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Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum β-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin

Aurora García-Fernández[1], Giuseppina Chiaretto[2], Alessia Bertini[1], Laura Villa[1], Daniela Fortini[1], Antonia Ricci[2] and Alessandra Carattoli[1]*

[1]*Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy;* [2]*Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy*

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***Objectives*: Plasmids belonging to incompatibility group I1 (IncI1) are widespread in Enterobacteriaceae and are characterized by the presence of a cluster of genes encoding the type IV pili, contributing to the virulence of Shiga-toxigenic *Escherichia coli*. Recently, IncI1 plasmids were identified in *E. coli* and *Salmonella* strains of animal origin as responsible for the dissemination of β-lactamase genes. Plasmid multilocus sequence typing (pMLST) was developed to discern naturally occurring IncI1 plasmids in homogeneous groups according to their allele assortment.**

***Methods*: pMLST was developed by selecting multiple target genes on the available complete IncI1 plasmid DNA sequences. Sixteen plasmids, all assigned to the IncI1 group by the PCR-based replicon typing method, were included in this study. They were analysed for β-lactamase genes and typed by restriction fragment length polymorphism (RFLP) and pMLST.**

***Results*: Sixteen plasmids identified in *E. coli* and *Salmonella* isolated from animals and humans in different countries carried bla_{CMY-2}, $bla_{CTX-M-15}$, $bla_{CTX-M-1}$, $bla_{CTX-M-14}$, bla_{TEM-52}, bla_{SHV-12} or bla_{TEM-1} β-lactamase genes. These plasmids were classified by RFLP in nine different groups corresponding to the nine sequence types determined by pMLST.**

***Conclusions*: The pMLST method was suitable for rapid and easy subtyping of IncI1 plasmids. This study demonstrates that the pMLST method can contribute to the epidemiological description of circulation of specific resistance plasmids among β-lactamase producers isolated from animals and humans.**

Keywords: pMLST, incompatibility group, CTX-M, CMY, SHV

Introduction

Plasmids belonging to incompatibility group I1 (IncI1) carrying extended-spectrum and AmpC β-lactamase genes have recently been described in *Escherichia coli* and *Salmonella*.[1,2] Three typical IncI1 plasmids were fully sequenced: R64 and colIb-P9, lacking β-lactamase genes, and pNF1358 carrying the bla_{CMY2} gene. These plasmids are characterized by the presence of a cluster encoding the type IV pili, contributing to adhesion and invasion of Shiga-toxigenic *E. coli*.[3] These peculiar pili are a virulence factor and the association of virulence and resistance determinants may favour the positive selection of plasmids belonging to the IncI1 family.[4] A study performed in the USA on a large collection of avian and human *E. coli* demonstrated that IncI1 plasmids were more frequent in pathogenic than in commensal strains.[4] The observation that IncI1 plasmids have been recently associated with highly widespread β-lactamase genes in *E. coli* and *Salmonella* from food animals is of concern for the potential spread of resistance determinants through the food chain.

The aim of this work was to analyse and characterize IncI1 plasmids identified in β-lactamase *E. coli* and *Salmonella* producers from animal and human sources in Europe and the USA. We set up a new plasmid multilocus sequence typing (pMLST) method to rapidly categorize plasmids belonging to the IncI1 family with different sequence types (STs).

*Corresponding author. Tel: +39-06-49903128; Fax: +39-06-49387112; E-mail: alecara@iss.it

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Materials and methods

Plasmids

Sixteen IncI1 plasmids were analysed for β-lactamases and plasmid scaffold. The bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY} and bla_{OXA} genes were searched for by PCR,[5] and amplicons were sequenced by fluorescent dye-labelled dideoxynucleotides using an ABI 3730 instrument (Applied Biosystems). The 16 plasmids were assigned to the IncI1 group by the PCR-based replicon typing (PBRT) method.[6] Twelve plasmids had been previously characterized: eight of them were kindly provided by L. Poirel (INSERM U914, Bicetre, France),[7] A. Cloeckaert (INRA, Nouzilly, France)[1] and K. Hopkins (HPA, Colindale, London, UK),[2] and four were from the collection of the Istituto Superiore di Sanità (18196T, 1358T, R144 and S82/10).[6,8–10] Four transconjugant strains (398T, 3115T, 3960T and 2392T) were obtained using *E. coli* K12 as a recipient strain from β-lactamase *E. coli* producers of animal origin isolated at the Istituto Zooprofilattico delle Venezie, Italy, in 2005–2006. Plasmids 398T and 3115T were positive for the bla_{CMY-2} gene, and 3960T and 2392T were positive for the $bla_{CTX-M-1}$ gene and the bla_{SHV-12} and bla_{TEM-1} genes, respectively (Table 1).

