Comparison of ESBL – And AmpC Producing Enterobacteriaceae and Methicillin-Resistant Staphylococcus aureus (MRSA) Isolated from Migratory and Resident Population of Rooks (Corvus frugilegus) in Austria

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Abstract

In order to test whether rooks (Corvus frugilegus) represent good indicators for the potential circulation of antibiotics in their native habitat, two populations with different migratory behavior were tested for the presence of beta-lactamase producing Enterobacteriaceae and methicillin-resistant Staphylococcus aureus (MRSA). In all, 54 and 102 samples of fresh feces of a migratory and a resident population were investigated. A total of 24 and 3 cefotaxime-resistant enterobacterial isolates were obtained from the migratory and resident population, respectively. In these isolates CTX-M-1 (n = 15), CTX-M-3 (n = 3), and CTX-M-15 (n = 3) genes were detected. TEM-1 and OXA-1 were associated with CTX-M in 3 and 2 isolates, respectively. In two E. coli isolates CMY-2 could be detected, where from one isolate displayed an overexpression of chromosomal AmpC as well. Among E. coli isolates the most common phylogenetic group was A (n = 11) and ST1683 (n = 5). In one E. coli of B2-ST131 the rfbO25b locus was detected. Three Enterobacter isolates were stably derepressed AmpC-producers. In five samples of the migratory population, PVL positive MRSA could be isolated. Two isolates were typed SCCmec IVa, spa type t127, and ST1. Three isolates carried a SCCmec type IVc, with spa type t852 and ST22. The highly significant difference of the occurrence of antibiotic resistance between the migratory population from eastern Europe compared to resident population in our study indicates that rooks may be good indicator species for the evaluation of environmental contamination with antibiotic resistant bacteria, especially due to their ecology, foraging behavior and differing migratory behavior.

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Introduction

Antibiotic resistance in bacteria of animal origin and its zoonotic potential has elucidated considerable attention worldwide. Microorganisms responsible for increased resistance are often those from the group of gram-negative rods, mostly beta (β)-lactamase producing members of the family Enterobacteriaceae. Emergence and dissemination of resistance among Enterobacteriaceae represent a serious threat to public health [1]. Therefore, numerous studies involving these bacteria in humans and domestic animals have been published. However, there is still a scarcity of information on the presence of resistant Enterobacteriaceae isolates in wildlife. Studies on the presence of Escherichia coli (E. coli), especially extended-spectrum beta-lactamase producing E. coli (ESBL) are performed to detect antibiotic resistance in populations of wild animals [2]. E. coli is a common inhabitant of the gastrointestinal tract of birds and mammals. It is also found in the environment and can therefore be used as indicator of antimicrobial resistance change [3–4]. To our knowledge there are no reports addressing isolation and characterization of beta-lactamase producing Enterobacteriaceae from wildlife in Austria. Nevertheless, in the last years a few sources regarding wildlife resistant enterobacterial isolates from neighboring countries are reported. Beta-lactamase producing E. coli isolates were found in various mammalian and avian hosts in central Europe [5–12]. Studies addressing the presence of beta-lactamase producing members of Enterobacteriaceae other than E. coli in wildlife are scarce [13]. Only few studies report the occurrence of another multi-drug resistant bacterial species: methicillin resistant Staphylococcus aureus (MRSA) in wildlife [14–15]. MRSA, first emerged in 1960s, is currently the most common multidrug-
resistant bacteria causing nosocomial infections in Europe [16]. In
the last years the transfer of MRSA isolates between animals and
humans gained specific attention, especially in the case of MRSA
multi locus sequence type (ST) 398 belonging to the clonal
complex CC398, which has been the most commonly reported
MRSA strain found in association with livestock (livestock-
associated [LA]-MRSA) [17]. While studies on MRSA in humans,
companion animals and livestock have been widely documented
the role of this particular pathogen in wildlife is rarely studied. The
emergence of MRSA in wild animals could represent a serious
threat to human health, companion animals and livestock. Corvids
and gulls feed on garbage in urbanized areas and are considered to
be important reservoirs and vectors of resistant isolates in the
environment [18]. Due to its wide distribution and gregarious
lifestyle the rook (Corvus frugilegus) is considered a good indicator for
the potential circulation of antibiotics in their habitat. The biggest
coherent population, comprising 5 to 10 million breeding pairs, is
located in Russia [19]. Wintering grounds or resident populations
range from the Czech Republic to France, mainly in the vicinity of
cities. The objective of the study was to evaluate the occurrence of
a broad-spectrum-β-lactam resistance determinants among Enterobacteriaceae and b) MRSA isolated from feces of rooks (Corvus frugilegus) collected from a small resident Austrian population in Burgenland as well as an eastern European migratory population with wintering grounds in the city of Vienna.

Methods

Ethics Statement

No specific permission was required to non-invasively collect fresh fecal samples from the ground on public land. While the rook is protected in the EU birds directive (79/409/EWG) and in the respective national legislation in Austria this study did not involve a protected species. This field study did not involve any contact or disturbance of actual birds as fresh fecal samples were collected from the ground and tree leaves in public areas used by rooks on a daily basis. No permissions are required for this type of activity.

No animals were used, disturbed or otherwise impacted by this study and therefore no Institutional Animal Care and Use Committee (IACUC) permission was required.

Bacterial isolates. In March 2013, 54 (1 k – 54 k) samples of fresh feces of a migratory population of rooks in the Lobau (40° 48’ 10.59’’N; 16° 30’ 15.03’’E) and 102 (55 k – 106 k) samples of fresh feces of a resident, non-migratory population in Wulkaprodersdorf (47° 47’ 18.30’’N; 16° 29’ 20.68’’E) were collected in sterile tubes and transported directly to the laboratory. Each fecal sample was suspended in 1.5 ml of 0.9% sterile saline. For isolation of Enterobacteriaceae, 200 μl of suspended feces was precultured at 37°C overnight in buffered peptone water (BPW) (Merck, Germany) supplemented with cefotaxime (1 mg/L) and then cultivated at 37°C overnight on MacConkey agar (MCA) (Oxoid, Basingstoke, United Kingdom) supplemented with cefotaxime (1 mg/L), which select for broad-spectrum-cephalosporin-resistant isolates. For MRSA isolation, another 200 μl suspended feces was incubated at 37°C overnight in Trypticase soy broth (TSB) (Becton Dickinson, Heidelberg, Germany) with 6.5% NaCl and then streaked in a BBLTM CHROMagarTM MRSA II (Becton Dickinson, Heidelberg, Germany) and on Columbia CNA Agar with 5% Sheep Blood, Improved II (Becton Dickinson, Heidelberg, Germany) and incubated for at 35°C for 24 h.

