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Nucleotide sequences of 16 transmissible plasmids identified in nine multidrug-resistant *Escherichia coli* isolates expressing an ESBL phenotype isolated from food-producing animals and healthy humans

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Objectives: Nine extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from healthy humans and food-producing animals were found to transfer their cefotaxime resistance marker at high frequency in laboratory conjugation experiments. The objective of this study was to completely characterize 16 transmissible plasmids that were detected in these bacterial isolates.

Methods: The nucleotide sequences of all 16 plasmids were determined from transconjugants using next-generation sequencing technology. Open reading frames were assigned using Rapid Annotation using Subsystem Technology and analysed by BLASTn and BLASTp. The standard method was used for plasmid multilocus sequence typing (pMLST) analysis. Plasmid structures were subsequently confirmed by PCR amplification of selected regions.

Results: The complete circularized nucleotide sequence of 14 plasmids was determined, along with that of a further two plasmids that could not be confirmed as closed. These ranged in size from 1.8 to 166.6 kb. Incompatibility groups and pMLSTs identified included IncI1/ST3, IncI1/ST36, IncN/ST1, IncF and IncB/O, and those of the same Inc types presented a similar backbone structure despite being isolated from different sources. Eight plasmids contained $bla_{CTX\text{-}M\text{-}1}$ genes that were associated with either IS*Ecp1* or IS*26* insertion sequence elements. Six plasmids isolated from humans and chickens were identical or closely related to the IncI1 reference plasmid, R64.

Conclusions: These data, based on comparative sequence analysis, highlight the successful spread of bla_{ESBL}-harbouring plasmids of different Inc types among isolates of human and food-producing animal origin and provide further evidence for potential dissemination routes.

Keywords: conjugation, CTX-M, TEM, resistance genes, IncI1

Introduction

The emergence and spread of antimicrobial resistance has become a major global public health concern. A component element of this problem is the spread of the plasmid-encoded extended-spectrum β-lactamase (ESBL) genes, which confer resistance to third-generation cephalosporins.[1] Third-generation cephalosporins are clinically significant antimicrobial compounds, licensed for use in human and veterinary medicine.[2,3] Plasmids expressing an ESBL phenotype often also carry genes encoding resistance to other commonly used antimicrobial drug classes (including aminoglycosides, fluoroquinolones, phenicols, tetracyclines or trimethoprim).[4–6]

The emergence and spread of antimicrobial resistance genes in microorganisms is a complex process and has been mainly driven by insertion sequences, transposons, integrons and plasmids, some of which are homologous in isolates from both food-producing animals and humans.[7,8] Thus investigation of the mobile genetic elements, especially plasmids, is a key component required for a better understanding of the dissemination of ESBL genes and others.

In an earlier study we reported on the characterization of higher generation cephalosporin-resistant *Escherichia coli*, which were isolated from food-producing animals and healthy humans in Switzerland.[6] Nine of these were selected for more detailed study as they were found to transfer their plasmids efficiently.

Sixteen transmissible plasmids were identified in the corresponding transconjugants,[6] and in this follow-up study the DNA sequence of all 16 plasmids was determined and a comparative analysis of these structures carried out.

Materials and methods

Selection of bacterial isolates for study

Nine ESBL-producing *E. coli* isolated from faecal samples of food-producing animals and healthy humans had previously been studied (Table 1).[9,10] Using a broth mating protocol, these isolates were found to transfer their cefotaxime resistance marker at high frequency in laboratory conjugation experiments.[6] Laboratory protocols applied for the maintenance of bacteria, conjugation experiments, purification of plasmid DNA and subsequent downstream methods were previously described in detail.[6]

Plasmid sequencing and annotation

Sixteen plasmids contained in the nine multidrug-resistant (MDR) *E. coli* isolates were sequenced commercially from their transconjugants by next-generation sequencing technology on the Roche 454 GS FLX platform (Eurofins MWG Operon, Ebersberg, Germany). Open reading frames (ORFs) were designated using the Rapid Annotation using Subsystem Technology (RAST) annotation pipeline.[11] Initial sequence analysis was carried out over the web, using the BLASTn and BLASTp programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf) was also used to identify features. Nucleotide sequences showing the highest similarity were identified in GenBank database using the BLAST search tool from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids of the IncI1 and IncN groups were further typed by plasmid multilocus sequence typing (pMLST) and the sequence types (STs) were obtained using the PubMLST database (http://pubmlst.org/plasmid). Plasmid structures were subsequently confirmed by the PCR amplification of selected regions using the primers shown in Table S1 (available as Supplementary data at *JAC* Online).

Nucleotide sequence accession numbers

The nucleotide sequences of all 16 plasmids were deposited in the GenBank database under accession numbers KJ484626–KJ484641.

Results and discussion

Plasmids identified in healthy human MDR E. coli isolates

Nine plasmids from isolates of healthy humans and belonging to different Inc groups were fully sequenced and annotated: pH2332-166 (IncFII and IncFIB), pH2332-107 (IncB/O), pH2291-144 (IncFII and IncFIB), pH2291-112 (IncI1), pH1519-88 (IncI1), pH1519-76 (IncFII), pH1519-7, pH1519-2 and a novel pH1038-142 (IncF and IncN).

Plasmids identified in chicken MDR E. coli isolates

Five conjugative plasmids from MDR *E. coli* cultured from chicken were sequenced and annotated. Four of these (pC60-108, pC59-112, pC49-108 and pC23-89) were determined to be IncI1 group plasmids, with one plasmid, pC59-153, being typed as IncF group. Among these, two plasmids, pC60-108 and pC23-89, were sequenced but not confirmed as closed by PCR (data not shown).

Plasmids identified in an MDR E. coli isolate from a lamb

Two conjugative plasmids from an MDR *E. coli* isolate of a healthy lamb and belonging to different Inc groups, IncB/O (pL2-87) and IncN (pL2-43), were fully sequenced and annotated.