Plasmid DNA was purified by the QIAGEN Plasmid Midi Kit (Qiagen Inc., Milano, Italy) and analysed by restriction fragment length polymorphism (RFLP) by *Pst*I digestion and Southern blot hybridization using the amplicons obtained from repI1 and bla_{CMY-2} and $bla_{CTX-M-1}$-group genes, respectively, as probes. Plasmids showing no more than two bands of difference were assigned to the same RFLP type (Table 1).

Plasmid multilocus sequence typing

Primers for pMLST are listed in Table 2. We selected 254 bp of *pilL*, 254 bp of *sogS* and 343 bp of *ardA* gene coding sequences. Moreover, a 104 bp sequence of repI1 and a 812 bp sequence including the 3′ end of the *trbA* gene, the intergenic region and the 5′ end of the *pndC* gene were also included as pMLST targets.

PCRs were performed as follows: 1 cycle at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min. The amplification was concluded by 1 cycle at 72°C for 5 min. The amplicons were purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI, USA) and fully sequenced.

Nucleotide accession numbers

The allele variants were assigned to the following EMBL accession numbers: repI1-1 (EU370458), repI1-2 (EU370459), repI1-3 (EU370460), ardA1 (EU370452), ardA2 (EU370453), ardA3 (EU370454), trbA-pndC1 (EU370465), trbA-pndC2 (EU370466), trbA-pndC3 (EU370467), trbA-pndC4 (EU370468), trbA-pndC5 (EU370469), trbA-pndC6 (EU370452), sogS1 (EU370461), sogS2 (EU370462), sogS3 (EU370463), sogS4 (EU370464), pilL1 (EU370455), pilL2 (EU370456) and pilL3 (EU370457).

Results

Sixteen IncI1 plasmids were purified from transconjugants/transformants and analysed by RFLP by *Pst*I restriction. They were categorized into nine different restriction patterns [A–I in Table 1 and Figure S1—available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. One strain (2392T) produced no RFLP pattern due to autolysis of the plasmid preparation.

The three available IncI1 complete DNA sequences were compared (R64-AP005147, colIb-P9-AB021078 and pNF1358-DQ017661). Six genes were selected as potential targets for the pMLST because they marked relevant maintenance and replication plasmid functions and were well conserved but also showed significant nucleotide differences potentially useful to the subtyping of plasmids. In particular, for this analysis, we selected *pilL* of the cluster for the type IV pilus biogenesis, *sogS*, encoding the primase that acts in the discontinuous DNA plasmid replication, and *ardA*, encoding a type I restriction-modification enzyme. We also analysed the RNAI antisense regulating the IncI1 replication system (repI1) and the intergenic region of the *trbA* and *pndC* genes, involved in maintenance and plasmid transfer, respectively (Table 2).

The 16 IncI1 plasmids of our collection were then tested by PCR, DNA sequencing and sequence comparison with the R64 DNA sequence (AP005147). Three allele variants for repI, *pilL* and *ardA*, four alleles for *sogS* and six alleles for *trbA-pndC* were identified. Insertion of the *finQ* gene (encoding the fertility inhibitor) within the 5′ end of the *pndC* gene occurred in five strains (C10-VLA, S.82/10, C1C-VLA, C12C-VLA and C13C-VLA) and characterized the *trbA-pndC* allele variants 5 and 6 (allele 6 was distinguished from allele 5 by 14 additional nucleotide changes in the *trbA* gene, Table 2).

The assortment of the different alleles defined nine different STs among the 16 IncI1 plasmids. These categories perfectly matched those obtained by RFLP (Table 1), indicating that pMLST has a comparable discriminatory power.

The three IncI1 bla_{CMY-2} plasmids identified in *E. coli* isolated from dogs in Rome (18196T) and in Padua (398T and 3115T) were assigned to the same pMLST and RFLP types (ST2, RFLP-A). They differ from the bla_{CMY-2} plasmid (1358T) identified by both RFLP (RFLP-B) and pMLST (ST4) in a *Salmonella* Thompson of human origin isolated in the USA in 1996.