After incubation on MCA one colony representing each a distinct colonial morphotype was regrown on the same plate and on Müller Hinton Agar II (Becton Dickinson, Heidelberg, Germany). For detection of members of Enterobacteriaceae standard bacteriological techniques were used. Isolates that were oxidase negative produced acid from glucose by oxidation as well as by fermentation were screened for ESBL production by combination disk tests using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson, Heidelberg, Germany) according to Clinical and Laboratory Standards Institute (CLSI) [20]. Furthermore, a cefoxitin disk (30 mg, Becton Dickinson, Heidelberg, Germany) was added to this test, to detect AmpC phenotypes. All isolates classified as intermediate or resistant using CLSI criteria (≥17 mm) to cefoxitin were suspected to be AmpC. ESBL as well as AmpC phenotype were confirmed by MASTDISCS™ ID AmpC and ESBL test (Mast Diagnostics, Merseyside, UK) and were subjected to further characterization. After incubation on BBL™ CHROMagar™ MRSA II the colonies showing typical colony appearance of MRSA were tested on production of β-lactamase using BBL™ DrySlide™ Nitrocefin (Becton Dickinson, Heidelberg, Germany).

Identification of enterobacterial isolates

E. coli-like colonial morphotypes were tested using species specific PCR [21]. For species characterization of other enterobacterial isolates differ in colonial morphology, 16S rDNA sequence analysis was performed [22]. The isolate was identified using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/). All obtained sequences of have been deposited in the GenBank.

Antimicrobial-susceptibility testing of enterobacterial isolates was performed by the agar disk diffusion method according to CLSI for the following antibiotics: ampicillin, piperacillin, aztreonam, doripenem, ertapenem, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, fosfomycin (all from Becton Dickinson, Heidelberg, Germany). Additionally, a minimal inhibitory concentration (MIC) was performed by Etest (bioMérieux, Marcy l’Etoile, France) for cefoxitin (ApampC-hyperproducing isolates (stably derepressed only) and ceftazidime, and M.I.C. Evaluation test (Thermo Fisher, Basingstoke, UK) for ciprofloxacin, gentamicin, meropenem and ceftozaxime. Escherichia coli ATCC 25922 was used as quality control strains for MIC tests. AmpC-hyperproducing isolates (stably derepressed) of Enterobacter sp. were defined as those with a ceftazidime and ceftazidime MIC of ≥32 mg/L, without ESBL production and a negative cefoxitin-ceftazidime disk antagonist test [23]. MRSA susceptibility testing were performed as described elsewhere [22].

Determination of genotypic resistance in enterobacterial isolates

Preliminary screening for the presence of blaOXA, blatem, blashv, blactXM genes in the ESBL-positive isolates was performed by multiplex-PCRs as described earlier [25–26]. The gene that responded positively in the preliminary screening were further analysed by PCR and sequencing using primers as described elsewhere [27–28]. All isolates displaying AmpC phenotypes were tested to detect plasmid mediated AmpC β-lactamases (pAmpC) by multiplex PCR [29] which resulted in a positive amplion corresponding to enzymes from the CIT group, which were further analyzed by PCR using CMY-F und CMY-R and sequenced as described elsewhere [27]. The sequences obtained were compared to those registered in the GenBank and compared with previously reported sequences for β-lactamases (http://www.lahey.org/studies/) - accessed August 2013). The E. coli isolates displaying AmpC phenotype were also tested for mutations in the chromosomal ampC promoter/attenuator region.
as described previously [30]. The presence of the genes non-β-lactamase genes tetA, tetB, tetC, tetD, tetE, tetG, cat, chlor, fluR, sul1, sul2, sul3, strA, int1, int2, variable regions of class 1 and class 2 integrons, and gene cassettes dfrA1, dfrA2, dfrA17, adaA1, adaA2, adaA3, estX, and sat1 and sat2 were tested with primers and under conditions as described elsewhere [31]. Isolates resistant to ciprofloxacin were tested for the plasmid-mediated quinolone resistance genes, qepA, qepB, qepC, qepD, qepS, and aac(6′)-Ib-cr by PCR [32–36].

Analysis of genetic environment of blaCTX-M

The genetic environment of blaCTX-M genes, such as upstream regions IS26 and IS26 was investigated by PCR and sequencing as previously described [31]. The IS26 region was searched using IS26 5′ primer binding in the 5′ region of IS26 and using IS26 UP primer binding to the transposase gene hpa of IS26 in the immediate blaCTX-M upstream region. The presence of IS26 linked to blaCTX-M was tested using hpa IS26 primer recognizing the transposase gene of the IS26 element.

Conjugations experiments

The transferability of bla genes was carried out by broth mating using a sodium azide-resistant E. coli J55 and sodium azide- and rifampicin resistant E. coli MT 102 as recipients. The strains were grown to the exponential phase and then mixed (1:2), and the donor and recipient mixture was incubated in Müller-Hinton (MH) broth overnight at 37°C. Transconjugants were selected on MH agar supplemented with sodium azide (200 mg/L), cefotaxime (1 mg/L), and MH supplemented with sodium azide (150 mg/L), rifampicin (50 mg/L), and cefotaxime (1 mg/L), respectively. Selected transconjugants were further characterized for their antimicrobial susceptibility, presence of the relevant bla genes, and co-transfer of other resistance genes as described above for the wild isolates.

Plasmid replicon typing

Plasmid DNAs were isolated using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Vienna, Austria) according to the manufacturer’s instructions. The presence of replicons was tested by PCR-based method as described previously [37].

Molecular typing methods

The phylogenetic group of each E. coli isolate was determined by recently described revised Clermont method [38]. Multilocus sequence typing (MLST) of E. coli isolates was performed as described previously [3]. Allelic profile and sequence type (ST) determinations were carried out according to the E. coli MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli. Accessed 2013 September 15.). In case of E. coli ST131, allele-specific PCR was performed to identify the O25 clone of E. coli [39]. Clonal diversity of enterobacterial isolates was assessed by enterobacterial repetitive intergenic consensus (ERIC)-PCR as described elsewhere [40].

