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A novel group of IncQ1 plasmids conferring multidrug resistance

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ABSTRACT

The IncQ is a group of non-conjugative but mobilisable plasmids that are found and stably maintained in a wide range of bacteria contributing to the spread of antimicrobial resistance genes and to the insurgence of multidrug resistant bacteria. Here we report the identification, in clinical *Salmonella* Typhimurium strains, of an IncQ1 plasmid (pNUC) which confers resistance to sulfamethoxazole, streptomycin and tetracycline through the presence of *sul2*, *strAB* and *tetA* genes, respectively. pNUC was detected in five multidrug resistant *S.* Typhimurium strains collected in Southern Italy from various hospitals and years of isolation. Bioinformatics analyses highlighted the presence of pNUC-like plasmids in pathogenic bacteria of various *Enterobacteriaceae* genera or species. Taken as a whole, these plasmids constitute a novel group of IncQ1 plasmids that might have originated through recombination events between a *tetR-tetA* gene cluster (possibly derived from a Tn*1721*) and a recipient IncQ1 plasmid related to RSF1010. Our findings raise concerns regarding the possible contribution of the newly identified group of IncQ1 plasmids to the spread of tetracycline resistance.

1. Introduction

Antimicrobial resistance is a persistent public health concern which has, in recent years, been the object of a number of studies. Resistance is determined, principally, by plasmid borne horizontally-transferred genes: where in conjugative plasmids undoubtedly play a key role. However, other genetic elements such as non-conjugative but mobilisable plasmids also contribute to the spread of antimicrobial resistance genes (ARGs). Transfer of mobilisable plasmids relies both on their encoding relaxase, that initiates and terminates the conjugative process, and the presence of a conjugative element encoding a protein export mechanism (type IV secretion system) (Llosa et al., 2002). Relaxase proteins have also been used to classify the conjugative systems into MOB families (MOB_C, MOB_F, MOB_H, MOB_P, MOB_Q and MOB_V) (Garcillan-Barcia et al., 2009). The MOB_Q family includes a number of relaxases found in mobilisable (IncQ-family) as well as conjugative plasmids from *Proteobacteria*, *Firmicutes*, *Nitrospirae* and *Acinectobacter* spp. (Garcillan-Barcia et al., 2009). The IncQ-family is of particular interest as plasmids of this family are stably maintained and transferred among a wide range of bacteria isolated from distinct environments. Initially plasmids of IncQ-family were identified as nonself-transmissible genetic elements encoding resistance to streptomycin and sulfonamide (Barth and Grinter, 1974). They were isolated in the 1960′s and early 1970′s from various Gram-negative bacteria and subsequently proved to belong to the same incompatibility group termed IncQ (Grinter and Barth, 1976). The RSF1010 plasmid, isolated from *Escherichia coli*, is one of the most extensively studied IncQ1 plasmids and is considered one of the prototypes of the IncQ incompatibility group (Diaz and Staudenbauer, 1982; Guerry et al., 1974; Loftie-Eaton and Rawlings, 2012; Sakai and Komano, 1996; Scherzinger et al., 1984; Yau et al., 2010). RSF1010 can be replicated in many diverse bacteria in that its replication is host-independent, it is maintained at high copy number and can use a variety of type IV transportes (Meyer, 2009). On account for this the IncQ plasmids are also referred as broad-host range plasmids (Loftie-Eaton and Rawlings, 2012).

Salmonella is a Gram-negative pathogen of global concern that is capable of infecting both humans and a broad range of animals (Valdezate et al., 2007). In humans, *Salmonella* can, depending on the serotype and host, cause diseases ranging from gastroenteritis to typhoid fever. Human salmonellosis is typically caused by consumption of contaminated food with *S.* Enteritidis and *S.* Typhimurium being the serovars most commonly isolated from humans in Europe and the USA (Weill et al., 2006). However, while in Europe as a whole, *S.* Enteritidis is the predominant serovar isolated from clinical cases, in Italy *S.* Typhimurium serovar

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is most prevalent (Frasson et al., 2016; Graziani et al., 2013; Graziani et al., 2008). Gastroenteritis caused by *Salmonella* is generally self-limiting and antimicrobial therapy is usually not indicated, except for patients with invasive risk (Sanchez-Vargas et al., 2011). Antimicrobial resistance has become more prevalent in *Salmonella* since the early 1990s, with the highest rate of resistance observed in *S.* Typhimurium (Chen et al., 2013). However, the contribution of non-conjugative but mobilisable plasmids to the insurgence of multidrug resistant (MDR) *Salmonella* strains remains an understudied subject.

