Veterinary Microbiology 173 (2014) 76–83

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Dissemination of plasmid-encoded AmpC β-lactamases in antimicrobial resistant *Salmonella* serotypes originating from humans, pigs and the swine environment

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ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form 23 July 2014
Accepted 24 July 2014

Keywords:
Salmonella
Plasmids
Antimicrobial resistance and pigs
Conjugation

ABSTRACT

The aim of this study was to characterize and determine the inter-serovar exchange of AmpC β-lactamase conferring plasmids isolated from humans, pigs and the swine environment. Plasmids isolated from a total of 21 antimicrobial resistant (AMR) *Salmonella* isolates representing human clinical cases ($n = 6$), pigs ($n = 6$) and the swine farm environment ($n = 9$) were characterized by replicon typing and restriction digestion, inter-serovar transferability by conjugation, and presence of AmpC β-lactamase enzyme encoding gene bla_{CMY-2} by southern hybridization. Based on replicon typing, the majority (17/21, 81%) of the plasmids belonged to the I1-Iγ Inc group and were between 70 and 103 kb. The potential for inter-serovar plasmid transfer was further confirmed by the PCR detection of AMR genes on the plasmids isolated from trans-conjugants. Plasmids from *Salmonella* serovars Anatum, Ouakam, Johannesburg and Typhimurium isolated from the same cohort of pigs and their environment and *S.* Heidelberg from a single human clinical isolate had identical plasmids based on digestion with multiple restriction enzymes (*EcoR*I, *Hind*III and *Pst*I) and southern blotting. We demonstrated likely horizontal inter-serovar exchange of plasmid-encoding AmpC β-lactamases resistance among MDR *Salmonella* serotypes isolated from pigs, swine farm environment and clinical human cases. This study provides valuable information on the role of the swine farm environment and by extension other livestock farm environments, as a potential reservoir of resistant bacterial strains that potentially transmit resistance determinants to livestock, in this case, swine, humans and possibly other hosts by horizontal exchange of plasmids.

1. Introduction

Salmonella enterica is a major zoonotic foodborne pathogen causing self-limiting gastrointestinal disease in humans and animals. *Salmonella* is a highly diverse pathogen with more than 2500 non-typhoidal *Salmonella* (NTS) serotypes, which are considered to be potential threat to humans (CDC, 2011). The development, propagation and dissemination of antimicrobial resistance (AMR) in bacterial pathogens, including *Salmonella*, in humans, pigs and swine farm environment, have been attributed to therapeutic and sub-therapeutic use of antimicrobials for treatment, prophylaxis and growth purposes (Dorr et al., 2009; Keelara et al., 2013, 2014). Development and spread of multidrug resistance (resistance to ≥3 antimicrobials; MDR) along with resistance to fluoroquinolones and cephalosporins, antimicrobials critical in treating *Salmonella*, are considered a significant public health concerns and can potentially lead

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http://dx.doi.org/10.1016/j.vetmic.2014.07.018
0378-1135/

to treatment failure (Vo et al., 2010; Boxstael et al., 2012). MDR *Salmonella* isolates, including those resistant to 3rd generation cephalosporins, associated with different serotypes from humans, pigs, and the swine environment have been reported worldwide, including in the US (Frye and Fedorka-Cray, 2007; Patchanee et al., 2008; Eller et al., 2013; Glenn et al., 2013; Keelara et al., 2014). These MDR isolates often carry resistance genes either on the chromosome or on mobile genetic elements, such as plasmids and integrons (Aarestrup et al., 2008). Localization of AMR determinants on plasmids and their subsequent horizontal transfer via conjugation can result in their rapid spread among the susceptible bacterial populations (Juhas, 2013).

Plasmid mediated resistance has been identified for the majority of clinically important antimicrobials, including cephalosporins and quinolones (Lindsey et al., 2009; Akiyama and Khan, 2012; Seiffert et al., 2013). Plasmids carrying the AmpC β-lactamases including the bla_{CMY-2} gene are predominant among *S.* Typhimurium, *S.* Heidelberg and *S.* Newport isolates (Lindsey et al., 2009). There is a lack of studies that have demonstrated the exchange of plasmids between distinct *Salmonella* serotypes isolated from temporally and spatially related isolates.

Previously we reported *Salmonella* prevalence, AMR profile, common MDR patterns, molecular characterization of resistance determinants and genotypic similarity among *Salmonella* isolates from humans, pigs and the swine environment at various stages of production (Keelara et al., 2013, 2014). Based on genotypic and phenotypic results, we were interested in determining whether different *Salmonella* serotypes with multiple resistance pattern (including MDR) harbor or exchange similar and/or different plasmids within temporally and spatially related MDR *Salmonella* serotypes in humans, pigs and the swine environment. To address our hypothesis, we isolated and characterized plasmids from different *Salmonella* serotypes representing three different sources, including, humans, pigs and the swine environment. We performed replicon typing, conjugation assays, plasmid restriction profile analysis and southern hybridization to identify conjugative plasmids and relatedness of plasmids isolated from these three different sources. Results from our study provide valuable information on the role of swine environment as a potential reservoir of these AMR bacterial strains that potentially transmit resistance determinants to humans and pigs by horizontal exchange of plasmids between the three sources.