MRSA characterization

MRSA isolates were confirmed by mecA PCR and further investigated by the PCR [41] targeting Panton-Valentine leukocidin (PVL) genes, staphylococcal cassette chromosome mec (SCCmec) typing using multiplex PCR methods with subsequent subtyping, staphylococcal Protein A (spa) typing, and multiplex locus variable-number tandem repeat analyses (MLVA) using multiplex-PCR [41]. Detection of antibiotic resistance genes [42–43] and detection of pyrogenic exotoxin genes were as described earlier [44]. MLST was carried out by PCR amplification and sequencing of seven housekeeping genes (arcC, aroE, gfpF, gnk,pta, tpi, yqiL) as previously described [45]. The allelic profiles and sequence types (ST) were assigned by the MLST web site (http://saureus.mlst.net/). Accessed 2013 September 15.

Statistical analysis

All statistical analyses were conducted using the statistical software R [46]. To test for differences of the frequencies of antibiotic resistances found in the Lobau population (lob) and the Wulkaprodasdorf population (wul) a poisson model (R function glm) was used (20 resistances). Since there was a clear difference in the frequencies between both populations, subsequently logistic regressions were conducted to post-hoc test every single antibiotic-resistance (156 samples each).

Results

Isolation of cefotaxime resistant isolates, phenotypic resistance in Enterobacteriaceae and species identification

Out of 34 samples, a total of 24 cefotaxime-resistant Enterobacteriaceae isolates were obtained from the migratory population, and 3 cefotaxime-resistant enterobacterial isolates were obtained from the resident population. 22 isolates displayed ESBL phenotype, and 5 isolates displayed AmpC phenotype, wherefrom three Enterobacter isolates were stably derepressed AmpC-producer. Apart from resistance to ampicillin and piperacillin antibiotics, resistance to tetracycline (85% of the isolates), trimethoprim-sulfamethoxazole (62%) and chloramphenicol (48%) was the most prevalent among isolates obtained from migratory population (Table 1). Three cefotaxime-resistant isolates retrieved from resident population showed phenotypic resistance to ampicillin and piperacillin (58 k, 69 k and 100 k) and tobramycin (isolate 58 k). All of enterobacterial isolates remained susceptible to carbapenems. The MICs of cefotaxime for all isolates ranged from 4 to >256 mg/L, the MIC of ceftazidime from 0.25 to 128 mg/L, the MIC for meropenem from 0.008 to 0.12 mg/L, the MIC for ciprofloxacin from 0.008 to >32 mg/L, and for gentamicin 0.5 and 64 mg/L (Table 1). Using species specific primers 19 isolates from migratory population as well as three from resident population were confirmed as E. coli. The highest 16S rDNA gene sequence similarity observed for the isolate 4.2 k (KF022262) was 99.65% with the type strain of Klebsiella pneumoniae subsp. azarum ATCC 11296T (Y17654). Isolates isolate 24 k (KF022261) (99.5%) and 44.1 k (KF022259) (99.1%) showed highest similarity to E. coli. Enterobacteriaceae and species identification, staphylococcal Protein A (spa) subtyping, staphylococcal cassette chromosome MecA (mecA) typing using multiplex PCR methods with subsequent subtyping, staphylococcal Protein A (spa) typing, and multiplex locus variable-number tandem repeat analyses (MLVA) using multiplex-PCR [41]. Detection of antibiotic resistance genes [42–43] and detection of pyrogenic exotoxin genes were as described earlier [44]. MLST was carried out by PCR amplification and sequencing of seven housekeeping genes (arcC, aroE, gfpF, gnk,pta, tpi, yqiL) as previously described [45]. The allelic profiles and sequence types (ST) were assigned by the MLST web site (http://saureus.mlst.net/). Accessed 2013 September 15.