Here we have identified and characterised an IncQ1 plasmid (termed pNUC) conferring resistance to streptomycin, sulphametoxazole and tetracycline and harboured by clinical MDR *S.* Typhimurium strains isolated in Southern Italy between 2006 and 2009. Strains harbouring pNUC were also characterised by Pulsed Field Gel Electrophoresis (PFGE) and PCR was used to detect ARGs. Bioinformatics analyses of the complete pNUC sequence revealed that this plasmid is part of a novel group of IncQ1 plasmids harbouring the *tetA* gene. Similar plasmids can be detected among *Enterobacteriaceae* strains from clinical or animal sources with a worldwide distribution.

2. Materials and methods

2.1. Bacteria isolates and antimicrobial susceptibility testing

One hundred and thirteen MDR clinical *S.* Typhimurium strains were isolated in Southern Italy from January 2006 to December 2012 (De Vito et al., 2015). Strains were isolated from six different hospitals scattered among the neighbouring Italian regions of Apulia and Basilicata. Biochemical identification of *Salmonella* was confirmed by an automated system (Microscan, dade Behring, Milan, Italy) and serotyping was performed by agglutination tests with specific antisera O and H antigens (Bio-Rad Laboratories, Italy). Classification was performed according to the Kauffmann-White scheme (Popoff et al., 2004). All isolates were included in the database of the Enternet Surveillance Network at the Institute of Hygiene of the University of Bari (Italy) coordinated by the Istituto Superiore di Sanità, Rome (Italy).

Antimicrobial susceptibility tests (disc diffusion method) were performed as reported previously (Scrascia et al., 2009).

2.2. Bacterial conjugation, gene detection and plasmid identification

Conjugation experiments were performed at 37 °C as described previously (Scrascia et al., 2009). The frequency of transfer of a genetic marker was expressed as the number of transconjugants per recipient cell. Plasmids were typed by the PCR Based Replicon Typing protocol (PBRT) using positive controls kindly supplied by A. Carattoli (Carattoli et al., 2005). Primers used in this study were as reported previously (Camarda et al., 2013a; Camarda et al., 2013b) or, if newly designed, as reported in Table S1. The presence of IncQ1 plasmids was assessed by PCR with specific primers that target the *mobA* gene also encoding for a relaxase protein required for plasmid mobilization and the *oriV* origin of replication (Loftie-Eaton and Rawlings, 2012). Positive PCR reactions for *mobA* and *oriV* indicated the potential presence of IncQ1 plasmids.

In mobilisation experiments performed to assess pNUC transfer mediated by conjugative plasmids IncFII or IncI1, 100 single colonies (transconjugants), isolated from plates added with antimicrobial selecting for the recipient strain and the antimicrobial selecting for either the conjugative IncFII or IncI1 or for the pNUC plasmid, were replicated onto plates supplemented with single antimicrobials. Transconjugants isolated (at the highest dilution of the conjugative mix) on plates supplemented with the antimicrobial selecting for the conjugative plasmids did not grow on plates supplemented with the antimicrobial selecting for pNUC. By contrast, transconjugants selected on plates with the antimicrobial selecting for pNUC grew on plates with antimicrobial selecting for the conjugative plasmids. Experiments were performed in triplicate and no difference was detected in either replication of transconjugants or frequency of transfer.

2.3. Strain molecular typing by PFGE

Genomic restriction was performed according to the standardised *Salmonella* protocol of the CDC PulseNet as described previously (De Vito et al., 2015). Digital images of the PFGE profiles were analysed using algorithms available in the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium).

All PFGE profiles were compared with those included in the PulseNet-Europe international database and named with a six letter code followed by a four-digit numerical identifier.