2. Materials and methods

2.1. Origin of Salmonella isolates

Salmonella isolates from temporally and spatially (originating from multiple counties in North Carolina) related pigs ($n = 386$), swine environment ($n = 536$) and human clinical cases ($n = 572$) were isolated and characterized for their antimicrobial susceptibility, resistance determinants and fingerprint profile (Keelara et al., 2013, 2014). The *Salmonella* isolates from pigs and their environment were collected as part of longitudinal study conducted from October 2008 to December 2011 on 30 conventional farms at different stages of production from farm to slaughter, including once at farrowing (7–10 day old), twice at each of nursery (4 and 7 weeks of age) and finishing stages (16 and 26 weeks of age), and finally once at slaughter in North Carolina. The environmental sampling from swine farms consisted of water, feed, soil and barn floor swabs. In addition, lagoon (repository of waste water draining from the barns), barn floor and inter-farm truck floor swab samples were also collected at farms. The details of the study design, sampling and microbiological methods, estimates of *Salmonella* prevalence in pigs and their environment at farm and slaughter, antimicrobial susceptibility profiles, and their phenotypic and genotypic characterizations have been reported elsewhere (Keelara et al., 2013, 2014). The conventional pigs were reared indoors and given antimicrobials for growth, prophylaxis and therapeutic purposes. The human clinical *Salmonella* isolates were temporally and spatially related and collected from North Carolina State Laboratory of Public Health (NCSLPH) during same period of longitudinal study period. Overall, we selected a total of 21 AMR *Salmonella* isolated from human clinical cases ($n = 6$), pigs ($n = 6$) and the swine environment ($n = 9$) which represented multiple resistance patterns, serotypes, stages of production and sources of origin, and were further analyzed for presence of plasmids and inter-serovar transferability in the present study.

2.2. Plasmid isolation and its characterization

The plasmids were isolated from the confirmed trans-conjugant (*Escherichia coli*) cultures following an alkaline lysis method (Sambrook and Russell, 2001). The purified plasmid DNA concentration and its purity were determined using NanoDrop (Thermo Scientific, Waltham, Massachusetts, USA). The restriction fragment analysis of the plasmid was performed using the *Eco*RI, *Hind*III and *Pst*I enzymes using a restriction enzyme kit following the manufacture guidelines (Roche Diagnostics, Mannheim, Germany). The restricted plasmid DNA was electrophoresed in a horizontal 0.7% agarose gel in Tris–acetate buffer and gels were run at 15 mA for 18 h, along with molecular marker and photographed under UV light. Polymerase chain reaction (PCR) was done to confirm the resistance determinants carried on the plasmid using purified plasmid from trans-conjugants as template. The primers of confirmed AMR genes includes ampicillin resistance encoding genes bla_{TEM} and bla_{PSE} (Carlson et al., 1999), ceftiofur and ceftriaxone resistance encoding gene bla_{CMY-2} genes (Zhao et al., 2001), kanamycin resistance coding *kn* (Frana et al., 2001), and tetracycline resistance coding *tet*(*A*), *tet*(*B*), *tet*(*C*) and *tet*(*G*) genes (Ng et al., 1999). Amplification reactions were carried out as described previously in the above studies.

2.3. Multiplex PCR for plasmid replicon typing

Plasmid replicon typing was completed to identify different plasmids types. Eighteen pairs of primers were used in five multiplex and three single PCRs to identify FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons. The PCR reaction mixture and

amplification was carried out as described previously (Carattoli et al., 2005). The purified plasmid DNA was used as template DNA. PCR running conditions used for amplifying the above replicons by multiplex PCR includes an initial denaturation for 5 min at 94.8 °C and 30 cycles of denaturation for 1 min at 95 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C, with a final extension of 7 min at 72 °C. The single PCR reactions were performed with the same amplification conditions with an annealing temperature of 52.8 °C for 30 s. The amplified products were electrophoresed in a horizontal 2% agarose gel in Tris–acetate buffer and gels were run at 90 mA for 6 h, along with molecular marker and photographed under UV light.