Comparison of plasmid backbones

In summary, plasmids showing the same incompatibility groups shared a common set of backbone modules even when they were isolated from different sources. Two plasmids from human sources, pH2332-166 and pH2291-144 (IncFII-IncFIB type plasmids; Figure 1a) were determined to be 99% identical at the nucleotide level. Similarly, pL2-87 (IncB/O) and pL2-43 (IncN) from the lamb isolate (Table 1) showed a high degree of nucleotide similarity (> 90% identical at the nucleotide level) when compared with the two plasmids from human isolates, pH2332-107 (IncB/O) and pH1038-142(IncF-IncN), respectively (Figure 1b and c). Five IncI1 plasmids containing a $bla_{CTX\text{-}M\text{-}1}$ gene identified in this study were isolated from human and chicken samples, and these were either identical or closely related to the reference IncI1 plasmid R64 (Figure 2). Furthermore, plasmid pC23-89 (IncI1 plasmid harbouring a $bla_{TEM\text{-}52}$ gene) was determined to be 99% similar at the nucleotide level to R64 (Figure 2). A detailed comparative description of all of these plasmids is provided below.

Comparative analysis of pH2332-166 and pH2291-144

The two largest plasmids sequenced, pH2332-166 (accession no. KJ484626) and pH2291-144 (accession no. KJ484628), were identified in MDR human *E. coli* isolates. These plasmids were 166594 and 144925 bp in size with an average G+C content of 50.9% and 50.6%, respectively. They contained 252 and 205 ORFs, respectively, as determined after annotation. Each plasmid also contained two replication systems, denoted as IncFII and IncFIB.

The backbone-containing regions of pH2332-166 and pH2291-144 can be divided into four functional modules consisting of antimicrobial resistance-encoding loci, along with plasmid replication, plasmid transfer and plasmid maintenance functions (Figure 1a). The plasmid transfer region in each case contained a complete *tra*-encoding locus of 32847 bp composed of 24 *tra* genes, 9 *trb* genes, 1 *artA* gene and 1 *finO* gene. These loci were similar to the transfer region of the typical *E. coli* F plasmid (accession no. U01159) and were 99% identical at the nucleotide level.

The *oriT*-encoding gene is 463 bp in size and is located proximal to *traM*. It is highly conserved in both pH2332-166 and pH2291-144 (Figure 1a). In plasmid pH2332-166 several replicons are recognized. The first is a 978 bp region (located between positions 78499 and 79476 bp) and this locus encodes a *repA* gene, synonymous with the IncFIB replicon type; two copies of IncFII (*repA1* and *repA2*, located at positions 11145 through to 12486 bp) mapped downstream of the F plasmid transfer region. These replication regions were completely conserved in pH2291-144 (Figure 1a).

BLASTn comparisons identified two completely novel scaffolds, wherein pH2332-166 was determined to have a 71% coverage and 99% nucleotide identity with pNRG857c (accession no. CP001856), whilst pH2291-144 had a 74% coverage and 99% identity with pTN48 (accession no. FQ482074). Plasmid pNRG857c was a non-β-lactam antimicrobial resistance-containing plasmid that had previously been isolated from a clinical isolate of adherent

Table 1. Summary of the features associated with all 16 sequenced plasmids purified from nine ESBL-producing *E. coli* isolates cultured from healthy human and food-producing animal origin previously reported in Switzerland

Isolate	Origin	Phylo-group	MLST	Plasmid	Accession no.	Plasmid size (bp)	Inc-type/ pMLST	β-Lactamase(s) identified	Other resistance genes	Toxin/antitoxin family
H-2332	Human	D	ST75/ST350 complex	pH2332-166	KJ484626	166594	IncFII-IncFIB	TEM-1	*tetR-tet*(A); *mph*(B); *catA1*; *sul1*; *aadA1b*; *dfrA1*; *sul2*; *strAB*	*phd/doc*; *vapBC*
				pH2332-107	KJ484627	107386	IncB/O	CTX-M-1	—	*relBE*
H-2291	Human	A	ST1638	pH2291-144	KJ484628	144925	IncFII-IncFIB	TEM-1	*tetR-tet*(A); *sul1*; *aadA1b*; *dfrA1*	—
				pH2291-112	KJ484629	112671	IncI1/ST3	CTX-M-1	*aadA5*; *dfrA17*	*phd/doc*
H-1519	Human	A	ST10 complex	pH1519-88	KJ484630	88678	IncI1/ST145	TEM-210; CTX-M-1	—	—
				pH1519-76	KJ484631	76197	IncFII	—	—	*phd/doc*
				pH1519-7	KJ484632	7036	—	—	—	—
				pH1519-2	KJ484633	1822	—	—	—	—
H-1038	Human	B1	New	pH1038-142	KJ484634	142875	IncF-IncN/ST1	TEM-1; CTX-M-1	*tetR-tet*(A); *strAB*; *catA1*; *sul3*; *aadA1*; *dfrA1*	*mazEF*
C-60	Chicken	B1	ST3174 complex	pC60-108	KJ484635	108662	IncI1/ST3	CTX-M-1	*aadA5*; *dfrA17*	*phd/doc*
C-59	Chicken	B1	ST155 complex	pC59-153	KJ484636	153231	IncFIIA-IncFIC-IncFIB	—	*catA1*; *macA*; *macB*	*relBE*; *vapBC*; *ccd*; *phd/doc*; *vagC/D*
				pC59-112	KJ484637	112330	IncI1/ST3	CTX-M-1	*aadA5*; *dfrA17*	*phd/doc*
C-49	Chicken	B1	ST446 complex	pC49-108	KJ484638	108660	IncI1/ST3	CTX-M-1	*aadA5*; *dfrA17*	*phd/doc*
C-23	Chicken	B1	ST1398	pC23-89	KJ484639	89513	IncI1/ST36	TEM-52	—	*vagC/D*
L-2	Lamb	D	ST295	pL2-87	KJ484640	87042	IncB/O	—	—	*phd/doc*
				pL2-43	KJ484641	43265	IncN/ST1	CTX-M-1	—	—

—, feature not identified.