The three plasmids carrying $bla_{CTX-M-15}$ and bla_{TEM-1} genes identified in *Salmonella* Anatum (C1-VLA) and Typhimurium (C12-VLA and C13-VLA) in the UK were identical by both RFLP and pMLST (RFLP-C, ST8). These IncI1 plasmids were characterized by the presence of the *finQ* gene within the *pndC* gene (Table 2).

Three of the four $bla_{CTX-M-1}$ plasmids were classified in the same RFLP-E and ST3 groups (Table 1). Two of them were identified in *E. coli* isolated from poultry in the district of Côtes d'Armor in France in 2005 (21T and 22T),[7] and one was from *E. coli* isolated from a dog in Italy in 2005 (3960T). Interestingly, the ST3 was also assigned to a plasmid identified in *E. coli* isolated from poultry in Italy in the same year (2392T), but this plasmid carried the bla_{SHV-12} and the bla_{TEM-1} genes. The fourth $bla_{CTX-M-1}$ plasmid (34T) was different by both restriction analysis (RFLP-D) and pMLST (ST9), and was identified in *E. coli* isolated from poultry in the district of Mayenne in France.[7]

Plasmids C10-VLA and 05-0001Tc1, carrying the $bla_{CTX-M-14}$ and the bla_{TEM-52} genes, respectively, and plasmids R144 and S.82/10, negative for β-lactamases and showing different RFLP patterns with respect to the other IncI1 plasmids, were assigned to different STs by pMLST (ST6, ST5, ST1 and ST7, respectively; Table 1).

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Table 1. Characteristics of the IncI1 plasmids analysed in this study

IncI1 plasmid[a]	Strain[b]	Country[b]	Year[b]	Origin[b]	β-lactamase	RFLP[c]	pMLST[d]					ST[d]	Reference
							repI1	*ardA*	*trbA-pndC*	*sogS*	*pilL*		
398T	*E. coli*	Italy	2006	dog	CMY-2	A	1	2	3	2	1	2	this study
3115T	*E. coli*	Italy	2005	dog	CMY-2	A	1	2	3	2	1	2	this study
18196T	*E. coli*	Italy	2003	dog	CMY-2	A	1	2	3	2	1	2	8
1358T	*Salmonella* Thompson	USA	1996	human	CMY-2	B	1	3	3	4	1	4	10
C1-VLA	*Salmonella* Anatum	UK	2001	human	CTX-M-15, TEM-1	C	3	2	6	3	3	8	2
C12-VLA	*Salmonella* Typhimurium	UK	2002	human	CTX-M-15, TEM-1	C	3	2	6	3	3	8	2
C13-VLA	*Salmonella* Typhimurium	UK	2003	human	CTX-M-15, TEM-1	C	3	2	6	3	3	8	2
34T	*E. coli*	France	2005	poultry	CTX-M-1	D	3	2	3	3	3	9	7
21T	*E. coli*	France	2005	poultry	CTX-M-1	E	2	1	4	1	2	3	7
22T	*E. coli*	France	2005	poultry	CTX-M-1	E	2	1	4	1	2	3	7
3960T	*E. coli*	Italy	2005	dog	CTX-M-1	E	2	1	4	1	2	3	this study
2392T	*E. coli*	Italy	2005	poultry	SHV-12, TEM-1	NA	2	1	4	1	2	3	this study
C10-VLA	*Salmonella* Enteritidis	UK	2001	human	CTX-M-14	F	1	2	5	4	2	6	2
05-0001Tc1	*Salmonella* Infantis	Belgium	2005	human	TEM-52	G	1	2	2	3	3	5	1
S.82/10	*Salmonella* Enteritidis	reference	1995	human	negative	H	2	1	5	4	2	7	6
R144	*Salmonella* Typhimurium	reference	NA	NA	negative	I	1	1	1	1	1	1	9

NA, not available.

[a]Plasmids were purified from transconjugants or transformants obtained from the original strains using *E. coli* K12 as recipient strain.

[b]Bacterial species, country, year and source of isolation of the organisms where the IncI1 plasmids were identified.

[c]Plasmid types were determined by restriction fragment length polymorphism (RFLP) by *Pst*I digestion and classified with capital letters.