2.4. Conjugation experiment, selection and confirmation of trans-conjugants

The MDR *Salmonella* isolates from humans, pigs and the swine environment (Table 1) were selected as donor strains while the nalidixic acid (NAL) resistant *Escherichia coli* K-12 strain MG1655 was used as the recipient. The mating experiments were carried out on Luria Bertani (LB) plates (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) with specific antibiotics as selective markers. The antibiotics and concentrations used in the selective plates were: ampicillin (100 μg/ml), ceftiofur (8 μg/ml), ceftriaxone (10 μg/ml), kanamycin (40 μg/ml), nalidixic acid (50 μg/ml) and tetracycline (20 μg/ml). The protocol used to carry out the plasmid analysis and conjugation experiments was carried out as described previously (Gebreyes and Altier, 2002). Briefly, loop full of overnight grown donor and recipient strains were mixed on LB agar plates for mating and incubated at 37 °C for 24 h. The mixed culture was streaked on LB agar containing nalidixic acid (50 μg/ml) and respective recipient selection antibiotic and incubated at 37 °C for another 24 h. Trans-conjugants were selected and re-streaked using tooth picks onto LB plates with antibiotics and *Salmonella* (Nontyphoidal) chromogenic plates (R & F, Downers Grove, IL, USA) for confirmation of trans-conjugant *E. coli*. The donor and recipient strains with respective antibiotic were grown separately as a negative control. Further confirmation of AMR profile of trans-conjugants was performed by antimicrobial susceptibility testing against a panel of 15 antimicrobials using the broth micro-dilution method in a 96-well Sensititre™ plate CMV1AGNF (Trek Diagnostic Systems, Inc., Cleveland, OH). The panel of antimicrobials with their concentration (μg/ml) tested include: amikacin (AMI; 0.5–64), ampicillin (AMP; 1–32), amoxicillin/clavulanic acid (AUG; 0.5–32/16), ceftriaxone (AXO; 0.25–64), cefoxitin (FOX; 0.5–32), ceftiofur (TIO; 0.25–8), chloramphenicol (CHL; 2–32), ciprofloxacin (CIP; 0.015–2), gentamicin (GEN; 0.25–16), kanamycin (KAN; 8–64), nalidixic acid (NAL; 0.5–32), sulfisoxazole (FIS; 16–256), streptomycin (STR; 32–64), trimethoprim/sulfamethoxazole (SXT; 0.12/2.38–4/76), and tetracycline (TET; 4–32). Testing procedure was carried out as described in the previous study (Keelara et al., 2013). The MICs (minimum

Table 1
MDR *Salmonella* serotypes from humans, pigs and the swine environment characterized for plasmids.

Serotype	Isolate/plasmid ID[a]	Cohort[b]	Stage[c]	Source	Sample type[d]	Resistance patterns and genes[e]	Plasmid size[f] (kb)
S. Anatum	S530/pS07	C3	FA	Environment	Lagoon	AMP AUG AXO FOX TIO FIS[2]	80–100
	S595/pS01	C5	FA	Pig	Fecal	AMP AUG AXO FOX TIO TET[2,4]	103
	S732/pS02	C5	N 1	Pig	Fecal	AMP AUG AXO FOX TIO TET[1,2,4]	103
	S813/pS12	C3	N 2	Pig	Fecal	AMP AUG AXO FOX TIO TET[2,4]	103
	S814/pS16	C3	N 2	Pig	Fecal	AMP AUG AXO FOX TIO TET[2,4]	103
	S1090/pS08	C3	Slaughter	Environment	Lairage	AMP AUG AXO FOX TIO[2]	103
S. Heidelberg	S607/pS14	C3	N 1	Environment	Lagoon	AMP KAN TET[1,3,5]	60–80
S. Johannesburg	S782/pS05	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX TIO TET[2,4]	103
S. Ouakam	S770/pS03	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX FIS TIO TET[2,4]	103
S. Rissen	S1004/pS17	C3	F 2	Environment	Floor swab	TET[4]	91
	S1005/pS18	C3	F 2	Environment	Floor swab	TET[4]	91
	S1006/pS19	C3	F 2	Environment	Floor swab	TET[4]	91
S. Typhimurium	S737/pS09	C5	N 1	Pig	Fecal	AMP FIS NAL STR TET[1,4]	60–90
	S773/pS04	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX TIO TET[1,4]	103
	S1128/pS20	C5	Slaughter	Pig	MLN	AMP CHL FIS NAL STR TET[1,4]	60–80
S. Heidelberg	HS1242/pHS01	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO TET KAN STR[1–3,5]	103
S. Muenchen	HS295/pHS03	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO[1,2]	80–100
S. Typhimurium	HS383/pHS04	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO[1,2]	80–100
	HS758/pHS06	N/A	N/A	Human	N/A	AMP AUG AXO FOX FIS TIO TET KAN STR[1–3,5]	80–100
	HS1014/pHS02	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO[2]	80–100
	HS1019/pHS05	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO[2]	80–100

Genes: [1]bla_{TEM}, [2]bla_{CMY-2}, [3]*kn*, [4] *tet*(*A*) and [5]*tet*(*B*).

[a] S, *Salmonella*; pS, plasmid *Salmonella*; HS, human; pHS, plasmid human *Salmonella*.

[b] C3 and C5: conventional pigs and their environment belongs to cohort 3 and cohort 5 respectively; N/A, not applicable.

[c] FA, farrowing; N 1, nursery 1; N 2, nursery 2; F 2, finishing 2; N/A, not applicable.

[d] MLN, mesenteric lymph node.

[e] AMP, ampicillin; AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; FIS, sulfisoxazole; FOX, cefoxitin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; TIO, ceftiofur.