Characterization of genotypic resistance in Enterobacteriaceae

The most prevalent genes in ESBL-producing isolates were those from the blaCTX-M group. In all but one isolate from both rook populations blaCTX-M was detected. The genes blaCTXM-1, blaCTXM-3, or blaCTXM-15 were detected solely in 15, 2, and 1 isolates, respectively. The gene blaCTXM-1 was detected in combination with blaTEM-1 in 2 isolates, blaCTXM-15 was detected in combination with blaOXA-1 in one isolate from the resident and in one from the migratory population. The gene blaCTXM-3 was detected in a Citrobacter isolate as well as in a Klebsiella isolate in combination with blaTEM-1. blaTEM-15 was detected in one E. coli
| Isolate | Species | blu gene | PG | ST | ISEcp1 IS26 | Replicon | Integrorn | Non-Î±-lactamase resistance pattern | Non-Î±-lactamase resistance genes | MIC (mg/L) |
|---------|---------|----------|----|----|-------------|----------|----------|----------------------------------|---------------------------------|-----------|
| 2 k    | E. coli | CTX-M-1  | B1 | ST162 | 1,7 | 0,25 | 0,6 | N(I)-ly+ | - | SXT(A), TET | tet(A)(+), sul1(+), sul2(-) | 16 1,008 0,015 0,5 n.a. |
| 7 k    | E. coli | CTX-M-1  | A  | ST744 | 0,25 | 0,6 | N(+), N(I)-ly+ | - | SXT(A), TET | tet(B)(+), cat(+), sul2(+), strA(+) | 32 0,008 8 0,5 n.a. |
| 8 k    | E. coli | TEM-15   | A  | ST23  | n.a. | n.a. | n.a. | F(+), FIB(+), N(I)-ly+ | Ila | TET(-) | sul2 | 4 3 0,008 0,008 0,5 n.a. |
| 19 k   | E. coli | TEM-1,   | B1 | ST58  | 0,25 | 0,6 | F(+), FIB(+), N(I)-ly+ | - | CIP, SXT, TET | sul2(+), strA(-) | 4 3 0,008 0,008 0,5 n.a. |
| 23,2 k | E. coli | TEM-1,   | B1 | ST58  | 0,25 | 0,6 | F(+), FIB(+), N(I)-ly+ | P | CIP, SXT, TET | sul2(+), strA(-) | 16 1 0,008 4 0,5 n.a. |
| 25 k   | E. coli | CTX-M-1  | A  | ST34  | 0,25 | 0,6 | F(+), FIB(+), N(+), N(I)-ly+ | P | CIP(+), SXT(+), TET | sul2(+), strA(-) | 64 0,75 0,008 0,008 32 n.a. |
| 28 k   | E. coli | CTX-M-1  | A  | ST1683 | 0,25 | 0,6 | F(+) | Ila | CHL(+), GEN(+), SXT(+), TET(+), TET(-) | tet(A), tet(B), cat(+), sul1(+), sul2(-), strA(-) | 8 0,25 0,008 0,008 64 n.a. |
| 31,2 k | E. coli | CTX-M-1  | A  | ST1683 | 0,25 | 0,6 | F(+) | Ila | CHL(+), GEN(+), SXT(+), TET(-) | tet(B)(+), cat(+), sul2(-), strA(-) | 16 1,5 0,008 0,015 64 n.a. |
| 32 k   | E. coli | CTX-M-15 | B2 | ST131 | 0,2 | 0,9 | F(+), FIA(+), FIB(+) | Ila | CIP(+), SXT(+), TET(+), TET(-) | sul2(+), strA(-) | >256 6 0,015 >32 1 n.a. |
| 33 k   | E. coli | CMY-2    | E  | NT    | n.a. | n.a. | NT | - | - | - | 8 8 0,015 0,015 0,5 n.a. |
| 38 k   | E. coli | OXA-1,   | A  | ST90  | 0,2 | 0,9 | F(+), FIA(+), FIB(+) | Ila | CIP(+), SXT(+), TET(+), TET(-) | tet(A)(+), sul1(+) | 64 12 0,008 >32 1 n.a. |
| 39 k   | E. coli | OXA-1,   | A  | ST90  | 0,2 | 0,9 | F(+), FIA(+), FIB(+) | Ila | CIP(+), SXT(+), TET(+), TET(-) | tet(A)(+), sul1(+) | 64 0,75 0,008 0,008 32 n.a. |
| 43,3 k | E. coli | CTX-M-1  | A  | ST1683 | 0,25 | 0,6 | F(+) | Ila | CHL(+), GEN(+), SXT(+), TET(-) | tet(A), tet(B), cat(+), sul1(+), sul2(-), strA(-) | 8 0,38 0,008 0,008 32 n.a. |
| 45,1 k | E. coli | CTX-M-1  | A  | ST1683 | 0,25 | 0,6 | F(+) | Ila | CHL(+), GEN(+), SXT(+), TET(-) | tet(A), tet(B), cat(+), sul1(+), sul2(-), strA(-) | 16 0,25 0,008 0,015 64 n.a. |
| 46,2 k | E. coli | CTX-M-3  | A  | ST744 | - | - | - | F(+) | AMK, CIP(+) | tet(B)(+), sul2, strA(-) | 8 1 0,008 8 0,5 n.a. |
| 47,1 k | E. coli | CTX-M-1  | C  | ST744 | 0,25 | 0,6 | N(+), N(I)-ly+ | - | CIP, TET | tet(B)(+), cat(+), sul2, strA(-) | 16 2 0,008 16 0,5 n.a. |
| 47,2 k | E. coli | CMY-2    | B1 | ST224 | n.a. | n.a. | n.a. | FIA, 11-ly+ | CHL, CIP, SXT, TET | tet(A), slor, sul2, strA(-) | 8 24 0,008 >32 1 n.a. |
| 48,1 k | E. coli | CTX-M-1  | B1 | ST162 | 0,25 | 0,6 | N(T) | NT | TET | tet(A) | 32 1 0,015 0,25 1 n.a. |
| 53 k   | E. coli | CTX-M-1  | A  | ST1683 | 0,25 | 0,6 | H11 | Ila | CHL(+), GEN(+), SXT(+), TET(-) | tet(A)(+), tet(B)(+), cat(+), sul1(+), sul2(-), strA(-) | 16 0,25 0,008 0,015 64 n.a. |
| 58 k   | E. coli | OXA-1,   | B2 | ST491 | 0,2 | 0,9 | F(+), FIA(+), FIB(+) | Ila | CIP, SXT, TOT | sul1 | 64 12 0,008 0,12 0,5 n.a. |
| 69 k   | E. coli | CTX-M-1  | D  | ST69  | 1,7 | 0,25 | - | F, FIB, 11-ly+ | - | - | - | 32 1 0,015 0,015 1 n.a. |
| 100 k  | E. coli | CTX-M-1  | D  | ST69  | 1,7 | 0,25 | - | F, FIB, 11-ly+ | - | - | - | 32 1 0,015 0,015 1 n.a. |
| Isolate | Species | $bla$ gene | PG | ST | ISEcp1 | ISEcp1 $UP$ | tnpA | IS26 | Non-$\beta$-lactam resistance pattern | Non-$\beta$-lactam resistance genes | MIC (mg/L)$^h$ |
|---------|---------|------------|----|----|---------|------------|------|------|---------------------------------|---------------------------------|-----------------|
| 4.2 k   | Klebsiella sp. | TEM-1, CTX-M-3 | n.a. | n.a. | 0.3 | - | FIA5 | - | CHL, TET | - | 32, 1.5, 0.008, 0.5, 0.5, n.a. |
| 22.1 k  | Citrobacter sp. | CTX-M-3 | n.a. | n.a. | 1.7 | 0.3 | NT | - | - | - | 16, 0.75, 0.06, 0.015, 0.5, n.a. |
| 11.2 k  | Enterobacter sp. | dAmpC | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | - | - | >256, 96, 0.12, 0.015, 0.5, >256 |
| 24 k    | Enterobacter sp. | dAmpC | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | - | - | >256, 128, 0.12, 0.03, 0.5, >256 |
| 44.1 k  | Enterobacter sp. | dAmpC | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | - | - | >256, 96, 0.12, 0.015, 0.5, >256 |

$^a$Phylogenetic group.
$^b$Sequence type.
$^c$ISEcp1 5', nexus between the 5' region of ISEcp1 and $bla_{CTX-M}$; ISEcp1 UP, nexus between ISEcp1 and $bla_{CTX-M}$ using forward primer for transposase gene of ISEcp1 and reverse primer for $bla_{CTX-M}$; tnpA IS26, nexus between IS26 and $bla_{CTX-M}$.
$^d$In parenthesis (+) means positive after testing of transconjugants.
$^e$I1a, class 1 integron 1.7 kb; dfr17-aadA5; I1b, class 1 integron 1.7 kb; dfr17; I1c2, class 1 integron 1.75 kb; none of cassettes could be detected.
$^f$In parenthesis (+) means positive after testing of transconjugants. Abbreviations: AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; FO, fosfomycin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TOB, tobramycin.
$^g$In parenthesis (+) means positive after testing of transconjugants.
$^h$Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; MEM, meropenem; CIP, ciprofloxacin; GEN, gentamicin; FOX, cefoxitin.

