1</i>            | c                      | —                                           | B2                 | —                             | —                                | —                                          | III                  | 80                | <i>int1</i> , 11, 2.0 kb, <i>dhfr12-aadA2</i>            |
| 17 LA CEF          | Herring gull    | ACISu                                        | <i>bla</i> <sub>CTX-M-1</sub>  | <i>su12</i>                                                                                                                      | e                      | +                                           | A                  | +                             | +                                | +                                          | IncI1                | 95                |                                                          |
| 11 LA CEF          | Herring gull    | ACISuSxt                                     | <i>bla</i> <sub>CTX-M-1</sub>  | <i>su11</i> , <i>int1</i> , 11, 2.0 kb, <i>dhfr12-aadA2</i>                                                                      | d                      | —                                           | B2                 | +                             | +                                | +                                          | ND                   | 80                |                                                          |
| 6 LA CEF           | Herring gull    | ACISuSxt                                     | <i>bla</i> <sub>CTX-M-9</sub>  | <i>bla</i> <sub>TEM-1b</sub> , <i>strA</i> , <i>aadA1</i> , <i>su12</i> , <i>tetD</i>                                            | ND                     | —                                           | D                  | —                             | —                                | —                                          | IncI1                | 95                |                                                          |
| 88                 | Waterbird feces | ACf                                          | <i>bla</i> <sub>SHV-12</sub>   | <i>int1</i> , 11, 2.1 kb, <i>aadA2</i>                                                                                           | g                      | —                                           | A                  | +                             | +                                | +                                          | IV                   | 95                |                                                          |
| 96                 | Waterbird feces | ACINaSuSxt                                   | <i>bla</i> <sub>CTX-M-1</sub>  | <i>bla</i> <sub>TEM-1b</sub> , <i>dhfr17</i> , <i>aadA5</i> , <i>su12</i> , <i>tetA</i> , <i>int1</i>                            | h                      | —                                           | B1                 | +                             | +                                | +                                          | V                    | 100               | <i>su12</i> , <i>int1</i> , <i>dhfr17</i> , <i>aadA5</i> |
| 8N <sup>b</sup>    | Waterbird feces | ACICpNaSuSxt                                 | <i>bla</i> <sub>CTX-M-15</sub> | <i>bla</i> <sub>CTX-M-15</sub> , <i>aac(6)-Ib-cr</i> , <i>su11</i> , <i>tetA</i> , <i>int1</i> , 11, 1.7 kb, <i>dhfr17-aadA5</i> | M                      | —                                           | B2                 | +                             | —                                | —                                          |                      |                   |                                                          |

<sup>a</sup> Two identical isolates (designed by PFGE) obtained from one bird using nonselective isolation and selective isolation on MacConkey agar with cefotaxime (2 mg/liter).

<sup>b</sup> The strain was isolated by selective cultivation on MCA with nalidixic acid (20 mg/liter).

<sup>c</sup> A, ampicillin; Ac, amoxicillin-clavulanic acid; C, chloramphenicol; Cf, cephalothin; Cip, ciprofloxacin; Gn, gentamicin; Na, nalidixic acid; S, streptomycin; Su, sulfonamides; Sxt, sulfamethoxazole-trimethoprim; T, tetracycline.

<sup>d</sup> Class 1 integron.

<sup>e</sup> ND, not detected.