[f] Plasmid size range: its approximate determination of plasmid size based on restriction profile.

inhibitory concentrations) were recorded and breakpoints were determined based on CLSI recommendations (CLSI, 2010).

2.5. Southern hybridization

Southern blot analysis was done in order to confirm the relatedness of plasmids from *Salmonella* serotypes of three different sources of origin by hybridizing the AmpC bla_{CMY-2} gene, which encodes resistance to ceftiofur/ceftriaxone. Representative isolates selected for the southern hybridization include *Salmonella* serovar Anatum (pig origin, S595/pS01, AMR pattern: AMP AUG AXO FOX TIO TET), *Salmonella* serovar Anatum (pig origin, S732/pS02, AMR pattern: AMP AUG AXO FOX TIO TET), *Salmonella* serovar Ouakam (swine farm environment origin, S770/pS03, AMR pattern: AMP AUG AXO FOX TIO TET FIS), *Salmonella* serovar Typhimurium (swine farm environment origin, S773/pS04, AMR pattern: AMP AUG AXO FOX TIO TET), *Salmonella* serovar Johannesburg (swine farm environment origin, S782/pS05, AMR pattern: AMP AUG AXO FOX TIO TET), *Salmonella* serovar Heidelberg (human origin, HS1242/pHS01, AMR pattern: AMP AUG AXO FOX TIO TET KAN STR). These *Salmonella* strains from pigs and their environment were isolated from same cohort (C5) at farrowing and nursery 1 production stage of conventional pigs (Table 1). The protocol was carried out as prescribed previously (Sandvang et al., 1998; Gebreyes and Thakur, 2005). Briefly, purified plasmid DNA was digested with a restriction enzyme (*EcoRI*) for 4 h and separated by gel electrophoresis on 1% agarose gel in 1X TAE (Tris–acetate–EDTA) buffer. Blotting was done by transferring restricted plasmid DNA onto a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN, USA) in transfer buffer with capillary action overnight at room temperature. After blotting, DNA on the nylon membrane was immobilized by UV cross-linking for 2 min in a Stratalinker apparatus (Stratagene, La Jolla, California, USA). A digoxigenin (DIG) labeled bla_{CMY-2} specific detection probe was generated using random primers according to the manufactures protocol. Prehybridization using DIG Easy Hyb solution followed and hybridization of the membrane with the denatured DIG labeled probe was carried out at 50 °C overnight with gentle agitation. After hybridization, the DIG labeled probe that was bound to the membrane was detected by chemiluminescence with anti-DIG alkaline phosphate and substrate after incubation at 37 °C for 10 min.

3. Results

3.1. Plasmid isolation and its characterization

Plasmids were isolated from all the 21 AMR *Salmonella* isolates in our study by the alkaline lysis method. The plasmids were restricted with the *EcoR*I enzyme to determine the size and relatedness of plasmids based on restriction profiles with different serotypes and AMR profile. Human clinical *Salmonella* isolates with a similar MDR pattern (AMP AUG AXO FOX TIO) including *S.* Muenchen (HS295/pHS03) and *S.* Typhimurium (HS383/pHS04; HS1019/pHS05; HS758/pHS06), had different plasmid profiles based on restriction analysis. The estimated size of the plasmids from these isolates was between 80 and 100 kb (Fig. 1). Similarly, plasmids from the TET resistant *S.* Rissen of swine farm environment origin had a different restriction profile and a 91 kb size plasmid (Fig. 1).