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resistant. E. coli MT 102 was demonstrated for all bla positive E. coli isolates. The bla<sub>CTX-M</sub> of the Citrobacter isolate and bla<sub>CTX-M-3</sub>/bla<sub>TEM-1</sub> of the Klebsiella isolate did not transfer. Resistance to non-β-lactamase genes was also cotransferred in some cases (Table 1). The replicon typing revealed 13 different replicon types. Replicon type F was the most common replicon among the isolates.

**Genetic environment of bla<sub>CTX-M</sub>**

PCRs applied different primer combination identified the insertion sequence IS<sub>Ep</sub>1 in its entirety or partially truncated in 20 isolates (Table 1). PCR using primer IS<sub>Ep</sub>1 <sup>5′</sup> indicated a ~1.7 kb amplicon of the entire IS<sub>Ep</sub>1 in two isolates (2 k and 100 k), 16 isolates contained the transposase gene of the insertion sequence IS26 as analyzed by PCR. As confirmed with sequencing all these isolates showed the presence of IS26 disrupted by IS<sub>Ep</sub>1. All CTX-M-1-positive isolates had a 48 bp region (W sequence) upstream of the bla<sub>CTX-M-1</sub> gene and an additional 32 bp X sequence, as observed in a previous sequence reported in the GenBank database (accession no. AM003904). The CTX-M-15-positive isolates had only the 48 bp W sequence, as previously described (AM040707).

**Enterobacterial molecular typing methods**

Eleven E. coli isolates belonged to phylogenetic group A, 4 to B1, 2 to B2, 1 to C, 2 to D and one to E. E. coli MLST typing identified 11 different types among the tested isolates (Table 1). Among E. coli isolates, the most commonly identified genotypes were ST1683 (n = 5), ST744 (n = 3), ST34 (n = 2), ST58 (n = 2), ST69 (n = 2), and ST162 (n = 2), whereas isolates belonging to the ST23, ST90, and ST131 genotypes were also identified. The CMY-2 isolate belonged to ST224. One CMY-2 isolate could not be assigned. Allele-specific PCR confirmed the presence of the <i>efbO25b</i> locus in E. coli of ST131 (phylogenetic group B2) and, showing that it belonged to the O25b type. ERIC-PCR analysis was used to analyze the molecular epidemiology of all enterobacterial isolates and showed that two ST162 (B1) isolates (2 k and 48 k), two ST1683 (A) (45 k and 53 k), two ST34 (A) (25 k and 39 k), two ST36 (B1) (19 k and 23 k), two ST69 (D) isolates from resident population (69 k and 100 k) as well as two derepressed AmpC Enterobacteriaceae sp. were clonally related due to identical ERIC fingerprint (data not shown).

**MRSA characterization**

In five samples of the migratory population, MRSA could be isolated, whereas all fecal samples of the resident population were isolate. In one E. coli (47.2 k) isolate displaying AmpC phenotypic resistance the combination of plasmidic AmpC (CMY-2) and overexpression of chromosomal AmpC was detected. Mutations in the promoter and attenuator region of the chromosomal ampC gene from E. coli were found at positions -18 (G→A), -1 (C→T), and +58 (C→T) of the ampC gene of E. coli K12. In one E. coli (33 k) isolate CMY-2 was solely detected. The non-β-lactamase genes strA, strB, strC, and tetB were solely detected. The non-β-lactamase genes

**Conjugations experiments and plasmid analysis**

The transfer of the ESBL and pAmpC producing isolates to sodium azide-resistant E. coli J53 and sodium azide- and rifampicin resistant E. coli J53 and sodium azide- and rifampicin resistant E. coli MT 102 was demonstrated for all bla positive E. coli isolates. The bla<sub>CTX-M</sub> of the Citrobacter isolate and bla<sub>CTX-M-3</sub>/bla<sub>TEM-1</sub> of the Klebsiella isolate did not transfer. Resistance to non-β-lactamase genes was also cotransferred in some cases (Table 1). The replicon typing revealed 13 different replicon types. Replicon type F was the most common replicon among the isolates.

**Table 2. Characteristics of Methicillin-resistant Staphylococcus aureus (MRSA) isolated from rooks.**

| Isolate | PVL<sup>a</sup> | SCC<sub>mec</sub> type | spa type | ST<sup>b</sup> | FOX, OX | MIC (mg/L)<sup>c</sup> | Resistance phenotype<sup>d</sup> | Resistance genotype<sup>d</sup> | Exotoxin genes |
|---------|----------------|------------------------|----------|----------|----------|----------------|-----------------|----------------|-----------------|
| 3msak   | +              | IVa                    | t127     | ST1      | 48, >256 | β-lactams, TET, CIP | tet(K)          | aacA-aphD, msr(A) | seh             |
| 5msak   | +              | IVa                    | t127     | ST1      | 48, >256 | β-lactams, TET, CIP | tet(K)          | aacA-aphD, msr(A) | seh             |
| 39msak  | +              | IVc                    | t852     | ST22     | 24, 32   | β-lactams, GEN, CIP | aacA-aphD, msr(A) | sed             |
| 41msak  | +              | IVc                    | t852     | ST22     | 24, 32   | β-lactams, GEN, CIP | aacA-aphD, msr(A) | sed             |
| 53msak  | +              | IVc                    | t852     | ST22     | 24, 32   | β-lactams, GEN, CIP | aacA-aphD, msr(A) | sed             |

<sup>a</sup>Panton-Valentine leukocidin.

<sup>b</sup>Sequence type.

<sup>c</sup>OX, oxacillin; FOX, cefoxitin.

<sup>d</sup>Antibiotics tested: penicillin, oxacillin, cefoxitin, cefovecin, cefquinome, tetracycline (TET), ciprofloxacin (CIP), gentamicin (GEN), chloramphenicol, erythromycin, clindamycin, teicoplanin, trimethoprim/sulfamethoxazole, linezolid, rifampicin and mupirocin.

<sup>d</sup>Table 2. Characteristics of Methicillin-resistant Staphylococcus aureus (MRSA) isolated from rooks.

**Figure 1. Proportion of antibiotic resistance in the two rook populations.** The percentage of antibiotic resistance rates is significantly (p<0.001) higher in the migrating (lob) population then in the resident (wul) population

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Differences in enterobacterial antibiotic resistance between the two rook populations

A total of 44.4% of the fecal samples (24 of 54) collected in the migrating population showed at least resistance to one of the tested antibiotics compared to 2.9% (3 of 102) in the resident population. As shown in Figure 1 the proportion of antibiotic resistances showed a significant difference (p<0.001) in the migrating (lob) population than in the resident (wul) population. Posthoc tests for each antibiotic showed significant differences for ampicillin, piperacillin, ceftazidime, cefotaxime, aztreonam and trimethoprim-sulfamethoxazole (ns indicates a non significant difference).