**Characterization of ESBL-producing *E. coli* isolates.** Nine ESBL-producing *E. coli* isolates were used for further characterization (see Table 2). The most prevalent gene in these ESBL-producing isolates was *bla*<sub>CTX-M-1</sub> (6 isolates). The gene *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-15</sub>, or *bla*<sub>SHV-12</sub> was detected in the remaining 3 isolates. In the isolate with *bla*<sub>CTX-M-15</sub>, the gene *aac(6)-Ib-cr*, responsible for resistance to aminoglycosides and fluoroquinolones, was detected. Most of the isolates were multiresistant, and four isolates contained a class 1 integron with gene cassettes. All the isolates showed unique XbaI-PFGE profiles. Most of the plasmids containing the *bla* genes for ESBL production were transferable by conjugation to *E. coli* and/or *Salmonella*. Transformants or transconjugants with single plasmids carrying the *bla*<sub>CTX-M-1</sub> gene were obtained from 5 isolates. Plasmid DNA purified from these transformants was used for replicon typing and RFLP performed by EcoRV (Fig. 2) and HincII digestions. The strain 5PL, from mallards, harbored the 35-kb conjugative plasmid (RFLP type I) of the IncN group. The gene *bla*<sub>CTX-M-1</sub>, in another 3 isolates from a herring gull (isolate 17 LA CEF) and waterbird feces (isolates 88 and 96), was located on the 90- to 100-kb conjugative plasmids of the IncI1 group. RFLP profiles of the IncI1 plasmids showed many band similarities. A plasmid 80 kb in size from the *E. coli* 11 LA CEF transformant was negative for the incompatibility group by the method used and showed the unique RFLP profile.

**Characterization of quinolone-resistant *E. coli* isolates.** Nine quinolone-resistant isolates with the *qnrS* genes were characterized (Table 3). Four isolates with high MIC levels of quinolones were examined for chromosomal mutations in the genes *gyrA* and *parC*. Mutation of Ser83 to Leu in GyrA and 80Ile to Ser in ParC were the most prevalent. A class 1 integron 1 kb in size was identified in one multiresistant isolate from a herring gull. All the isolates showed unique XbaI PFGE profiles and belonged to the A (6 isolates) or B1 (3 isolates) phylogenetic group as determined by multiplex PCR. Most of the *qnrS* plasmids were transferable by conjugation to *E. coli* and/or *Salmonella*. Transformants or transconjugants with a single *qnrS* plasmid were obtained only from 4 isolates and were used for further testing of the Inc groups and EcoRV (Fig. 2) and HincII plasmid DNA profiling. The isolates 18 PL CIP from a mallard and 6 LA CIP from a herring gull harbored the same 45-kb conjugative plasmid (RFLP type VI) of the X1 incompatibility group. The gene *qnrS* was associated with the Tn3-like transposon on this IncX1 plasmid together with *bla*<sub>TEM-1</sub> in both the isolates. A plasmid 30 kb in size from a transformant of *E. coli* strain 11 belonged to the IncX2 group and showed the unique RFLP profile. The *qnrS* gene in the isolate 58 PL CIP from a mallard was located on a 45-kb conjugative IncN plasmid. The RFLP profile of this plasmid differed from the profiles of other IncN plasmids harboring *bla*<sub>CTX-M-1</sub> genes detected in this study.

## DISCUSSION

Even if populations of wild birds are not directly influenced by antibiotic practice, the acquired resistance in bacteria isolated from wild waterbirds occurs in various prevalences. Studies of resistant *E. coli* isolates have been mostly based on phenotypic characterization of these isolates (20, 24, 26, 51, 58,



TABLE 3. Characterization of *Escherichia coli* with *qnr* genes from wild birds on the Baltic Sea coast of Poland