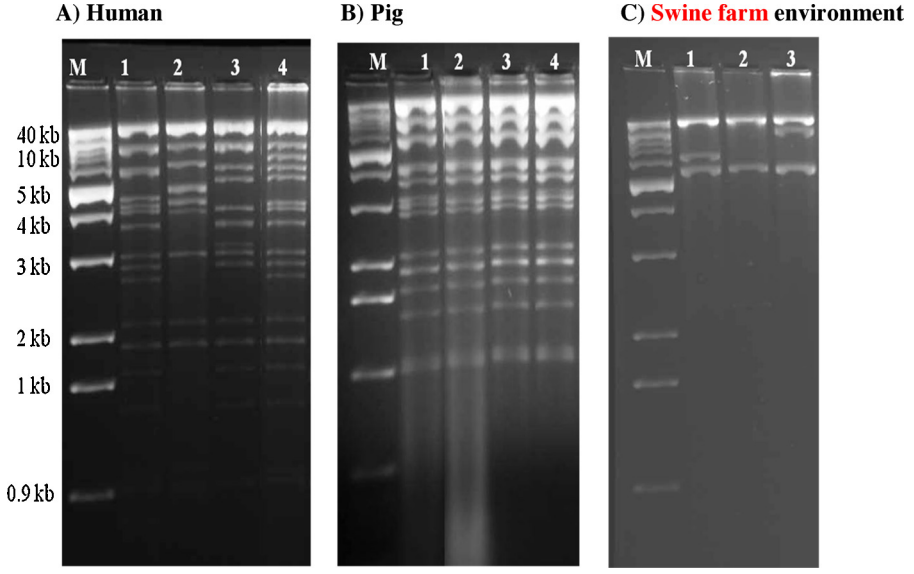

Fig. 1. Plasmid restriction analysis with *EcoR*I enzyme of *Salmonella* serotypes. (A) Plasmid restriction analysis of *Salmonella* isolated from human clinical cases with AMR pattern AMP AUG AXO FOX TIO. Lane M: 40 kb marker, lane 1, *S.* Muenchen (HS295/pHS03); lane 2, *S.* Typhimurium (HS383/pHS04); lane 3, *S.* Typhimurium (HS1019/pHS05); lane 4, *S.* Typhimurium (HS758/pHS06). (B) Pig *Salmonella* Anatum isolates with AMR pattern AMP AUG AXO FOX TIO TET. Lane M: 40 kb marker, lane 1, S595/pS01; lane 2, S732/pS02; lane 3, S813/pS12; lane 4, S814/pS16. (C) Plasmids restriction analysis of *Salmonella* Rissen isolated from the swine farm environment which are resistant to TET. Lane M: 40 kb marker, lane 1, S1004/pS017; lane 2, S1005/pS018; lane 3, S1006/pS019).

Plasmids isolated from *Salmonella* serotypes belonging to the same cohort of pigs and their environment including *S.* Anatum (pig origin, S595/pS01 and S732/pS02), *S.* Ouakam (swine farm environment origin, S770/pS03), *S.* Typhimurium (swine farm environment origin, S773/pS04), *S.* Johannesburg (swine farm environment origin, S782/pS05) and human clinical *S.* Heidelberg strain (HS1242/pHS01) were approximately 103 kb size. The restriction analysis of these plasmids with the *Eco*RI enzyme exhibited a similar restriction profile, suggesting the presence of the same plasmid (Fig. 2). Further analysis of these isolates with two additional restriction enzymes (*Hind*III and *Pst*I) confirmed the similar restriction profile, except the human origin *S.* Heidelberg isolate which exhibited a different pattern when restricted with the *Hind*III enzyme (Fig. 2). The remaining *Salmonella* isolates had different restriction profiles when restricted with the *Eco*RI enzyme and the approximate sizes of plasmids isolated from these serotypes are listed in Table 1. The presence of AMR genes on all the 21 plasmids isolated in this study were detected by PCR including ampicillin resistance encoding gene bla_{TEM}, ceftiofur and ceftriaxone resistance encoding gene bla_{CMY-2} gene, kanamycin resistance coding *kn* and tetracycline resistance coding *tet*(*A*) and *tet*(*B*) (Table 1).

3.2. Multiplex PCR for plasmid replicon typing

A PCR based replicon typing consisting of five multiplex and three single PCRs reactions were carried out to identify different types of plasmids. All 21 plasmids isolated from different *Salmonella* serotypes (Table 1) were screened. The predominant replicon type identified in our study was I1-Iγ Inc group (17/21, 81%), characterized by an amplicon size of 137 bp (Fig. 3). A plasmid from the *S.* Typhimurium isolate with the AMR pattern AMP FIS NAL STR TET was typed as the FIA Inc group with an amplicon size of 462 bp (Fig. 3). Three plasmids isolated from the *S.* Rissen isolates