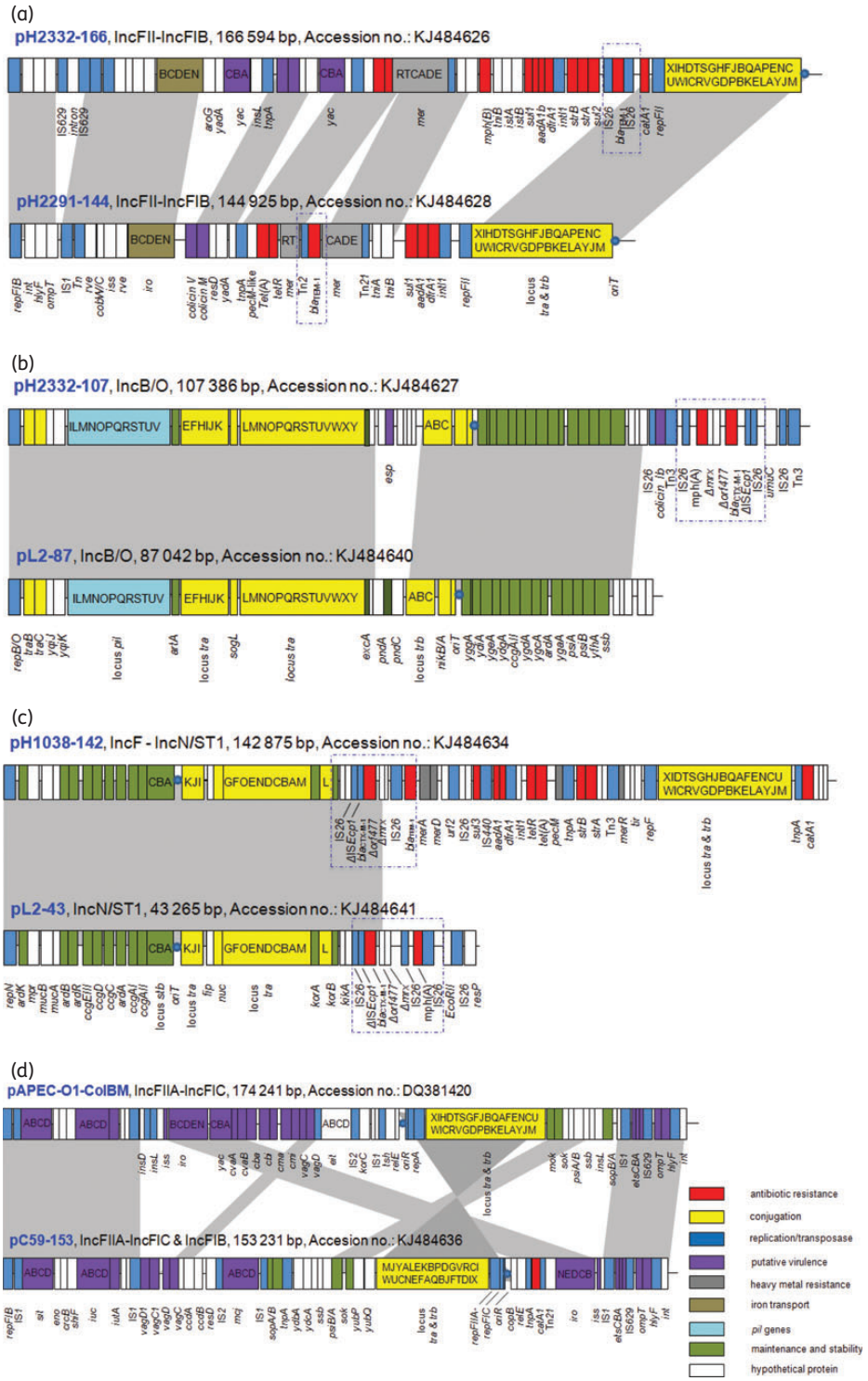

Figure 1. Comparison of major structural features of (a) plasmids pH2332-166 and pH2291-144; (b) plasmids pH2332-107 and pL2-87; (c) plasmids pH1038-142 and pL2-43; and (d) pC59-153 in comparison with plasmid pAPEC-O1-ColBM (accession no. DQ381420). Areas shaded in grey indicate homologies identified across the plasmid scaffold regions. Antibiotic resistance genes are indicated by red boxes. The individual conjugation-related genes (associated with *tra* and *trb*) are indicated with capital letters inside the yellow boxes. Blue boxes denote transposon-, integron- or replication-associated genes. The putative virulence-related genes are indicated by violet boxes. Green boxes indicate maintenance- and stability-related genes. Grey boxes indicate heavy metal resistance-related genes and the brown box shows the iron transport genes. White boxes indicate hypothetical proteins or mobile element proteins. The *pil* genes are indicated in light blue. The origin of transfer *oriR* is indicated by a blue circle. The figure is not drawn to scale. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