2.4. Plasmid DNA sequencing, assembly and annotation

Plasmids were purified using Pure-Yield (Promega). The sul2-F (5′-3′ GACAGTTTCTCCGATGGAGGCCGGTA)/tetA-R (5′-3′ CACCCGTTCCA CGTTGTTATAGAAGC) sul2-R (5′-3′ GTGTGCGGATGAAGTCAGCTCCAC CT)/tetA-F (5′-3′ CCTGATCGTAATTCTGAGCACTGTCG) primer pairs were used to generate 2 overlapping PCR products of approximately 5 and 8 Kb respectively. 1 ng of each product was used for sequencing library construction (Nextera XT DNA Library Preparation Kit Illumina Inc.). Libraries were indexed using the Nextera XT Index Kit (96 Index, Illumina Inc.). Equimolar quantities of each library were pooled and subjected to 2 × 250 bp paired-end sequencing on the Illumina MiSeq platform. In order to increase the sample genetic diversity, the phage PhiX genomic DNA library was added as a spike-in. Sequences reads from the distinct PCR products (a total of 627,266 pairs of 2x250bp reads) were pooled, trimmed using Trimmomatic (Bolger et al., 2014) (112,462 pairs of reads for a total of 83,269,561 bases remained after trimming) and assembled using SPAdes v 3.51 (Nurk et al., 2013). Annotation was performed using the Rapid Annotation Server (RAST) (Aziz et al., 2008).

2.5. Bioinformatics analyses

Blast searches were performed using the blastN algorithm (http://ncbi.nlm.nih.gov/Blast.cgi), against the whole-genome shotgun contigs (wgs) database, limited by Bacteria (taxid:2). The pNUC sequence was analysed and visualised using SnapGene software (from GLS Biotech, available at snapgene.com).

3. Results

3.1. Plasmid detection, antimicrobial susceptibility and resistance genes

Using PCR with *mobA* and *oriV* specific primers, five clinical MDR *S.* Typhimurium strains (ST1004, ST1014, ST1016, ST1102 and ST1104) isolated from different patients and hospitals in Southern Italy (between 2006 and 2009) were found to harbour IncQ1 plasmids (Table S2). As well as IncQ1 these strains (ST1004, ST1014, ST1016, ST1102 and ST1104) also harboured a plasmid of the IncFIIs group (named pST1004, pST1014, pST1016, pST1102 and pST1104, respectively). The strains characterised by PFGE were: ST1004 pulsotype STYMXB.0179; ST1014 and ST1016 pulsotype STYMXB.0233; while ST1102 and ST1104 were not assignable to any known pulsotype (Fig. S2).

The strains ST1004, ST1014 and ST1016 were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim (termed R-type ACSSuTTp); while ST1102 and ST1104 to ampicillin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim (termed R-type ASSuTTp).

The R-type ACSSuTTp was encoded by bla_{TEM}, *cmlA1*, (*aadA1*, *aadA2*, *strAB*), (*sul2*, *sul3*), *tetA* and *dfrA12* loci respectively as determined by PCR; the R-type ASSuTTp was encoded by bla_{TEM}, (*aadA2*, *strAB*), *sul2*, *tetA* and *dfrA12* loci respectively. *sul3* is the most recently described

sulfonamide-resistance gene and has been found linked to a specific class1 integrons termed atypical *sul3*-associated integrons (Antunes et al., 2007; Perreten and Boerlin, 2003). *sul3*-associated integrons have been classified into three distinct gene cassette organizations. Specific PCRs and restriction analysis of the amplicons demonstrated that the atypical *sul3*-associated integrons in strains ST1004, ST1014 and ST1016 were of type I (5′CS-*dfrA12-orfF-aadA2-cmlA1-aadA1-qacH*-IS*440-sul3*) (Fig. S1). The *sul3*-type I integrons are present in various conjugative plasmids (Montero et al., 2013; Antunes et al., 2007). Mating experiments between the donors ST1004, ST1014, ST1016 as well as for ST1102 or ST1104 and the recipient *E. coli* CSH26 NAL strain (resistant to nalidixic acid) were performed to investigate if markers identified in these donors were transferred by conjugation. No resistance marker was transferred (detection frequency less than 1×10^{-9}). The absence of any detectable transconjugant in our experimental conditions implied that the some or all the identified resistance markers might be located either on the chromosome, on non conjugative plasmids or on non conjugative but mobilisable plasmids.

3.2. Identification and general features of pNUC

In the light of the absence of conjugative elements harbouring any of the investigated resistance markers we explored, by transformation, the linkage between the identified IncQ1 plasmids with some of the detected resistance markers. *E. coli* DH5α transformants from the five donors were obtained only on agar plates supplemented with either tetracycline or streptomycin. A plasmid of about 11 Kb, conferring resistance to streptomycin, sulfamethoxazole and tetracycline was identified from each transformant (named BA144, BA145, BA146, BA169 and BA170, respectively). Restriction analysis performed with *Eco*RI, *Eco*RV and *Pst*I did not differentiate between the plasmids harboured by the transformants (Fig. S3). Complete plasmid sequencing was performed for one isolated plasmid (from BA144) through shotgun sequencing of two overlapping PCR products, reads from which were pooled and assembled to produce a single 11.168 nt contig with over 1100 kmer coverage depth (GenBank Acc. No KU852461) with flanking direct repeats of 99 nucleotides suggesting a circular molecule of 11.069 bp. The sequence confirmed that this plasmid was of the IncQ1 group and harboured *sul2*, *strAB* and *tetA* organised as a gene cluster. The plasmid (named pNUC) was further confirmed from comparison with the plasmid database (http://cge.cbs.dtv.dk/services/PlasmidFinder/), to be consistent with the IncQ plasmid subgroup Q1.