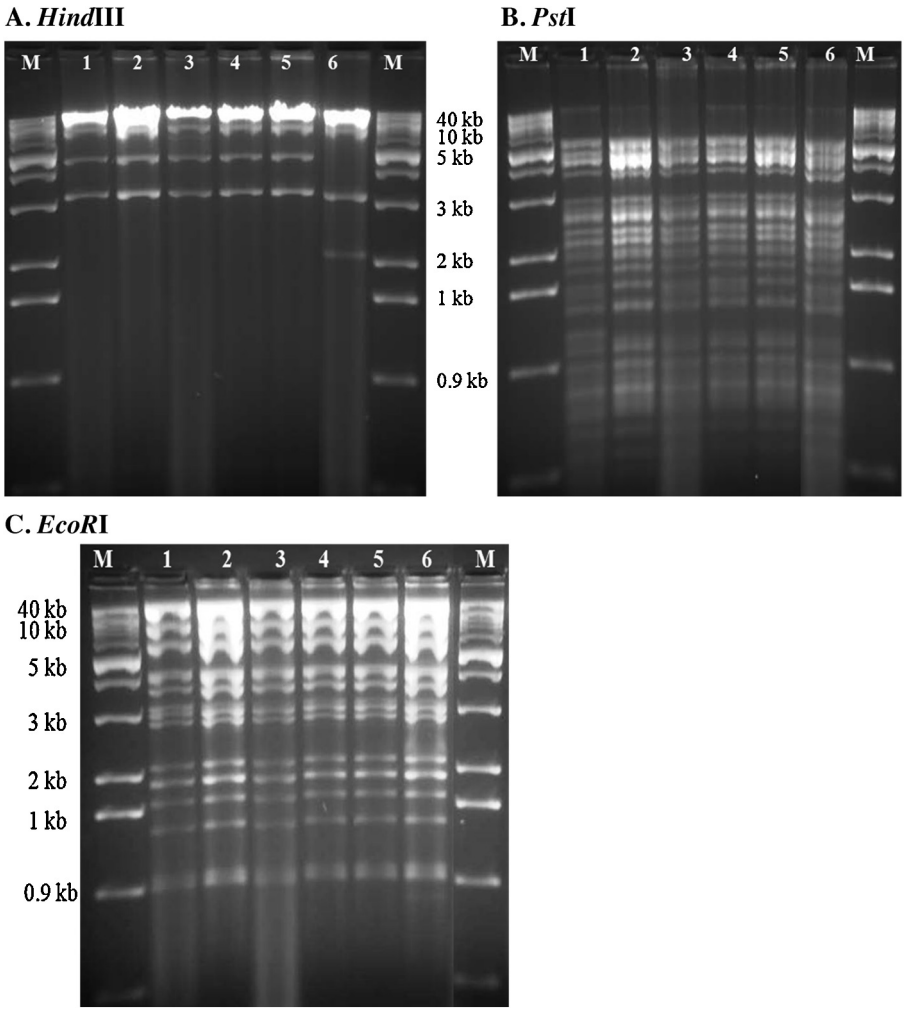

Fig. 2. Plasmid restriction of β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and the swine farm environment. Plasmid restriction analysis of *Salmonella* isolated from humans, pig and the swine environment (belongs to same cohort and farm) with following restriction enzymes. (A) *Hind*III; (B) *Pst*I; (C) *Eco*RI. Lanes M: 40 kb marker; lane 1, *S.* Anatum (pig origin, S595/pS01); lane 2, *S.* Anatum (pig origin, S732/pS02); lane 3, *S.* Ouakam (swine farm environment origin, S770/pS03); lane 4, *S.* Typhimurium (swine farm environment origin, S773/pS04); lane 5, *S.* Johannesburg (swine farm environment, S782/pS05); lane 6, *S.* Heidelberg (human origin, HS1242/pHS01).

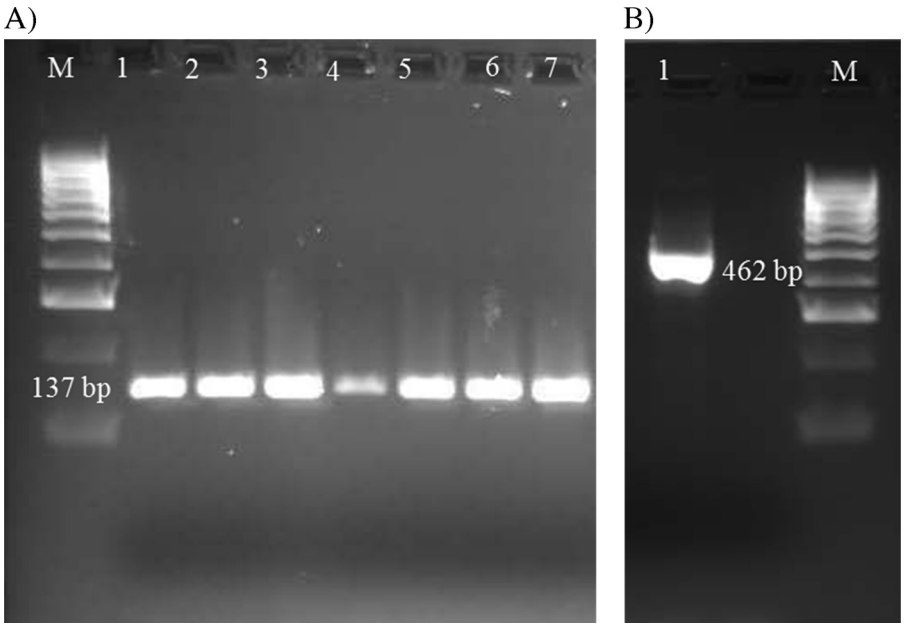

Fig. 3. Multiplex PCR for plasmid replicon typing. (A) Lane M: 1 kb DNA marker; lane 1–7: *Salmonella* serotypes amplifying 137 bp amplicon corresponding to Inc I1-Iγ plasmid type. (B) Lane M: 1 kb DNA marker; lane 1: *S.* Typhimurium plasmid with an amplicon size of 462 bp corresponding to Inc FIA plasmid type.

(TET Pattern) did not belong to any Inc group we tested in this study.