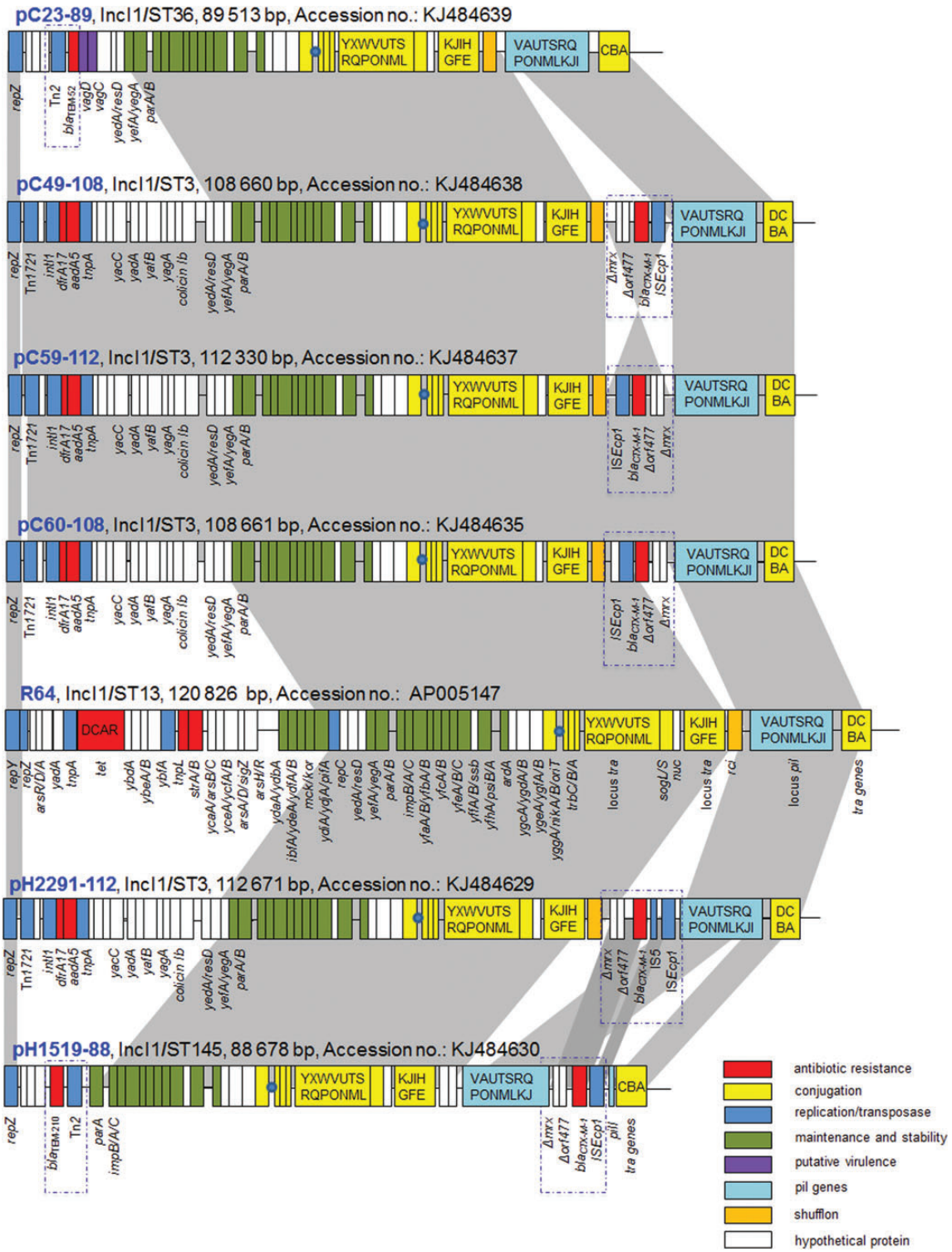

Figure 2. Major structural features of pC23-89, pC49-108, pC59-112, pC60-108, pH2291-112 and pH1519-88 in comparison with the IncI1 reference plasmid R64 (accession no. AP005147). Areas shaded in grey indicate homologies in plasmid scaffold regions. Antibiotic resistance genes are indicated by red boxes. The individual conjugation-related genes (associated with *tra* and *trb*) are indicated with capital letters inside the yellow boxes. Blue boxes denote transposon-, integron- or replication-associated genes. The putative virulence-related genes are indicated by violet boxes. Green boxes indicate maintenance- and stability-related genes and white boxes indicate hypothetical proteins. The origin of transfer *oriT* is indicated by a blue circle. The *pil* genes are indicated by light blue boxes. The figure is not drawn to scale. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and invasive *E. coli*.[12] In contrast, pTN48 was characterized as a multiresistance IncFII/FIB plasmid, which contained a $bla_{CTX\text{-}M\text{-}14}$-encoding gene.[13]

The accessory regions of the two plasmids, containing the antimicrobial resistance encoding genes, were highly similar when comparing pH2332-166 and pH2291-144. Uniquely, the former plasmid contained a single copy of *catA1* and *mph*(B), encoding resistance to chloramphenicol and macrolides, respectively. In addition, two streptomycin resistance-encoding genes (*strA* and *strB*) and one sulphonamide resistance gene (*sul2*) were identified and found to be located downstream of a class 1 integron in pH2332-166 only. Both plasmids contained a single copy of a $bla_{TEM\text{-}1}$ gene, along with a *tetR-tet*(A) locus and a class 1 integron, containing two gene cassettes in a classical head-to-tail arrangement with the *dfrA1-aadA1b* and *dfrA1-aadA1* genes, respectively. In pH2332-166, the $bla_{TEM\text{-}1}$-encoding gene was flanked by a putative transposon along with two identical copies of IS*26*, whilst in pH2291-144 this same gene was associated with a remnant of transposon Tn*2*, which was truncated within a mercury resistance cassette (Figure 1a).

Comparative analysis of pH2332-107 and pL2-87

Plasmid pH2332-107 (accession no. KJ484627) was identified in an MDR *E. coli* isolate cultured from a human source and designated as human-2332 of ST57/ST350 complex; pL2-87 (accession no. KJ484640) was purified from a lamb *E. coli* isolate, and the latter was an ST295 type. Plasmid pL2-87 was devoid of any resistance-encoding genes. Both plasmids were identified as IncB/O types, which were found to be closely related to the IncI1 family of replicons[14] and the mechanism of replication control of this group of plasmids is similar to that of IncFII plasmids.[15,16] The DNA sequence of pH2332-107 consists of a circular double-stranded DNA molecule of 107 386 bp and contained 149 putative ORFs as identified after RAST annotation. The G+C content of this plasmid was 52.4%. Plasmid pL2-87 was 87 042 bp in size, encoding 113 predicted ORFs and with a 52.9% G+C content. The RepA proteins of pH2332-107 and pL2-87 shared 99% amino acid identity with one residue difference (data not shown). As these two plasmids were from the same Inc group (IncB/O), the core regions of pH2332-107 and pL2-87 showed a high degree of sequence similarity, having a 99% nucleotide identity (Figure 1b).

The pH2332-107 sequence exhibited a highly organized structure consisting of five major functional regions of replication, drug resistance, stability, transfer leading and conjugative transfer (Figure 1b). The transfer region of pH2332-107 comprises 19 *tra* genes, 7 *trb* genes and 1 *finO* gene. It also contains 12 genes (*pilI*, *pilL*, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, *pilR*, *pilS*, *pilT*, *pilU* and *pilV*) encoding a functional type IV pilus that is thought to be involved in pilus-mediated conjugal transfer.[17,18] These pili are considered a virulence factor that, in association with resistance determinants, may support their successful dissemination.[19] The transfer leading and conjugative transfer regions in pH2332-107 and pL2-87 were 44 671 and 44 668 bp in size, respectively, and were 99% identical at the nucleotide level (Figure 1b).