| Isolate no. | Origin          | Antibiotic resistance phenotype <sup>a</sup> | Antibiotic resistance gene(s) <sup>b</sup>                                                                           | <i>qnr</i> gene | MIC (g/liter) of: |      | Mutation in <i>gyrA</i> and <i>parC</i>          | XbaI PFGE | Presence of colicin ( <i>cib</i> gene) | Phylogenetic group | Conjugation to <i>E. coli</i> | Conjugation to <i>Salmonella</i> | Plasmid incompatibility group | RFLP pattern | Plasmid size (kb) | Additional antibiotic resistance gene(s) on <i>qnr</i> plasmid |
|-------------|-----------------|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-----------------|-------------------|------|--------------------------------------------------|-----------|----------------------------------------|--------------------|-------------------------------|----------------------------------|-------------------------------|--------------|-------------------|----------------------------------------------------------------|
|             |                 |                                              |                                                                                                                      |                 | NAL               | CIP  |                                                  |           |                                        |                    |                               |                                  |                               |              |                   |                                                                |
| 71 PL CIP   | Mallard         | AGn                                          | <i>bla</i> <sub>TEM</sub>                                                                                            | <i>qnrS</i>     | 8                 | 0.25 |                                                  | D         | +                                      | A                  | +                             | +                                |                               |              |                   |                                                                |
| 67 PL CIP   | Mallard         | AAcCf                                        | <i>bla</i> <sub>TEM</sub>                                                                                            | <i>qnrS</i>     | 8                 | 0.25 |                                                  | C         | +                                      | A                  | +                             | +                                |                               |              |                   |                                                                |
| 18 PL CIP   | Mallard         | ACipNaT                                      | <i>bla</i> <sub>TEM</sub> , <i>tetA</i> , <i>int1</i>                                                                | <i>qnrS</i>     | >256              | 8    | <i>gyrA</i> : 83Ser→Leu                          | A         | +                                      | A                  | +                             | +                                | IncX1                         | VI           | 45                | <i>bla</i> <sub>TEM-1</sub>                                    |
| 58 PL CIP   | Mallard         | ACipNaSSuSxtT                                | <i>bla</i> <sub>TEM</sub> , <i>strA</i> , <i>aadA1</i> , <i>sul2</i> , <i>tetA</i> , <i>int1</i>                     | <i>qnrS</i>     | >256              | >8   | <i>gyrA</i> : 83Ser→Leu, 87Asp→Asn               | B         | +                                      | B1                 | +                             | +                                | IncN                          | VIII         | 45                | <i>bla</i> <sub>TEM-1</sub> , <i>sul2</i> , <i>tetA</i>        |
| 19 LA CIP   | Herring gull    | A                                            | <i>bla</i> <sub>TEM</sub>                                                                                            | <i>qnrS</i>     | 8                 | 0.25 |                                                  | G         | +                                      | A                  | +                             | +                                |                               |              |                   |                                                                |
| 6 LA CIP    | Herring gull    | ANaT                                         | <i>bla</i> <sub>TEM</sub> , <i>tetB</i>                                                                              | <i>qnrS</i>     | 256               | 1    | <i>gyrA</i> : 83Ser→Leu, <i>parC</i> : 80Ile→Ser | F         | +                                      | B1                 | +                             | +                                | IncX1                         | VI           | 45                | <i>bla</i> <sub>TEM-1</sub>                                    |
| 1 LA CIP    | Herring gull    | ANaSSuT                                      | <i>bla</i> <sub>TEM</sub> , <i>sul1</i> , <i>sul2</i> , <i>tetA</i> , <i>int1</i> , <i>II</i> , 1.0 kb, <i>aadA1</i> | <i>qnrS</i>     | 256               | 1    | <i>gyrA</i> : 83Ser→Leu, <i>parC</i> : 80Ile→Ser | E         | +                                      | A                  | +                             | +                                |                               |              |                   |                                                                |
| 11          | Waterbird feces | T                                            | <i>tetA</i>                                                                                                          | <i>qnrS</i>     | 16                | 0.25 |                                                  | I         | +                                      | B1                 | +                             | +                                | IncX2                         | VII          | 30                | <i>tetA</i>                                                    |
| 1           | Waterbird feces | AST                                          | <i>bla</i> <sub>TEM</sub> , <i>strA</i> , <i>tetA</i>                                                                | <i>qnrS</i>     | 16                | 0.25 |                                                  | H         | +                                      | A                  | +                             | +                                |                               |              |                   |                                                                |

<sup>a</sup> A, ampicillin; Ac, amoxicillin-clavulanic acid; Cf, cephalothin; Cip, ciprofloxacin; Gn, gentamicin; Na, nalidixic acid; S, streptomycin; Su, sulfonamides; Sxt, sulfamethoxazole-trimethoprim; T, tetracycline. Determined by the disk diffusion method.  
<sup>b</sup> II, class 1 integron.