3.3. Conjugation experiment, selection and confirmation of trans-conjugants

The conjugation experiment was carried out to isolate and determine the presence of conjugative plasmids from MDR *Salmonella* strains representing humans, pigs and the swine environment. The MDR *Salmonella* isolates represented in Table 1 were selected as donor strains and NAL^R *E. coli* K-12 strain MG1655 was used as the recipient strain. All the 21 AMR *Salmonella* isolates from the three sources of origin had conjugative plasmids and were isolated from confirmed trans-conjugants *E. coli* (Table 1). We also performed the same conjugation experiment to evaluate inter-serovar transmission of resistance determinants among *Salmonella* serotypes represented in Fig. 2. Plasmids from *Salmonella* isolates with MDR pattern AMP AUG AXO TIO TET (*S.* Anatum, $n = 4$; *S.* Johannesburg, $n = 1$and *S.* Typhimurium, $n = 1$) were successfully transferred to the recipient *E. coli* strain. The trans-conjugants were selected on LB plates with NAL, AMP, TIO and TET antibiotics as the marker. Similarly, *Salmonella* serotypes with the MDR pattern AMP AUG AXO FOX TIO (*S.* Anatum, $n = 1$; *S.* Muenchen, $n = 1$ and *S.* Typhimurium, $n = 3$) were selected on the above mentioned marker plates, without TET. The plasmid from *S.* Rissen (TET resistant) isolated from trans-conjugants was selected on NAL and TET marker LB plates. The antimicrobial susceptibility testing of trans-conjugants was conducted on 96 well-sensititre plates against a panel of 15 antimicrobials. The susceptibility results matched 100% with AMR profiles and MIC values of donor strains confirming effective transfer of plasmids from the donors to the recipient strain.

3.4. Southern hybridization

Southern blot analysis was carried out to confirm and compare the relatedness of plasmids of six *Salmonella* strains including a *S.* Heidelberg from a human clinical case ($n = 1$), *S.* Anatum from pig fecal at farrowing and nursery stage, and *S.* Ouakam, *S.* Typhimurium, and *S.* Johannesburg from swine farm environment samples (lagoon) at the nursery stage. These *Salmonella* strains from pigs and their environment were isolated from same cohort (C5) at farrowing and nursery 1 production stage of conventional pigs. Southern hybridization was performed using a DIG labeled gene probe specific bla_{CMY-2} gene, which hybridized the gene on all the six *Salmonella* plasmids at the same location (4 kb), confirming relatedness of plasmids as shown in Fig. 4.

4. Discussion

Plasmids are considered as backbone of AMR determinant persistence and propagation in the bacterial population. Identification and characterization of plasmids carrying multiple resistance determinants and their exchange among multiple *Salmonella* serotypes from humans, pigs and the swine environment is a basic element in understanding the spread and persistence of AMR in the food chain. Therefore, the aim of this study was to characterize and determine the inter-serovar exchange of plasmids isolated from temporally and spatially related humans, pigs and the swine environment. In this study, we selected 21 AMR *Salmonella* strains from humans, pigs and the swine environment that were representative of multiple resistance patterns, serotypes, stages of production, and genotypic characteristics. The AMR *Salmonella* strains characterized in this study were part of longitudinal

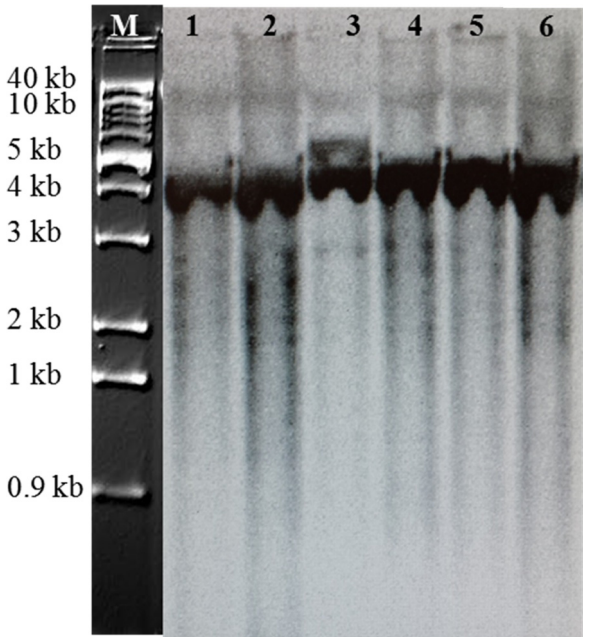

Fig. 4. Southern hybridization of bla_{CMY-2} gene in β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and the swine farm environment. Southern hybridization of all six MDR *Salmonella* isolates with DIG labeled bla_{CMY-2} gene. Lanes M: 40 kb marker; lane 1, *S.* Anatum (pig origin, S595/pS01); lane 2, *S.* Anatum (pig origin, S732/pS02); lane 3, *S.* Ouakam (swine farm environment origin, S770/pS03); lane 4, *S.* Typhimurium (swine farm environment origin, S773/pS04); lane 5, *S.* Johannesburg (swine farm environment, S782/pS05); lane 6, *S.* Heidelberg (human origin, HS1242/pHS01). All the serotypes irrespective of sources had the gene on the same restriction fragment, which was 4 kb.

study by our group involving temporally and spatially related *Salmonella* isolates from humans, pigs and the swine environment (Keelara et al., 2013, 2014). Briefly, the frequency of AMR and MDR was higher in *Salmonella* isolates of pig origin (AMR: 82.6%; MDR: 36%) followed by the swine environmental (AMR: 67.6%; MDR: 21.4%) and human clinical isolates (AMR: 28%; MDR: 21.3%). We found identical resistance patterns, resistance determinants, and fingerprint profiles among *Salmonella* strains among the three sources. In addition, we detected the mobile genetic elements, Class I and Class II integrons in MDR *Salmonella* isolates, potentially suggesting their importance in the transmission of AMR to susceptible population.