pNUC shares three genes required for replication (*repA*, *repB′* and *repC*), those for mobilization (*mobA*, *mobB* and *mobC*) and the resistance genes *strAB* and *sul2* with RSF1010 (GenBank Acc. No M28829), a prototype for the IncQ group (Loftie-Eaton and Rawlings, 2012; Yau et al., 2010; Sakai and Komano, 1996). It also retains the IR of Tn*5393* and the *ter* end of CR2; however, the *rcr2Δ* present in RSF1010 is missing in pNUC. The additional *tetA* and *tetR* genes present in pNUC are located between the IR of Tn*5393* and the *ter* end of CR2 (Fig. 1).

IncQ1 plasmids from each transformant BA144, BA145, BA146, BA169 or BA170 were used in conjugation experiments to assess their mobilisation mediated by conjugative plasmids. All IncQ1 plasmids were mobilised by the conjugative IncFII or IncI1 plasmids employed in these experiments. Since no difference was detected in frequency of mobilisation for any of the five IncQ1 plasmids, we report data only for BA144 (Table 1). *E. coli* CSH26 NAL BA130-4 and BA132 that harboured distinct conjugative plasmids (IncFII and IncI1, respectively) acquired from *S.* Typhimurium clinical strains (unpublished data), were transformed with pNUC (from BA144). The resulting transformants, named BA131 and BA133, and the respective parental strains, BA130-4 and BA132, were used as donors in conjugation experiments with an *E. coli* DH5α rifampicin resistant mutant as recipient strain. pNUC was co-transferred with a frequency two or three orders of magnitude lower than that detected for the respective conjugative IncI1 and IncFII plasmids.

3.3. Bionformatics analyses

In-silico comparison of the pNUC sequence versus the NCBI GenBank and bacterial whole genome shotgun sequence databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed a high similarity (99% with a query cover range from 96 to 100%) with contigs from several genome assemblies from Gram-negative bacteria belonging to different *Enterobacteriaceae* genera or species (Fig. 1) as well as to plasmid pSTU288-2. A detailed analysis of the shared region from the IR of Tn*5393* to the *ter* end of CR2 highlighted the presence of three features distinguishing pNUC and a group of plasmids (termed class A) harboured by *S. enterica* subspecies enterica serovar Muenchen strains isolated from turkeys in USA 2011–2013 (e.g. GenBank Acc. No LHKR01000005 and LHJA01000017) (Fig. 1): i, a shorter DNA segment (473 rather than