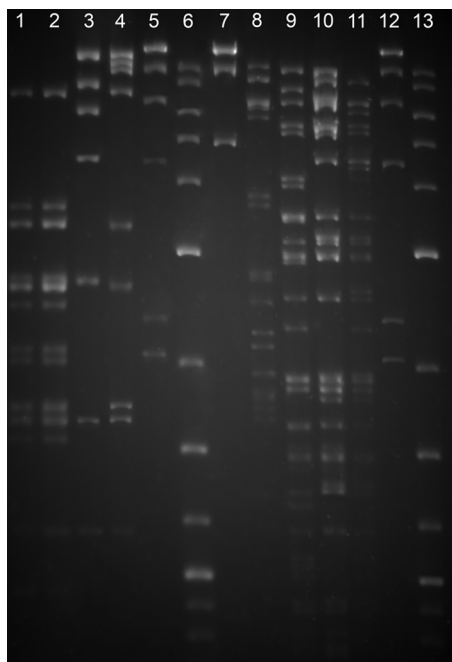


FIG. 2. RFLP analysis of plasmid DNA harboring genes for ESBL or quinolone resistance designed by EcoRV. Lane 1, *E. coli* 18 PL CIP (*qnrS*); lane 2, *E. coli* 6 LA CIP (*qnrS*); lane 3, *E. coli* 11 (*qnrS*); lane 4, *E. coli* 58 PL CIP (*qnrS*); lanes 5 and 12,  $\lambda$  DNA-HindIII Digest (Biolabs, New England); lanes 6 and 13, 2-log DNA marker (Biolabs); lane 7, *E. coli* 5 PL (*bla*<sub>CTX-M-1</sub>); lane 8, *E. coli* 11 LA CEF (*bla*<sub>CTX-M-1</sub>); lane 9, *E. coli* 17 LA CEF (*bla*<sub>CTX-M-1</sub>); lane 10, *E. coli* 88 (*bla*<sub>SHV-12</sub>); lane 11, *E. coli* 96 (*bla*<sub>CTX-M-1</sub>).

71, 75, 76, 82), and the genes determining resistance to old-generation antibiotics were sporadically detected (17, 21, 22, 29). Isolates of *E. coli* from birds on the Polish coast of the Baltic Sea were commonly resistant to old-generation antibiotics, and they were highly variable in phenotype and genotype characteristics. Resistances to streptomycin, ampicillin, and tetracycline were recently found in waterbirds elsewhere in Europe (4, 21, 23, 29). In the United States, resistance to streptomycin and ampicillin has been frequently seen in *E. coli* isolates from Canada geese (17, 51).

Isolates of *E. coli* with class 1 integrons and gene cassettes were found in waterbirds on the Polish coasts of the Baltic Sea. The gene cassettes contained the genes *aadA1*, *aadA2*, *dhfr1-aadA1*, *dhfr12-aadA2*, and *dhfr17-aadA5*. In Portugal, *E. coli* strains with class 1 and 2 integrons have been isolated from gulls (66). In Italy, *E. coli* isolates with class 1 integrons have been found in Mediterranean herring gulls (29). In the Czech Republic, *E. coli* isolates with class 1 and 2 integrons have been found in black-headed gulls (21, 23). We may summarize that the integrons are commonly found in *E. coli* isolates from waterbirds living in European areas inhabited by people and that the gene cassettes detected within these integrons are highly variable even though the genes *aadA1* and *dhfr1* are found regularly.

The emergence and wide dissemination in recent years of *E. coli* resistant to broad-spectrum cephalosporins due to production of ESBL and/or *E. coli* resistant to fluoroquinolones due to a presence of plasmid-encoded quinolone resistance genes

are of great concern and represent a serious problem for the treatment of infectious diseases (59, 79). These strains are also spread into the environment and can colonize wild mammals as well as wild birds, especially omnivorous gulls (4, 18, 23, 29, 47, 66, 67). We have proven the occurrence of *E. coli* isolates producing ESBL, as well as isolates with plasmid-encoded fluoroquinolone resistance, in mallards, herring gulls, and waterbird feces on the Polish coast of the Baltic Sea. Moreover, most isolates showed resistance to multiple antibiotics and contained class 1 integrons. Coresistance to non-beta-lactam antibiotics in the ESBL-producing *Enterobacteriaceae* is commonly described (60). Such multiresistant ESBL-producing strains are a serious problem since they severely limit therapeutic options against human and animal infections (69).