Using pH2332-107 as the query sequence, BLAST searching revealed a 99% nucleotide identity with one recently reported multiple antimicrobial resistance virulence plasmid, pO26-CRL_{125} (accession no. KC340960). Similarity for pL2-87, BLAST comparisons showed 99% identity with pO111-CRL_{115} (accession no. KC340959). When plasmids pH2332-107 and pL2-87 were compared with plasmids pO26-CRL_{125} and pO111-CRL_{115}, the core regions, including the transfer and stability region of both, exhibited a high degree of sequence similarity. The transfer modules in pO26-CRL_{125} and pO111-CRL_{115} were also virtually identical and contained type IV conjugative transfer operons.[20] Interestingly, plasmid pO26-CRL_{125} and pO111-CRL_{115} contained two separate replication regions consisting of a complete IncZ replicon and a partial IncQ replicon, respectively.[20] These two MDR plasmids belonging to different pathotypes (Table 1) were originally purified from a human O26:H- enterohaemorrhagic *E. coli* and a bovine O111 atypical enteropathogenic *E. coli* respectively.[20]

In a more detailed assessment of pH2332-107, a segment of 5331 bp that contained two copies of IS*26* and a single copy of the $bla_{CTX\text{-}M\text{-}1}$ gene was identified and this region exhibited significant homology with pHHA45 (accession no. JX065630) and pKCT398 (accession no. GQ274931). This locus contained one $bla_{CTX\text{-}M\text{-}1}$ gene flanked by the characteristic insertion sequence (IS) element IS*Ecp1* and which interestingly in this case was disrupted by an IS*26* element. A similar arrangement was also noted in plasmids pH1038-142 and pL2-43 and analogously in the case of a recently reported $bla_{CMY\text{-}2}$ gene of equine origin.[21] Plasmid pH2332-107 exhibited mosaic features distal to the $bla_{CTX\text{-}M\text{-}1}$ gene. A macrolide resistance gene cluster was identified, containing a truncated *mrx* gene (denoted as *Δmrx*) followed by an *mph*(A) gene, which encodes a macrolide 2′-phosphotransferase, and then followed by a second IS*26* element. This gene module IS*26*-ΔIS*Ecp1*-$bla_{CTX\text{-}M\text{-}1}$-*Δorf477*-*Δmrx*-*mph*(A)-IS*26* was recently identified in plasmids purified from *E. coli*, including those from the successful clone ST131 and from isolates from pigs in Germany.[22] This block of genes is more often encountered in IncN type plasmids,[23] whilst the arrangement noted here is the first report of this structure in an IncB/O type plasmid background (Figure 1b). Sequence comparisons as shown in Figure 1b indicate that the *Δmrx*-*mph*(A) junction in the $bla_{CTX\text{-}M\text{-}1}$ gene module may serve as a hotspot for the insertion of IS elements and other antimicrobial resistance-containing transposons.

Comparative analysis of pH1038-142 and pL2-43

A plasmid of 142 875 bp with 191 ORFs and 52.2% G+C content was identified in an *E. coli* of undefined ST and denoted as pH1038-142 (accession no. KJ484634). This *E. coli* isolate was originally cultured from a healthy human. Plasmid pH1038-142 was transferred by conjugation and contained two incompatibility-group-encoding genes, IncF and IncN. Furthermore, two replicons were identified on pH1038-142, the first of which was contained in a 1410 bp region with *repA1* and *repA2*; these were 100% identical at the nucleotide level to the subgrouped IncFII region. In addition, a *repA* locus (related to IncN plasmids) was located upstream of the *ardK* gene (Figure 1c).

Plasmid pL2-43 (accession no. KJ484641) was a 43 265 bp structure and the smallest conjugative plasmid among those characterized in this study (Table 1). It was identified in a lamb faecal sample from which an *E. coli* ST295 isolate was cultured. Plasmid pL2-43 had 59 predicated ORFs with an average G+C content of 50.9%. The backbone region was 37 209 bp and this encoded plasmid replication, horizontal transfer functional

regions, maintenance and stability functions, and an additional continuous 6056 bp variable region, which included the $bla_{CTX\text{-}M\text{-}1}$ resistance gene, insertion sequences or transposons. Comparing pL2-43 with pH1038-142, the former exhibited 99% nucleotide identity with pH1038-142 (Figure 1c). However, pL2-43 was determined to be IncN type and was devoid of the IncF transfer region and $bla_{TEM\text{-}1}$ gene cassettes noted in the human-derived plasmid (Figure 1c).

The conjugative transfer system of pH1038-142 was the most complicated region identified in this study. The transfer region was composed of two separate elements. Plasmid pH1038-142 contained the complete sequence of the typical F transfer region (*E. coli* F sex factor transfer region, accession no. U01159) and comprised 24 *tra* genes and 8 *trb* genes (Figure 1c). In comparison with the IncFII-IncFIA plasmid pEC_L46 (accession no. GU371929), which contained a $bla_{CTX\text{-}M\text{-}15}$ gene and was originally isolated from a human in Belgium,[24] it showed 66% coverage and 98% nucleotide identity with pH1038-142. The IncN-type transfer locus on pH1038-142 was identified and found proximal to the bla_{ESBL}-gene-containing region on this plasmid (Figure 1c). It included 14 *tra*-encoding genes. In general the structure of pH1038-142 could be divided into four main functional modules, involved in plasmid replication, transfer, stability and antimicrobial resistance. BLAST analysis revealed two β-lactam resistance genes, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}1}$, which were located on pH1038-142 (Figure 1c). It also carried eight other resistance-encoding genes—*strA*, *strB*, *tet*(A), *tetR*, *catA1*, the *dfrA1-aadA1* gene cassette array (linked to the *intI1* gene of the class 1 integron integrase) and *sul3* coding for resistance to streptomycin, tetracycline, chloramphenicol, trimethoprim, streptomycin/spectinomycin and sulphonamide, respectively.