The number of resistant individuals per group respectively the percentage of resistant individuals per study site (in brackets) differs significantly between in the two populations for ampicillin, piperacillin, ceftazidime, cefotaxime, aztreonam and trimethoprim-sulfamethoxazole (ns indicates a non significant difference).

Dicussion

In the present study, the phenotypic and genotypic characteristics of β-lactamase producing Enterobacteriaceae as well as methicillin resistant *Staphylococcus aureus* isolates from two different rooks populations were investigated. The isolation rates (44.4%) of cefotaxime resistant enterobacterial isolates was significantly higher in migratory population (2.9%) than in the resident population.

Genotypic characterization of the isolates revealed that the majority of ESBL-*E. coli* harbored *blaCTX-M-1* genes and belonged to six different STs: ST34 (phylogenetic group A), ST58 (B1), ST69 (D), ST162 (B1), ST744 (A and C), and ST1683 (A). CTX-M-1, a major ESBL type in livestock in Europe [47] has also been detected in different wildlife avian host in Europe such as different birds of prey from Portugal, seagulls from Portugal and Sweden, black headed gull from Czech Republic and yellow legged gull from France [6]. Very recently the *E. coli* harboring *blaCTX-M-1* and *blaTEM-1* belonged to ST744 (A) were isolated from different birds of prey (*Milvus migrans*, *M. m. melius* and *Buteo buto*) in Germany [52]. In our study two *E. coli* CTX-M-1 clonally related isolates also harbored *blaTE(M-1)* and belonged to ST58. ST58 isolates harboring the same genes were also isolated from seagulls in Portugal [53]. Two CTX-M-1 *E. coli* isolates retrieved from the resident rook’s population belonged to the phylogenetic group D and ST69. This is especially interesting because previous studies have reported the association of *E. coli* isolates of group D with extra-intestinal infections in humans [54]. ST69 has been also identified among CTX positive *E. coli* isolates from seagulls [53]. Three isolates, one *E. coli* and two isolates, identified based on their 16S rDNA similarity as a members of genera *Citrobacter* and *Klebsiella* from migratory population carried *blaCTX-M-3* genes. The *Klebsiella* sp. isolate harbored also the *blaTEM-1* gene. To the authors knowledge, there are no reports describing isolation of *E. coli, Citrobacter* sp. and/or *Klebsiella* sp. harboring CTX-M-3 type alone or in combinations with other β-lactamas from rooks. Two *E. coli* (32 k and 38 k) isolates from the migratory as well as one
Furthermore, two isolates. The presence of pAmpC Enterobacter coli aceae PLOS ONE | www.plosone.org 8 December 2013 | Volume 8 | Issue 12 | e84048 and companion animals [47] [63]. The information on the majority of non-bacterial Commander Islands in Russia [61]. Association of IS norvegicus harbouring the CTX-M-9 type in a feral urban brown rat (B2-O25b-ST131 has also been reflected in wildlife. This clone was in infections, bacteraemia, and sepsis [58–59]. Pandemic spread of pAmpC Enterobacteriaceae and MRSA from Rooks (58 k) from the resident population harbored the blaCTX-M-15 gene, which is the dominant ESBL gene in strains isolated from humans and is rarely found in animals [55–57]. CTX-M-15 isolates from this study belonged to ST90 (A), ST491 (B2) and ST131 (B2), wherefrom isolates 38 k and 38 k carried a blaOXA-1 gene as well. CTX-M-15 producing ST90 (A) E. coli variant has been detected previously from yellow legged gulls in the South of France [51], whereas ST491 (B2) has not yet been detected among wildlife. In one fecal sample of the migratory population, the currently spreading pandemic CTX-M-15 producing E. coli clone B2-O25b-ST131 population was isolated. The B2-O25b-ST131 clone presents an anthropogenic zoonotic pathogen [58] with high extraintestinal virulence and is described to cause urinary infections, bacteraemia, and sepsis [58–59]. Pandemic spread of B2-O25b-ST131 has also been reflected in wildlife. This clone was isolated not only from wildlife in urban areas, as detected in E. coli harbouring the CTX-M-9 type in a feral urban brown rat (Rattus norvegicus) [60], but also in Glaucous-winged gull on the remote Commander Islands in Russia [61]. Association of IS26 and blactx-m-15 in isolate 32 k has been observed previously in the B2-O25b-ST131 clone [31] [62]. One cefotaxime-resistant E. coli isolate (3 k) harboring the blactx-m-15 gene belonged to ST23 (A). This type of E. coli has not been detected yet in rooks.

During this study three kind of AmpC-producing Enterobacteriaceae were isolated, plasmid mediated AmpC (pAmpC) genes in E. coli isolates, combination of pAmpC and overexpression of chromosomal AmpC, and derepressed AmpC in three Enterobacter sp. isolates. The presence of pAmpC β-lactamases have been found worldwide but are less common than ESBLs [63]. Furthermore two E. coli isolates harbored pAmpC. Among the AmpC β-lactamases, blactx-M-2 is by far the most common worldwide, which was detected in isolates from humans, livestock and companion animals [47] [63]. The information on the presence of AmpC producing Enterobacteriaceae in wildlife is scarce. Recently, E. coli harboring CMY-2 belonged to different phylogenetic groups and STs were isolated from wild coastline birds in Miami Beach, Florida, USA [13]. Among others, one E. coli isolate belonged to B1-ST224. E. coli isolate 47.2 k analyzed in this study belonged also to the same phylogenetic group and shared the same ST with those from USA. Based on the results obtained, three Enterobacter isolates most closely related to type strain of Enterobacter hormaechaei, were detected as a derepressed AmpC mutants. Derepressed production of AmpC β-lactamase has been documented to be a predominant mechanism of extended-spectrum cephalosporin resistance in clinical isolates of Enterobacter spp. [64–66]. To the authors’ knowledge there are no current reports describing the isolation of stably derepressed AmpC-producing Enterobacter isolates from wildlife. In the present study, for all E. coli isolates, the bla genes could be transferred by conjugation to both E. coli recipient strains. Additionally, the majority of non-β-lactamase genes were co-transferred. Our results indicate that the horizontal dissemination of bla genes is due to multiple plasmids, because the majority of replicon types were detected both on conjugative plasmids as well as in the wild isolates. Association of ISEsp1 and blactx-M-15 was observed in all but one blactx-M isolates. The presence of ISEsp1 element upstream of blactx-M in E. coli isolates originated from wildlife has been observed previously [12]. Genotyping of the enterobacterial isolates by ERIC-PCR showed that some are genetically homogeneous. These isolates have all other characteristics in common (bla genes, in case of E. coli belonged to the same phylogenetic group and ST type, replicon combination, resistance pattern) indicating that resistances to antibiotics spread, not only due to dissemination of different β-lactamases, but also due to clonal transmission. This is probable, as they originated from rooks sharing the same habitat.