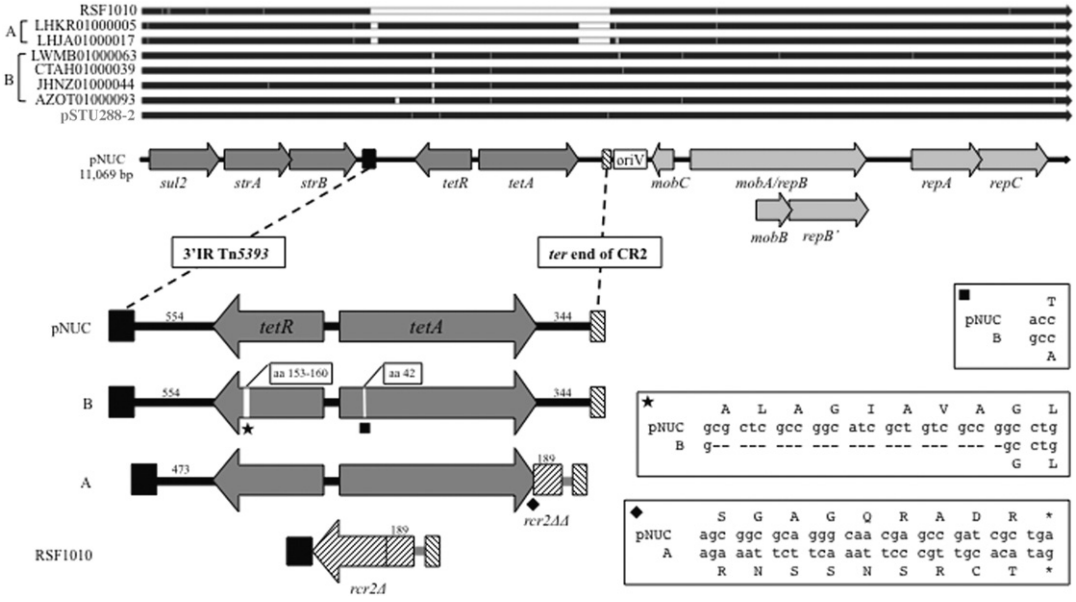

Fig. 1. Scaled linear map of pNUC. The linear map of pNUC was generated using SnapGene Viewer. Arrowheads indicate the direction of transcription. Resistance genes are shown in dark grey arrows; genes required for mobilization and replication are in light grey. *oriV* indicates the origin of replication.

Table 1
Horizontal transfer of pNUC.

Strain	Resistance(s)[a]	Resistance gene clusters	Genomic localization	Genes transferred by:		Frequency of conjugation
				Transformation	Conjugation	
ST1004	ACSSuTp	bla_{TEM}, *dfrA12-aadA2-cmlA1-aadA1-sul3*	ND	None	None	None
	SSuT	*sul2-strAB-tetA*	pNUC	*sul2-strAB-tetA*	None	None
BA130-4	ASSu	bla_{TEM}-*strAB-sul2*	IncFII	ND	bla_{TEM}-*strAB-sul2*	1×10^{-2}
BA131	ASSu	bla_{TEM}-*strAB-sul2*	IncFII	ND	bla_{TEM}-*strAB-sul2*	1×10^{-2}
	SSuT	*sul2-strAB-tetA*	pNUC	ND	*sul2-strAB-tetA*	1×10^{-5}
BA132	CSSuTp	*dfrA12-aadA2-cmlA1-aadA1-sul3*	IncI1	ND	*dfrA12-aadA2-cmlA1-aadA1-sul3*	1×10^{-2}
BA133	CSSuTp	*dfrA12-aadA2-cmlA1-aadA1-sul3*	IncI1	ND	*dfrA12-aadA2-cmlA1-aadA1-sul3*	1×10^{-2}
	SSuT	*sul2-strAB-tetA*	pNUC	ND	*sul2-strAB-tetA*	1×10^{-4}

[a] A, ampicillin; C, chloramphenicol; S streptomycin; Su, sulfamethoxazole; T, tetracycline; and Tp, trimethoprim.

554 bp) between the IR of Tn*5393* and the *tetR* gene.; ii, a new 3′ end of *tetA* (last 10 codons) overlapping the *rcr2Δ* sequence present in RSF1010 and absent in pNUC. The new 3′ end was probably due to an event of recombination generating a variable sequence of the last 9 aminoacids at the C-terminus of TetA that changed (underlined) from -L W S G A G Q R A D R- of pNUC to -L W R N S S N S R C T- in *S.* Muenchen (Hartman et al., 2003); iii, a shorter region from *tetA* to the *ter* of CR2. This region retains only 189 bp (*rcr2ΔΔ*) out of the 406 bp of *rcr2Δ* present in RSF1010; while in pNUC this region was constituted by 344 bp not found in the *S.* Muenchen.

In the same region (from the IR of Tn*5393* to the *ter* end of CR2) two other features distinguish pNUC from IncQ1 plasmids (termed class B) harboured by: the *S.* Typhimurium DT104 strain H67 (isolated from human in the UK in 1996; GenBank Acc. No CTAH01000039), the *S. enterica* strain 12 (isolated from poultry in Malaysia in 2015; GenBank Acc. No LWMB01000063), the clinical *Shigella* strain Shi06HN244 (isolated in China in 2006; GenBank Acc. No AZOT01000093) (Zhang et al., 2014) and the *E. coli* 081:NM strain 02-3012 isolated in USA (source and year of isolation were not available) (GenBank Acc. No JHNZ01000044). In detail, 8 codons were missing in the *tetR* core domain (aa 153 to 160) of the aforementioned group of strains and a missense variant in *tetA* determined the substitution (aa 42) of threonine (T) in pNUC to alanine (A).