A sequence analysis of pL2-43 showed a high degree of similarity with three other IncN antimicrobial resistance-containing plasmids: pHHA45 (accession no. JX065630), pVQS1 (accession no. JQ609357) and R46 (accession no. AY046276). Plasmid pHHA45 was identified from a pig *E. coli* containing $bla_{CTX\text{-}M\text{-}1}$ in Denmark and pVQS1 originated from a *Salmonella* Virchow isolate cultured from a human in Switzerland, which contained the $bla_{TEM\text{-}1}$ and *qnrS1* genes.[23,25] A comparative analysis of pL2-43 and pH1038-142 with the IncN reference plasmid R46 demonstrated that they both contained the typical IncN plasmid scaffold, including the replicon gene (*repA*), the *stbA-stbB-stbC* genes required for plasmid stability, the *mucA-mucB* genes involved in mutagenesis enhancement, the *ardA-ardB* and *ardK-ardR* genes providing antirestriction functions, the *ccg* genes that encode products protecting plasmid DNA from the type I restriction system and the regions composing the conjugative apparatus (*traL*, *traM*, *traA*, *traB*, *traC*, *traD*, *traN*, *traE*, *traO*, *traF*, *traG*, *traI*, *traJ* and *traK*).[23,26] These regions showed 99% nucleotide identity in plasmids pH1038-142 and pL2-43. It is interesting to note that plasmid pH1038-142 could be considered to be a structural derivative of plasmid pL2-43. The former contained all the main function modules identified in pL2-43, including the IncF-type transfer region together with an array of accessory genes, inserted as an IS*26*-Δ*ISEcp1*-$bla_{CTX\text{-}M\text{-}1}$-Δ*orf477*-Δ*mrx*-IS*26* gene module into pH2332-142 (Figure 1c).

Molecular characterization of pC59-153

The complete sequence of a 153 231 bp plasmid, denoted as pC59-153 (accession no. KJ484636), from a ST155 complex *E. coli* was determined. The bacterial isolate was cultured from a chicken faecal sample (Table 1). The plasmid contained 193 putative ORFs with G+C content 49.8% and IncF replicons (*repFIIA-repFIC* and *repFIB*). It was found to contain virulence-associated genes, antimicrobial resistance genes, plasmid transfer genes, plasmid maintenance genes, mobile genetic elements and genes encoding hypothetical proteins of unknown function (Figure 1d).

Plasmid pC59-153 contained a transfer region spanning 37 189 bp, which was similar to those of the previously characterized virulence plasmid pAPEC-O1-ColBM (accession no. DQ381420), the virulence plasmid pVir68 (accession no. CP001162) and the typical F sex factor transfer region (accession no. U01159). The transfer region of pC59-153 shared 99% nucleotide sequence similarity with the previously published sequences of pAPEC-O1-ColBM (Figure 1d). Downstream of the transfer region was one of the two plasmid-encoded replicons. The first of these shared the highest homology with the RepFIIA and RepFIC replicons, albeit with some interesting variations. For example, the first 600 bp of this region contained the *copB* repressor and flanking DNA sequences from the RepFIC region, but not the *repA1* gene of this replicon that it has been found to repress.[27,28] Plasmid pC59-153 also appeared to be a member of the IncFIB incompatibility group, based upon BLAST homology and alignment with proteins of the RepFIB replicon (data not shown). Within this region the *repA* replication gene and a site-specific integrase (*int*) were involved in recombination.[29] This region shared 99% nucleotide homology with the RepFIB replicons from pAPEC-O1-ColBM (accession no. DQ381420), which contained a putative virulence cluster and had been previously isolated from an avian pathogenic *E. coli* (APEC) (Figure 1d).[30]

APEC strains cause a complex of diseases in birds, and because of their phylogenetic background they are also suspected to be a potential zoonotic risk for humans.[31,32] Large plasmids are commonly associated with virulence in APEC isolates. According to recent studies, most virulence genes associated with APEC are often located on IncF plasmids,[33] namely the ColV plasmids because of their ability to code for the production of colicin V, a small protein from the microcline family.[34,35] ColV plasmids are associated with *E. coli* in general and with APEC in particular. Interestingly, pC59-153 showed high levels of similarity with several other ColV plasmids, such as pAPEC-O1-ColBM (accession no. DQ381420) with 79% coverage and 99% nucleotide identity, pJIE186-2 (accession no. JX077110) with 71% coverage and 99% nucleotide identity, and pAPEC-O2-ColV (accession no. AY545598) with 69% coverage and 99% nucleotide identity. However, pC59-153 belonged to the non-ColV plasmids as the multiple bacteriocin-producing modules (*cvaABC*) for the production of colicin V were absent.[36]

Plasmid pC59-153 was found to contain several genes that have previously been associated with APEC virulence. The comparison of pC59-153 with one ColV plasmid pAPEC-O1-ColBM is shown in Figure 1d. The virulence factors of both were located to a 60 kb region (Figure 1d) that included the following: *sitABCD*, genes of a putative ABC transport system involved in iron and manganese transport;[30] *iucABCD* and *iutA*, genes of the aerobactin siderophore system;[37] *iroBCDEN*, genes of the salmochelin siderophore system;[38] *iss*, which functions to promote serum survival by resisting complement-mediated killing;[39] *estABC*, genes of another ABC transport system;[30] *ompT* and

hlyF.[28,36] Additionally, pC59-153 also contained one bacteriocin module, *mcjABCD* encoding microcin J25, which is known to be involved in inhibition of the RNA polymerase and operates on the cell membrane, which was absent from pAPEC-O1-ColBM (Figure 1d). The presence of this module appears to be unique as it has only been reported once from completely sequenced plasmids from Enterobacteriaceae deposited in GenBank.[36] Two copies of *vagCD* with only 86% nucleotide identity were located on pC59-153 and were designated *vagC1D1* and *vagC2D2*. These were 100% identical to the same genes on plasmid pJIE186-2 (accession no. JX077110).[36] All the virulence factors identified on pC59-153 are reported to be present among APEC isolates and furthermore these share a high degree of homology with the corresponding regions of ColV plasmids, suggesting that they may be derived from the same ancestral multiple virulence plasmid backbone.

It is interesting to note that no antimicrobial resistance gene(s) have been reported on ColV virulence plasmids. Plasmid pC59-153 contained a *catA1* gene conferring resistance to chloramphenicol that was flanked by Tn*21*. No other antimicrobial resistance-related genes were identified.

Molecular characterization and comparative analysis of pH1519-76, pH1519-7 and pH1519-2

The MDR *E. coli* isolate human-1519 (Table 1) was identified as a member of the ST10 complex and contained four transmissible plasmids ranging in size from 2 to 88 kb. Following an examination of the annotation in each case, only the 88 kb plasmid was found to contain antimicrobial resistance genes and this plasmid showed a high degree of similarity when compared with R64 (outlined below). Plasmid pH1519-76 (accession no. KJ484631) was the second largest plasmid from the human-1519 *E. coli* isolate and was found to be 76197 bp in size and belongs to the IncFII type. Interestingly, no antimicrobial resistance gene(s) was detected on this structure. The smaller plasmids, pH1519-7 (accession no. KJ484632) and pH1519-2 (accession no. KJ484633), were 7036 and 1822 bp in size, respectively, and were most likely transferred in an *in trans* mode.