Together with the isolation of pandemic CTX-M-15-producing E. coli clone B2-O25b-ST131, the isolation of MRSA isolates, even in a low prevalence (9.4% in migratory population and none in the resident), is an important finding as there are only few reports regarding this issue. In 2012, three MRSA isolates from lesser yellowlegs (Tringa flavipes) and eastern cottontails (Sylvilagus floridanus) in Central Iowa, USA have been isolated [14]. These isolates were spa typed as t002 and t008, whereas from t008 was PVL positive. MRSA was also detected in brown rats (Rattus norvegicus), chaffinch (Fringilla coelebs), common seals (Phoca vitulina) [67], and from a hedgehog [68] but isolates from these wild animals were MRSA carrying the novel mec gene. Recently, our working group also detected mec positive MRSA isolates in wildlife, particularly in European brown hare (Lepus europaeus), European otter (Lutra lutra) and a European hedgehog (Erinaceus europaeus) [22]. Very recently, 12 out of 1342 tested wild animals from Spain (Red deer, Iberian ibex, Wild boar, Eurasian Grifon vulture) were tested MRSA positive. Among them, two isolates belonging to t127, ST1 were recovered from two wild boars [15]. MRSA isolates 3mrsak and 5mrsak MRSA had all characteristics in common, carried SCCmec IVa, belonged to spa type t127 and ST1, were PVL positive. MRSA belonging to SCCmec IVa, t127, ST1 are commonly isolated from humans as well as from animals [69]. From Austria, a MRSA with SCCmec IVa, t127, ST1 has been reported. Three SCCmec IVa, t127, ST1 MRSA isolates originated from a veterinary hospital, (University of Veterinary Medicine, Vienna) and caused infections in horses. Two isolates originated from horse-associated personnel. All five isolates were PVL negative [70]. Unlike reports from other European countries [69] [71] there are no studies describing the isolation of MRSA belonging to t127, ST1 from livestock in Austria. The other three PVL positive MRSA isolated during this study belonged to spa type t852 and ST22 and carried SCCmec type IVc are a variant of epidemic meticillin-resistant Staphylococcus aureus (EMRSA-15) [72]. EMRSA-15 variants occur worldwide and were isolated from humans as well as from animals [73–74]. In Europe, t852, ST 22 PVL-MRSA strains are mainly reported from North-West Europe [72] [75–76]. Interestingly, there are no reports on presence of t852 MRSA in Austria, and no entries in the Ridom spaserver database (http://spaserver.ridom.de/, Accessed 2013 August 26.). To the authors’ knowledge there are no reports on presence of these MRSA variants in rooks.

Based on the significant differences recorded between the migratory and non-migratory populations, it is the authors’ opinion that rooks may be good indicator species for the evaluation of environmental contamination with antibiotic resistant bacteria. Populations originating from North and Central-Russia to the Ural, travel the longest distance by migrating along the Baltic to Western Europe (Ukraine, Poland, Czech Republic, Slovakia over Germany, South- West France until North-England). Rook-flocks wintering in Vienna mostly originate from Russia, Belarus, Poland and the region North-East of the Carpathian mountains and arrive regularly every winter with the first small flocks of rooks usually being observed in the beginning of October followed by a peak in the middle of the month [19]. Insufficient degradation of antibiotics in wastewater treatment plants results in antibiotic residues entering the environment in moderate concentrations, and over time, selection pressure enhances survival of organisms bearing resistance traits [77–78]. The spread of manure containing resistant organisms onto crop fields is another path of how antibiotics or resistant bacteria can enter the environment [52]. The highly significant difference in
the occurrence of antibiotic resistance between the migratory population from eastern Europe compared to the non-migratory, resident population in our study indicates that rooks may provide a sufficiently good quality data for the evaluation of environmental contamination with antibiotic resistant bacteria, especially due to the rooks foraging behavior and different migratory behavior.

In conclusion, our study contributes to the growing evidence that multiresistant members of Enterobacteriaceae as well as MRSA can be found in wildlife. The presence of pandemic isolates in wildlife emphasizes the complexity in the dissemination of antimicrobial drug resistance. Moreover, the present study confirmed that rooks might represent an important vector not only of β-lactamase producing enterobacteria including pandemic zoonotic isolates but also as a vector of MRSA.

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Author Contributions

Conceived and designed the experiments: GLS CW. Performed the experiments: IL KM. Analyzed the data: IL FK FH KM RR. Contributed reagents/materials/analysis tools: IL GLS FH. Wrote the paper: IL GLS FH CW.