We isolated plasmids from all 21 selected AMR *Salmonella* strains, resulting in plasmids ranging in size from 60 kb to 103 kb based on restriction analysis. These results support the previous studies reporting relatively larger plasmids from *Salmonella* strains (Giles et al., 2004; Mataseje et al., 2010; Carattoli, 2011). Previous studies have reported plasmids from *S.* Muenchen and *S.* Typhimurium of human origin that exhibited a different plasmid profile even though they shared an identical MDR pattern (AMP AUG AXO FOX TIO), suggesting diversity of plasmids carrying similar resistance determinants (Winokur et al., 2000). However, we found a similar plasmid profile among *Salmonella* serotypes with the same MDR pattern AMP AUG AXO FOX TIO TET from pig and their environmental origin (Fig. 1). We isolated plasmids from three *S.* Rissen isolates of swine farm environment origin that were resistant to a single antimicrobial (TET). The plasmid size from *S.* Rissen was 91 kb, which was in contrast with a recent study reporting a 30 kb plasmid from *S.* Rissen strains resistant to multiple antimicrobials, including tetracycline (Antunes et al., 2011). To our knowledge, this is first report of plasmid-mediated resistance in a *S.* Rissen isolate from a farm environment origin in the United States. *Salmonella* Rissen is rarely found in the US; less than 20 isolates per year were isolated from humans from 1999 to 2007, and there were no reports of its occurrence in food animals in the US (CDC, 2011). It was reported to have entered the US in late 2008 and early 2009 through imported white pepper, resulting in a human outbreak in northern California and Nevada (CDC, 2010). This serotype has been reported to rapidly disseminating in the swine environment as reported previously (Keelara et al., 2013). Identification of plasmid mediated resistance mechanism in this serotype is a concern and could potentially have helped this serotype to adapt and establish itself in the food animal production systems in the US. Further sequence analysis of this plasmid is necessary to determine its structure and identify other AMR determinants that could be present on it.

Replicon typing by PCR clustered the plasmids in the Inc group I1-Iγ. The majority of the plasmids encoding resistance to cephalosporins (bla_{CMY-2} gene) identified in *Salmonella* in the US are categorized in the Inc A/C group and this plasmid is broadly disseminated among zoonotic foodborne pathogens (Zhao et al., 2001; Giles et al., 2004; Mulvey et al., 2009; Lindsey et al., 2009). However, in our study the AmpC β-lactamase encoding bla_{CMY-2} gene associated with Inc I1-Iγ plasmid suggests a diversified population of plasmids encoding resistance to the β-lactam group of antimicrobials.

All the plasmids isolated in this study have the potential to transfer resistance determinants among different *Salmonella* serotypes as reported previously (Gebreyes and Thakur, 2005; Moodley and Guardabassi, 2009; Akiyama and Khan, 2012). We evidenced inter-serovar exchange of resistance determinants among *Salmonella* strains including *S.* Anatum (pig origin) *S.* Typhimurium, *S.* Ouakam and *S.* Johannesburg (swine environmental origin) belonging to same cohort of pigs with multiple AMR patterns and a human clinical *S.* Heidelberg with a similar plasmid profile as reported previously (Gebreyes and Thakur, 2005; Patchanee et al., 2008; Moodley and Guardabassi, 2009). Identification of similar conjugal plasmids among different *Salmonella* serovars highlights the establishment of plasmids in different serotypes. In addition, plasmids of swine farm environment origin and exchange between human and pig strains highlight the key role of the farm environment as a source of resistant bacteria and resistance genes for both animals and humans. These plasmids carried resistance determinants encoding for resistance to 3rd cephalosporins. PCR identification and confirmation of similar resistance determinants on these plasmids highlights the role of plasmids in the transmission of AMR determinants encoding resistance to critically important antimicrobials used in human, veterinary medicine. In addition, makes it difficult for clinicians to treat infections and public health agencies to control spread of resistance determinants to susceptible

bacterial populations thereby impacting human health (Mataseje et al., 2010; Carattoli, 2011; Eller et al., 2013). Southern hybridization of a DIG labeled bla_{CMY-2} gene on the plasmid confirmed the relatedness and inter-serovar exchange of identical plasmids among three different sources of origin as reported for kanamycin gene (Gebreyes and Thakur, 2005). In conclusion, we identified and characterized identical conjugal plasmids responsible for inter-serovar exchange of resistance determinants among humans, pigs and the swine environment. The study results underscore the role of swine environment as a reservoir for resistance determinants which contributes to rapid dissemination of antimicrobial resistance in the food chain. The detection of trans-conjugal plasmids in emerging and predominant *Salmonella* serotypes of human, pig and the swine environment is concerning since these serotypes are commonly associated with foodborne outbreaks and human sporadic illness.

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