pNUC was highly similar (99% identity over the entire sequence) to an IncQ1 plasmid (pSTU288-2) found in the *S.* Typhimurium strain U288, an emerging pathogen of pigs isolated in UK (GenBank Acc. No CP004059) (Hooton et al., 2014). This strain harboured two other plasmids in addition to pSTU288-2: a conjugative IncFIIs of 148.711 bp (pSTU288-1) and a small cryptic plasmid of 4.674 bp (pSTU288-3). pSTU288-1 is a hybrid plasmid since it is relatively closely related to the virulence plasmid pSLT of *S.* Typhimurium LT2 but harboured multiple horizontally acquired regions including an atypical *sul3*-associated integron (type I) and a *spvR-spvA* deletion following insertion of bla_{TEM} coupled by a transposon. We verified, by PCR, that such a genetic organization was not present in the IncFIIs plasmids carried by the strains ST1004, ST1014 or ST1016 and also harbouring the atypical *sul3*-associated integron (type I).

Above the linear map of pNUC (Fig. 1), a schematic diagram of linear alignment with the pNUC-like plasmids is shown as black arrows. White segments within the black arrows indicate DNA regions or single nucleotide different from pNUC. Plasmids name or the relative accession numbers and the groups of pNUC-like plasmids with distinguishing features (A and B) are reported on the left side.

Below the linear map of pNUC, the region between the 3′ IR of Tn*5393* (black box) and the *ter* of CR2 (white box with downward diagonals) is enlarged on scale. CR2 (also reported as IS*CR2*) is a small mobile genetic element that plays an important role in mobilizing several of the resistance genes found in Gram-negative bacteria (Toleman et al., 2006, Partridge and Hall, 2003).

The length of specific DNA segments is reported in number of bp. The box and the arrow white with upward diagonals represent the partial sequences of *rcr2* (189 bp for *rcr2ΔΔ* and 406 bp for *rcr2Δ*). The black diamond indicates the variable 3′ end of *tetA* present in the pNUC-like plasmids of group A. The black square and star indicate the point mutation in *tetA* and the 8 codons missing in *tetR* of the group B pNUC-like plasmids, respectively. The distinguishing features of *tetA* and *tetR* are shown in details within boxes.

4. Conclusions

Multidrug resistant bacteria have been increasingly isolated from clinical cases and the progressive diffusion of MDR pathogens or potentially pathogen bacteria has emerged as a cause of public health concern all over the world. The rate by which MDR can be acquired relays on several factors among which horizontal gene transfer plays a key role and wherein non-conjugative but mobilisable plasmids contribute to the diffusion and/or maintenance of ARGs.

In this study we report the detection of the IncQ1 plasmid pNUC conferring resistance to sulphametoxazole, streptomycin and tetracycline in five clinical MDR *S.* Typhimurium strains isolated in Southern Italy. The strains were characterised by PFGE and their pulsotypes differed from each other for up to 5 *Xba*I fragments arguing for the diffusion of a group of clonally or possibly clonally related strains (Tenover et al., 1995).

To date, only an IncQ1 plasmid (pSTU288-2) similar to pNUC has been described (Hooton et al., 2014). Here we highlight the diffusion of this new group of IncQ1 plasmid in *Enterobacteriaceae* strains of clinical or animal origin. The emergence of this group seems to have originated by a recombination between a *tetR-tetA* gene cluster (possibly derived from a Tn*1721*) and a recipient IncQ1 plasmid like RSF1010. Recombination may have occurred in the DNA region spanning from the IR of Tn*5393* and the *ter* end of CR2. The presence of distinguishing features within this region in pNUC and pNUC-like plasmids are consistent with independent recombination events generating the variability among the members of this group of plasmids.

Interestingly, the IncQ1 plasmid found in the *E. coli* 081:NM strain 02-3012 harboured an additional *dfrA14* gene (conferring resistance to trimethoprim) inserted within the *strA* gene (Trees et al., 2014). This datum highlights the potential evolution of this group of IncQ1 plasmids within the *Enterobacteriaceae* family.

It is worth mentioning that pNUC identified in our strains was not associated with the presence of any conjugative plasmids (as in the strain U288) or other self-transmissible elements (i.e. ICEs) which might have been involved with its acquisition and/or diffusion.

Tetracycline is an antibiotic discovered more than 60 years ago. It has been extensively used in clinical and non-clinical fields and the increased number of tetracycline resistant strains has mainly been the result of horizontal genes exchange carried by mobile genetic elements. The finding of this novel group of mobilisable plasmids highlights their potential contribution to the widespread of tetracycline resistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plasmid.2016.11.005.

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