Plasmid pH1519-76 contained 119 putative ORFs and its genome consisted of three major regions: replication, conjugative transfer regions and stability regions. BLASTn comparison revealed a novel plasmid scaffold. Some regions of plasmid pH1519-76 showed 78% coverage and 99% nucleotide identity with a large virulence plasmid pTC (accession no. CP000913), which encoded the STa and STb heat-stable enterotoxins along with a tetracycline resistance gene.[40] Plasmid pHK01 (accession no. HM355591) also shared a high degree of homology with pH1519-76, with 76% coverage and 99% nucleotide identity. The former belonged to the IncFII type and carried a $bla_{\text{CTX-M-14}}$ gene, which was related to the $bla_{\text{CTX-M-14/24}}$ gene currently being disseminated across mainland China and Vietnam.[41]

Non-transfer associated genes were identified on pH1519-7 and pH1519-2, both of which were non-conjugative plasmids. In this case, such plasmids may parasitize a conjugative plasmid, availing itself of the transfer mechanisms *in trans* and transferring at high frequency.

Twelve ORFs were detected on pH1519-7. A comparative analysis of pH1519-7 with the current database showed that it exhibited 99% nucleotide identity to a non-conjugative colicinogenic plasmid ColE1 (accession no. J01566) that can be transmitted efficiently from cells containing a conjugative plasmid such as an F or R factor.[42] The plasmids pH1519-7 and ColE1 both harboured an *imm* gene that encoded the colicin E1 immunity protein. Colicin E1 acts by forming membrane channels leading to a depolarization of cell membrane potential and a dissipation of the cell's proton electrochemical gradient.[43] In pH1519-2 only two ORFs were detected, one related to replication and the other encoding a hypothetical protein.

Analysis of six plasmids with high similarity to R64

Six large plasmids showed a high degree of nucleotide similarity with the reference plasmid for the IncI1 group, R64 (accession no. AP005147). These could be divided into two groups based on their original bacterial sources. The first group, containing four plasmids—pC23-89 (accession no. KJ484639), pC49-108 (accession no. KJ484638), pC59-112 (accession no. KJ484637) and pC60-108 (accession no. KJ484635) (Figure 2)—were from different chicken *E. coli* isolates. The remaining two plasmids, pH2291-112 (accession no. KJ484629) and pH1519-88 (accession no. KJ484630) (Figure 2), were from human *E. coli* isolates (Table 1).

Plasmid pC23-89 was 89513 bp in size, and in this structure 125 putative ORFs were predicted. The G+C content was determined to be 50.2%. Plasmid pC23-89 shared a 99% identity of its DNA sequence with R64, albeit with considerable rearrangements, particularly relating to the stability and drug resistance regions (Figure 2). When compared with R64, pC23-89 lacked the arsenic, tetracycline and streptomycin resistance genes; this region was substituted by another resistance element including Tn*2*-$bla_{\text{TEM-52}}$. The collinearity between the pC23-89 and R64 plasmid scaffolds was conserved across the transfer functional region. The R64 transfer region was highly organized into four major functional gene clusters.[44] The *tra*ABCD regulatory gene cluster was located at the distal end of the type IV pili locus region as shown in Figure 2, and was located immediately upstream of the replication region in the R64 circular genome. In contrast, in pC23-89, a *traD* gene was absent and was substituted by a gene encoding an ORF for a hypothetical protein of 183 amino acid residues. Based on previous studies, two genes from this cluster, *traB* and *traC*, were reported to be essential for conjugative transfer, not only in liquid, but also in solid medium.[44,45] The *tra*/*trb* gene cluster contained 21 *tra* genes and 3 *trb* genes required for conjugation in pC23-89. Located towards one end of the pC23-89 and R64 transfer loci was an *oriT* operon consisting of the origin of transfer, *oriT* and *nikAB* genes, which were required to initiate the DNA transfer.[46] Furthermore, the *pil* gene cluster that encodes a type IV pilus locus in pC23-89 and R64 were 95% identical at the nucleotide level.

Comparing the stability region of pC23-89 with that of R64, two types of phage growth inhibition systems recognized in the latter were absent in pC23-89, and similarly the *mck* and *kor* genes known to be involved in a toxin–antitoxin system for the maintenance of R64 were absent in pC23-89.[44] Nonetheless, pC23-89 carried several plasmid maintenance and partitioning modules (*yefA*, *parAB*, *impABC*, *ssb*, *yfhA*, *psiAB*, *ardA*), ensuring stable plasmid inheritance. In addition, two virulence associated genes, *vagC* and *vagD*, were also detected on pC23-89, and these were flanked by a $bla_{\text{TEM-52}}$ gene on the proximal side (Figure 2).

The sizes of three plasmids, pC49-108, pC59-112 and pC60-108, were 108 660 bp, 112 330 bp and 108 661 bp, respectively (Figure 2). They all possessed an overlapping region of 61 145 bp and were 99% identical with R64. The G+C content for these was determined to be 50.9%, 51.1% and 50.9%, respectively. All three plasmids shared a common backbone module design that encoded initiation of plasmid replication, conjugative transfer, plasmid maintenance and stability, and antimicrobial resistance regions (Figure 2). They all possessed 14 *pil* genes encoding a group of proteins corresponding to a type IV pilus that is known to be involved in the formation of donor–recipient cell aggregates during the conjugation in liquid media.[47]

The conjugative transfer and stability regions in pC49-108, pC59-112 and pC60-108 were 99% identical at the nucleic acid level when compared with pC23-89 and R46 (Figure 2). The transfer regions consisted of 21 *tra* genes and 3 *trb* genes. The *traABCD* regulatory gene cluster was 99% identical with the same locus in pC23-89. Similarly for pC23-89 described above, these plasmids differed primarily in their accessory regions when compared with plasmid R64. These differences were mainly associated with insertion sequences and transposases, class 1 integrons and antimicrobial resistance determinants. An analysis of these regions revealed the presence of several accessory gene clusters implicated in resistance to multiple antimicrobial agents. One of these regions occurred between *pilVA* and *rci* (in plasmid R64) and consisted of a 2.9 kb module containing IS*Ecp1*-$bla_{\text{CTX-M-1}}$-*Δorf477*-*Δmrx* encoding resistance to broad-spectrum cephalosporins. Another accessory region was located distal to the *repZ* gene and was identified as a partial class 1 integron containing *dfrA17*-*aadA5*, encoding trimethoprim and streptomycin/spectinomycin resistance, but lacking the complete 3′-conserved structure. These accessory regions were conserved across all three plasmids.