Isolates of *E. coli* producing ESBL found in mallards, in herring gulls, and in waterbird feces had the genes *bla*<sub>CTX-M-1</sub> (most isolates), *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-15</sub>, and *bla*<sub>SHV-12</sub>. The extended-spectrum beta-lactamase CTX-M-1 seems to be the most prevalent ESBL in animal *E. coli* strains throughout Europe (6, 30, 77). The various ESBLs have been found in *E. coli* from wild waterbirds on the Atlantic coast of Europe (66), on the coast of the Mediterranean Sea (4), and in the European inland (23). The spectrum of genes associated with the production of ESBL in Polish isolates was closer to the spectrum of genes in isolates from birds originating from the Mediterranean Sea and the European inland than to the spectrum of genes found in *E. coli* isolates from birds originating on the European Atlantic coast. Both gulls and mallards occurring in European areas inhabited by people are commonly colonized with *E. coli* strains resistant to broad-spectrum cephalosporins and having various *bla* genes responsible for the ESBL phenotype.

In the present study, *bla*<sub>CTX-M-1</sub> was found harbored on conjugative plasmids of the incompatibility groups IncN and IncI1. The association between *bla*<sub>CTX-M-1</sub> and IncN plasmids was recently described (49, 52, 55). IncN plasmids are broad-host-range plasmids that might contribute to the further spread of isolates producing CTX-M-1-like enzymes among other members of the *Enterobacteriaceae*. The *bla*<sub>SHV-12</sub> gene in the *E. coli* isolate from a waterbird was harbored on an IncN conjugative plasmid. The association of SHV-12 and plasmids of this incompatibility group has been previously described (28, 49). In addition to CTX-M-1 and SHV-12, other types of ESBL have been found to be connected with IncN plasmids (28, 33). This indicates their role in disseminating ESBL genes. The IncI1 plasmids have been found to be more frequent in pathogenic than in commensal avian and human *E. coli* strains (35). These plasmids are also characterized by association with virulence and antibiotic resistance genes that can be involved in positive selection of these plasmids (35). Plasmids belonging to IncI1 carrying *bla*<sub>CTX-M-1</sub> have recently been found in *E. coli* and *Salmonella* isolates throughout Europe (19, 28, 30, 47, 49).

The beta-lactamase CTX-M-15 is one of the most important ESBLs in human medicine. *E. coli* isolates producing this enzyme have emerged worldwide as an important cause of community-onset urinary tract and bloodstream infections (65). CTX-M-15 is often associated with OXA-1, TEM-1 beta-lactamases, and Aac(6')-Ib-cr, a variant of an aminoglycoside-modifying enzyme that is responsible for reduced susceptibility to both aminoglycosides and fluoroquinolones (70). Similar

findings were seen in the waterbird isolate from our study. This ESBL has been recently identified in the clone ST131, which is responsible in large measure for an international epidemic caused by CTX-M-15-producing *E. coli* (72). The pandemic dissemination of the clone ST131 producing CTX-M-15 was linked to the epidemic's narrow host range of IncFII plasmids (49, 62).

We found *E. coli* isolates with plasmid-encoded quinolone resistance associated with the *qnrS* gene in mallards, herring gulls, and waterbird feces on the Polish coast of the Baltic Sea. Moreover, one *E. coli* isolate from waterbird feces producing ESBL harbored the gene *aac(6)-Ib-cr*. All these findings of *E. coli* with genes responsible for fluoroquinolone resistance in wild birds are described here for the first time. Only recently, *E. coli* strains determined to be resistant to ciprofloxacin based on their resistance phenotype have been noted in waterbirds (23, 29, 76). The *qnrS* gene was first found in *Shigella flexneri* during 2003 in Japan (32), and together with the *qnrB* gene it has been identified as the most prevalent plasmid-mediated fluoroquinolone resistance gene (42). This gene has been detected in quinolone-resistant human, poultry, and swine *E. coli* and *Salmonella* isolates (10, 31, 34, 86).