[d]Plasmid multilocus sequence typing (pMLST). Allele variants for each sequenced gene (repI1, *pilL*, *sogS*, *ardA and trbA-pndC*) were identified and numbered. Different sequence types (STs) were assigned to the different combinations of allele variants observed among the IncI1 plasmids.

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Table 2. Primers used on the IncI1 plasmids and pMLST allele variants identified in this study

Primer[a]	Primer sequence	Amplicon size	Primer position[c]	Allele DNA sequence position[c]	Allele name (nt changes versus R64/total nt)
repI1 FW	5′-CGAAAGCCGGACGGCAGAA-3′	142	198–216	236–339	repI1-1 (0/104), repI1-2 (1/104), repI1-3 (2/104)
repI1 RV	5′-TCGTCGTTCCGCCAAGTTCGT-3′		339–319		
ardA FW	5′-ATGTCTGTTGTTGCACCTGC-3′	501	61 426–61 445	61 469–61 811	ardA1 (0/343), ardA2 (11/343), ardA3 (12/343)
ardA RV	5′-TCACCGACGGAACACATGACC-3′		61 926–61 906		
trbA FW	5′-CGACAAATGCTTCCGGGGT-3′	883[b]	74 198–74 216	74 221–75 032[b]	trbA-pndC1 (7/812), trbA-pndC2 (7/812), trbA-pndC3 (7/812), trbA-pndC4 (10/812), trbA-pndC5 (8/752+*finQ*), trbA-pndC6 (24/752+*finQ*)
pndC RV	5′-CGAATCCCTCACCATCCAG-3′		75 080–75 062		
sogS FW	5′-TTCCGGGGCGTAGACAATACT-3′	291	93 088–93 108	93 125–93 378	sogS1 (0/254), sogS2 (2/254), sogS3 (8/254), sogS4 (9/254)
sogS RV	5′-AACAGTGATATGCCGTCGC-3′		93 378–93 360		
pilL FW	5′-CCATATGACCATCCAGTGCG-3′	316	114 765–114 784	114 804–115 057	pilL1 (0/254), pilL2 (5/254), pilL3 (22/254)
pilL RV	5′-AACCACTATCTCGCCAGCAG-3′		115 080–115 061		

[a]DNA sequence of the amplicons is obtained using the forward primer.
[b]Amplicon size and allele variant sequence length vary in alleles showing the insertion of the *finQ* gene in the *pndC* gene (2305 bp).
[c]EMBL AP005147 R64 plasmid.

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Discussion

The pMLST method was suitable for the rapid and easy typing of the IncI1 plasmids. The variants can be discriminated by significant and stable nucleotide divergence, matching results obtained by restriction analysis. pMLST can be applied as a second line of plasmid typing after they have been assigned to incompatibility groups by the PBRT method or other methods. A DNA sequence-based method allows recognition of similar plasmids and their detection among isolates from different countries and laboratories without exchange of strains and direct comparison of the plasmids. pMLST can also be developed for other plasmid families and can contribute to the epidemiological description of plasmid circulation in animal reservoirs and humans by describing the spread of virulence and resistance plasmids.

Our results demonstrated that indistinguishable IncI1 plasmids carrying the bla_{CMY-2} gene circulated in pets living in different towns in Italy in the period 2003–06, but they were different from the bla_{CMY-2} plasmid identified in *Salmonella* in the USA. Those carrying the $bla_{CTX-M-15}$ gene from *Salmonella* isolated in the UK were identical to each other and different from the other IncI1 plasmids. Variable associations among the plasmids and the β-lactamase genes were observed: the same plasmid scaffold (ST3) was associated with different genes such as $bla_{CTX-M-1}$ and bla_{SHV-12}–bla_{TEM-1}, and different plasmid scaffolds (ST3 and ST9) were associated with the $bla_{CTX-M-1}$ gene.

From this study and the current literature, the prevalence of plasmids belonging to IncI1 seems to be linked to a particular reservoir of *E. coli* and *Salmonella* from poultry. Previous studies demonstrated that the IncI1 plasmids are significantly associated with avian pathogenic *E. coli* in poultry populations analysed in the USA.[4] There could be a particular niche favouring these plasmids in certain specific *E. coli* populations, probably due to the contribution of the virulent type IV pili. This characteristic could promote the rapid spread in poultry and other food sources of the β-lactamase producers carrying IncI1 plasmids.

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Transparency declarations

None to declare.

Supplementary data

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

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