The second group of plasmids was composed of pH2291-112 and pH1519-88, which were 112 671 and 88 678 bp in size (Figure 2). The overall G+C content was determined to be 50.7% and 49.7%, respectively. Both of the plasmids were 99% similar to R64 at the nucleotide level and overlapped by 44 904 and 46 998 bp with R64, respectively (Figure 2). The structure of these plasmids can be divided into four functional modules related to replication, plasmid transfer and maintenance, stability and antimicrobial resistance.

As with the first group described above (shown in Figure 2), the plasmids in the second group also showed a high degree of nucleotide similarity with R64 (99% identity) within their conjugative transfer regions. pH2291-112 and pH1519-88 both contained the *tra/trb* cluster for conjugation, the *pil* gene cluster encoding the type IV pilus biogenesis system, and the *oriT* and *nikAB* gene clusters for conjugative DNA processing. Similar to pC23-89, the *traD* gene of unknown function was substituted by a hypothetical protein in pH2291-112. In pH1519-88 the *traC* was flanked by a *pilI* gene, and no *traD* was identified (Figure 2).

Another interesting finding within pH2291-112 and pH1519-88 was the presence of the accessory regions. On plasmid pH2291-112 there were two antimicrobial resistance regions, which showed 99% identity with those identified on pC49-108. A class 1 integron was located at the distal end of the *repZ* gene, containing *dfrA17*-*aadA5* encoding trimethoprim and streptomycin/spectinomycin resistance. A second integron was located between *pilVA* and *rci*, but the cluster consisting of IS*Ecp1*-IS*5*-$bla_{\text{CTX-M-1}}$-*Δorf477*-*Δmrx* was rearranged in pH2291-112 with respect to pC49-108 by the insertion of an IS*5* element (Figure 2). Plasmid pH1519-88 showed 99% identity to R64 and contained two resistance genes, $bla_{\text{CTX-M-1}}$ and a novel $bla_{\text{TEM-210}}$ gene. Furthermore, similar to pC49-108, pH1519-88 also contained the IS*Ecp1*-$bla_{\text{CTX-M-1}}$-*Δorf477*-*Δmrx* module, but in this case the cluster was located between the *pilJ* and *pilI* genes instead of between *pilVA* and *rci* (as in pC49-108; Figure 2). The $bla_{\text{TEM-210}}$ was identified as a new bla_{TEM} variant with two amino acid substitutions, at positions 49 and 69, when compared with the progenitor $bla_{\text{TEM-1}}$ sequence (accession no. J01749).

A comparative analysis of these IncI1 plasmids purified from *E. coli* isolates of human and poultry origin clearly highlights a high degree of structural conservation. These plasmids carry one or more antimicrobial resistance genes including those related to the bla_{ESBL} class. These data also provide evidence of the dissemination of an R64-type IncI1-like plasmid between animal and human sources.

Concluding remarks

In this study we determined the sequences of 16 transmissible plasmids, including eight containing a $bla_{\text{CTX-M-1}}$-encoding gene together with five others carrying a bla_{TEM}-encoding resistance gene. These plasmids ranged in sizes from 1.8 through 166.6 kb and were purified from nine MDR *E. coli* of different STs in Switzerland. All eight $bla_{\text{CTX-M-1}}$ genes were located within various genetic environments, with either a complete or a truncated IS*Ecp1* gene identified proximal to each of these resistance genes. Three of the five bla_{TEM} genes were detected within the complex transposon Tn*2*, whilst the other two were found to be flanked by IS*26* elements. These data highlight the range of genetic contexts within which *bla*-encoding genes can be found.

In this study *bla* genes were identified on plasmids with similar genetic backbones and located in unrelated human, poultry and lamb *E. coli* isolates. This observation points towards a plasmid transfer that had occurred historically among these bacterial isolates of human and animal origins. This is potentially suggestive of a common gene pool capable of contributing to the transfer of *bla* resistance genes between bacteria of the same (and unrelated) genera. Furthermore, it underscores the need for continued monitoring for these mobile genetic elements.

From the bioinformatics analysis of the plasmid structures in each case, those of the same plasmid incompatibility types shared a high degree of similarity across their backbone scaffolds. Of note, two plasmids purified from two MDR *E. coli* isolates cultured from healthy humans were very similar structurally when compared with two plasmids purified from an *E. coli* cultured from a lamb sample. Furthermore, six plasmids from human and chicken sources shared the R64 backbone structure. These features can be expected to contribute to the mosaic nature of plasmids themselves and to facilitate the dissemination of *bla* and other resistance-associated integrons/transposons, as previously reported in other microorganisms.[48,49] Interestingly, the well-characterized features of R64, including its replication locus, the *pil* gene cluster and its associated conjugative transfer genes, were highly conserved among all six R64-like plasmids characterized in this study. Based on these observations, it is tempting to speculate that the R64-like elements are contributing

to the genetic exchange of resistance-encoding genes including β-lactamase genes along with other resistance/virulence-associated loci between humans and animals in Switzerland. Further studies will be necessary to extend our understanding of the precise role played by this element in particular.

In conclusion, the evolution of different plasmid structures and their potential for dissemination among bacteria between and in different ecological niches represent a significant challenge to the control of antimicrobial resistance. Carefully monitoring the selective pressure imposed, through the use of these chemotherapeutic compounds in food-producing animals and in humans, may limit the dissemination, and it will be important to quantify success here. In the future, larger scale molecular epidemiological studies allied to an in-depth analysis of drug usage should be considered as a means of uncovering those factors contributing to these dynamics and providing the basis for an improved understanding of the driving forces associated with horizontal gene transfer.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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