In our study, *qnrS* genes were carried by plasmids of the N, X1, and X2 incompatibility groups. The association of IncN conjugative plasmids with *qnrS* genes was recently described (28, 37). IncX plasmids have been implicated in the acquisition and dissemination of transferable resistance in pathogenic bacteria (81).

IncX1 plasmids have recently been found to be associated with ESBL genes, such as *bla*<sub>TEM-52</sub> (1, 47), and with increased production of biofilm (54, 56), which could impart to the strains carrying them a greater potential for spreading and surviving in the environment. In our study, two *E. coli* isolates with identical conjugative IncX1 plasmids harboring the *qnrS* and *bla*<sub>TEM-1</sub> genes inside the Tn3-like transposon, from a mallard and a herring gull sampled in different areas of the Polish coast (the distance between the areas is about 20 km), were found. Both isolates showed high levels of resistance to quinolones and contained chromosomal mutations in the *gyrA* and/or *parC* gene. However, the isolates showed different macrorestriction profiles by PFGE and belonged to different phylogenetic groups, A and B1. This suggests a possible role for horizontal spreading of the IncX1 plasmid in the *E. coli* population of wild birds on the Polish coast. The association of plasmid-mediated *qnrS* genes with plasmids of this incompatibility group was recently described (13). The *qnrS1* gene has been found to be located on a 45-kb nonconjugative IncX1 plasmid in one poultry *E. coli* isolate from Italy. The *qnrS1* gene of this isolate was associated with a Tn3-like transposon containing the *bla*<sub>TEM-1</sub> gene, as previously described for an animal isolate of *Salmonella enterica* serovar Infantis (39, 40). Analysis of the IncX1 plasmid carrying this element showed it to resemble that of a plasmid identified in *Salmonella* Dublin (14). Those authors have suggested that genetic exchange among *Salmonella* and *E. coli* has occurred, thus indicating the existence of a potential animal reservoir for the *qnr* genes. Further investigation of our IncX1 plasmids harboring *qnrS* and *bla*<sub>TEM-1</sub> in *E. coli* from wild birds needs to be undertaken to find a possible association with the results presented by the studies mentioned above.

In one waterbird *E. coli* isolate, the *qnrS* gene was found to be located on a 30-kb conjugative plasmid of the IncX2 group. To our knowledge, this is the first report of *qnrS* genes on plasmids of the X2 incompatibility group. We think that our IncX2 plasmid harboring the *qnrS* gene could be related to the pR6K plasmid (36). Plasmids of this group are not very common, and the pR6K plasmid is the only IncX2 member described to date (36).

Mallards and gulls are identified as important bioindicators, as reservoirs and vectors of *E. coli* strains resistant to old-generation antibiotics, broad-spectrum cephalosporins, and fluoroquinolones, and as a potential melting pot for the development of new resistance types. We regard the common occurrence of these isolates as alarming. It provides evidence that these *E. coli* isolates, selected for in humans and domestic animals, are colonizing wild waterbirds and that via these birds, *E. coli* isolates important from a public health viewpoint can be spread into the environment. Although waterbirds do not naturally come into contact with antimicrobials, they are omnivorous and often search for food in agricultural, rural, and urban areas and in communal garbage dumps and sewage water treatment plants. Thus, they can easily be infected with resistant bacteria from domestic animal and human sources. Urban waters, too, can possibly be a source of antibiotic-resistant isolates inasmuch as these waters harbor higher percentages of resistant *E. coli* strains than do rural waters (38).

Waterbirds excrete large quantities of fecal coliforms, and they are theoretically capable, through their feces, of affecting drinking and recreational water quality (27, 43, 44). It is important to keep these birds away from swimming areas by limiting food supplies. Further, it is important to educate owners of beaches and restaurants, as well as the general public. Keeping beaches clean and prohibiting the feeding of birds are simple ways of limiting potential problems. The occurrence of resistant bacteria in populations of wild waterbirds where there is no selective pressure can imply that such resistance will be difficult to displace.

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