References

1. Pitout JD, Laupland KB (2008) Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis 8: 159–166.
2. Gaukler S, Evers C, Wieler LH (2011) Extended-Spectrum Beta-Lactamases Producing Enterobacteriaceae in wildlife, yet Another Form of Environmental Pollution? Front Microbiol doi: 10.3389/fmicb.2011.00829.
3. Wirth T, Falahu D, Lan R, Colles F, Mensa P, et al. (2006) Sex variability in Escherichia coli: an evolutionary perspective. Mol Microbiol 60: 1136–1151.
4. Silva N, Igrejas G, Figueiredo N, Gonçalves A, Radhouani H, et al. (2010) Molecular characterization of antimicrobial resistance in enterococci and Escherichia coli isolated from European wild rat (Rattus norvegicus). Sci Total Environ 408: 4871–4876.
5. Doleželka M, Cízek A, Literák I (2007) High prevalence of antimicrobial-resistant genes and integrons in Escherichia coli isolates from Black-headed Gulls in the Czech Republic. J Appl Microbiol 103: 11–19.
6. Doleželka M, Birsova B, Kohoútoka L, Literák I, Cízek A (2009) Antibiotic-resistant Salmonella and Escherichia coli isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. J Appl Microbiol 10: 1941–1950.
7. Literák I, Vana řek R, Doleželka M, Cízek A, Karpísková R (2007) Antibiotic resistant Escherichia coli and Salmonella in Russian rooks (Corvus frugilegus) wintering in the Czech Republic. Let Appl Microbiol 45: 616–621.
8. Literák I, Doleželka M, Janoušovska D, Hrusáčková J, Meissner W, et al. (2010) 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. Appl Environ Microbiol 76: 8126–8134.
9. Literák I, Doleželka M, Radimský T, Klimes J, Friedman M, et al. (2010) Antimicrobial-resistant fecal Escherichia coli in wild mammals in central Europe: multiresistant Escherichia coli producing extended-spectrum beta-lactamases in wild boar. J Appl Microbiol 108: 1702–1711.
10. Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, et al. (2010) Determination of qnrS, a novel gene encoding a novel plasmid-mediated quinolone resistance gene, found in enterobacteria. J Antimicrob Chemother 67: 1103–1107.
11. Poirel L, Potron A, De La Cuesta C, Cleary T, Nordmann P, et al. (2012) Wild Enterobacteriaceae possess a new AmpC promoter and qnrS gene, isolated from wildlife. J Antimicrob Chemother 68: 2222–2225.
12. Wardyn SE, Kauffman LK, Smith TC (2012) Methicillin-resistant Staphylococcus aureus (MRSA): a prokaryotic 16S rRNA Gene sequence database with phylogenotypes that represent uncultured species. Int J Syst Evol Microbiol 62: 716–721.
13. Guenther S, Grobbel M, Beutlich J, Bethe A, Friedrich ND, et al. (2010) Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J Antimicrob Chemother 65: 490–5.
14. Doleželka M, Frolkova P, Florek M, Janoušková L, Purgertová M, et al. (2011) CTX-M type extended-spectrum beta-lactamases-producing Escherichia coli from wild birds in Germany. Environmental Microbiology Reports 2: 641–643.
15. Tausova D, Doleželka M, Cízek A, Hanusova L, Hrusáčková J, et al. (2012) Escherichia coli with extended-spectrum β-lactamase and plasmid-mediated quinolone resistance genes in great cormorants and mallards in Central Europe. J Antimicrob Chemother 67: 2153–2162.
16. Dierick CM, van Duijkeren E, Schoormans AH, van Essen-Zandbergen A, Veldman K, et al. (2012) Occurrence and characteristics of extended-spectrum β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. J Antimicrob Chemother 67: 1368–1374.
17. Kiratisin P, Apisarnthanarak A, Laserga C, Saifon P (2008) Molecular characterization and epidemiology of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae isolates causing health care-associated infection in Thailand, where the CTX-M family is endemic. Antimicrob Agents Chemother 52: 2818–2824.
18. Perez-Perez FJ, Hanson ND (2002) Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 40: 2158–2163.
19. Carro N, Espave E, Bérard I, Richet H, Reynaud A (1999) Mutations in the ampC promoter of Escherichia coli resistant to oxyiminocephalosporins without extended spectrum beta-lactamase production. FEMS Microbiol Lett 173: 459–465.
20. Doleželka M, Frolkova P, Florek M, Jambarova I, Purgertová M, et al. (2011) CTX-M-15-producing Escherichia coli clone B2-O25b-ST131 and Klebsiella spp. isolates in municipal wastewater treatment plant effluents. J Antimicrob Chemother 67: 2794–2797.
21. Cavaco LM, Hasman H, Xia S, Aarestrup FM (2009) qnrD, a novel gene conferring transferable quinolone resistance in Salmonella enterica serovar Kentucky and Bovismorbiirfs strains of human origin. Antimicrob Agents Chemother 53: 603–608.
22. Gay K, Robicsek A, Strahilevicz J, Park CH, Jacoby G, et al. (2006) Plasmid-mediated quinolone resistance in non-TypH serotypes of Salmonella enterica. Clin Infect Dis 43: 297–304.
23. Wang M, Guo Q, Xu X, Wang X, Ye X, et al. (2009) New plasmid-mediated quinolone resistance gene, qnrC, found in a clinical isolate of Proteus mirabilis. Antimicrob Agents Chemother 53: 1364–1366.
24. Yamanaka N, Wachino J, Suzuki S, Arakawa Y (2008) Plasmid-mediated qepA gene among Escherichia coli clinical isolates from Japan. Antimicrob Agents Chemother 52: 1564–1566.
25. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC (2006) Prevalence in the United States of mecA(-f)-l-ar encoding a ciropodoxin-modifying enzyme. Antimicrob Agents Chemother 50: 3953–3953.
26. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, et al. (2005) Identification of plasmid-borne qnr genes by PCR-based methods. J Microbiol Methods 63: 219–223.
27. Clermont O, Christensen JK, Denamur E, Gordon DM (2013) The Clermont Escherichia coli phyto-type method revised: identification of specificity and detection of new phyto-groups. Environmental Microbiology Reports 5: 38–65.
44. Løvseth A, Loncarevic S, Berdal KG (2004) Modified multiplex PCR method for detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. J Clin Microbiol 41: 4099–4101.

47. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH (2012) Extended-spectrum beta-lactamase-producing and AmpC-producing Enterobacteriaceae and MRSA from Rooks. PLoS ONE 7(10): e48374. doi: 10.1371/journal.pone.0048374.

50. Wallensten A, Hernandez J, Bonnefoi D, Eiasson I, Wallensten A, Comstedt P, et al. (2013) Globally disseminated human pathogenic Escherichia coli O25b-ST131 clone harboring theCTX-M-9 extended-spectrum beta-lactamase type in a feral urban brown rat (Rattus norvegicus). J Antimicrob Chemother 68: 582–584.

59. Rogers BA, Saulsbury WM, Paterson DL. (2011) Interspecies transmission of methicillin-resistant Staphylococcus aureus sequence type 398. J Clin Microbiol 51: 1625–1628.

39. Clermont O, Dhanji H, Upton M, Gibreel T, Fox A, et al. (2009) Rapid detection of the O25b-ST131 clone of Escherichia coli encompassing the CTX-M-15-producing strain. J Antimicrob Chemother 64: 274–277.

52. Nicolaou C, Theodorakis E, Zervoudakis S, Karyotis D, Karamanos K, et al. (2011) Characterization of selected Gram-negative non-fermenting bacteria isolated from honey bees (Apis mellifera carnica). Apidologie 42: 312–25.

53. Løvseth A, Brunnhaler R, Spengler J (2013) Suspected goat to human transmission of meticillin-resistant Staphylococcus aureus sequence type 398. J Clin Microbiol 51: 1625–1628.