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Impact of co-carriage of IncA/C plasmids with additional plasmids on the transfer of antimicrobial resistance in *Salmonella enterica* isolates

Jing Han[a,*,1], Sean J. Pendleton[a,1], Joanna Deck[a], Ruby Singh[b], Jeffrey Gilbert[b], Timothy J. Johnson[c], Yasser M. Sanad[a,d], Rajesh Nayak[a], Steven L. Foley[a,*]

[a] Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States
[b] Division of Human Food Safety, Office of New Animal Drug Evaluation, Center for Veterinary Medicine, U.S. Food and Drug Administration, Rockville, MD 20855, United States
[c] Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, United States
[d] Department of Agriculture, University of Arkansas at Pine Bluff, Pine Bluff, AR 71601, United States

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ABSTRACT

Background: Antimicrobial resistance in *Salmonella enterica* is often plasmid encoded. A key resistance plasmid group is the incompatibility group (Inc) A/C plasmids that often carry multiple resistance determinants. Previous studies showed that IncA/C plasmids were often co-located with other plasmids. The current study was undertaken to evaluate the impact of plasmid co-carriage on antimicrobial resistance and plasmid transfer.

Methods: A total of 1267 *Salmonella* isolates, representing multiple serotypes and sources were previously subjected to susceptibility testing and 251 isolates with resistance to at least 5 antimicrobial agents were identified for further study. Each isolate was subjected to PCR-based replicon typing, and those with IncA/C plasmids were selected for plasmid isolation, PCR-based mapping of IncA/C plasmid backbone genes, and conjugation assays to evaluate resistance plasmid transferability.

Results: Of the 87 identified IncA/C positive isolates, approximately 75% carried a plasmid with another identified replicon type, with the most common being I1 (39%), FIA, FIIA, FIB and HI2 (each 15%). PCR-based mapping indicated significant diversity in IncA/C backbone content, especially in regions encoding transfer-associated and hypothetical proteins. Conjugation experiments showed that nearly 68% of the isolates transferred resistance plasmids, with 90% containing additional identified plasmids or larger (> 50 kb) non-typeable plasmids.

Conclusions: The majority of IncA/C-positive strains were able to conjugally transfer antimicrobial resistance to the recipient, encoded by IncA/C and/or co-carried plasmids. These findings highlight the importance of co-located plasmids for resistance dissemination either by directly transferring resistance genes or by potentially providing the needed conjugation machinery for IncA/C plasmid transfer.

1. Introduction

Salmonella enterica is one of the major causes of foodborne illnesses throughout the world. According to the Centers for Disease Control and Prevention (CDC), the top laboratory-confirmed serotypes causing human *Salmonella* infections over the last half century have been Typhimurium, Enteritidis, Newport, Heidelberg, Infantis and Javiana (Centers for Disease Control and Prevention, 2013b). These serovars comprised over 47% of the human infections caused by *Salmonella* in 2013 (Centers for Disease Control and Prevention, 2016). The emergence and spread of antimicrobial-resistance strains of *Salmonella*, especially multidrug resistant strains, has become an important health concern worldwide and complicated antibiotic treatments (Centers for Disease Control and Prevention, 2015). According to the 2013 National Antimicrobial Resistance Monitoring System (NARMS) report from CDC, the prevalence of isolates of nontyphoidal *Salmonella* resistant to one or more classes of antimicrobials was higher in 2013 than from 2008 to 2012 (Centers for Disease Control and Prevention, 2015). Infections caused by antimicrobial-resistant strains are usually more severe and have higher hospitalization rates, resulting in higher economic

* Corresponding authors at: Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, United States.
E-mail addresses: Jing.han1@fda.hhs.gov (J. Han), steven.foley@fda.hhs.gov (S.L. Foley).
[1] Authors contributed equally to the project.

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cost (Centers for Disease Control and Prevention, 2013a).

Many antimicrobial resistance genes of Gram-negative bacteria are encoded on transmissible plasmids, which provide a means for rapid dissemination of multidrug resistance (MDR) phenotypes among different bacteria (Chen et al., 2005). Several MDR-encoding plasmids representing different incompatibility (Inc) groups have been identified in *Salmonella* (Couturier et al., 1988; Han et al., 2012a, 2012b; Rankin et al., 2005; Welch et al., 2007); among them, a key group that has attracted increasing attention is the IncA/C plasmids (Fricke et al., 2009; Garcia et al., 2011; Han et al., 2012b; Lindsey et al., 2009). The widespread distribution of IncA/C plasmids among *S. enterica* isolates has caused considerable concern in the public health community because many carry resistance genes such as $bla_{CMY\text{-}2}$, which encodes a beta-lactamase that confers resistance to medically important extended-spectrum cephalosporins (Han et al., 2012a, 2012b; Hopkins et al., 2006; Wiesner et al., 2011, 2013).

Many plasmids carry genes that facilitate their own conjugal transfer within bacteria communities. Others plasmids may lack necessary conjugal transfer genes; however, they can be transferred with the help of another conjugative plasmid co-residing in the bacterial cell (Bennett, 2008). The conjugative transferability and transfer rates of IncA/C plasmids vary significantly (Poole et al., 2009; Welch et al., 2007; Wiesner et al., 2013). In previous studies from our group and others, it appeared that IncA/C plasmids were often co-located with additional plasmids, which may also play a role in antimicrobial resistance, virulence or ability to transfer plasmids (Evershed et al., 2009; Han et al., 2012a, 2012b; Poole et al., 2009). Understanding the factors that contribute to antimicrobial resistance and their transfer is important for identifying strategies to limit the further development and spread of antimicrobial resistance among enteric pathogens. Therefore, the current study was undertaken to evaluate co-localization of IncA/C plasmids with other plasmids in antimicrobial resistant *Salmonella*, their transferability, and the conjugation efficiencies of strains with the different plasmid configurations.

2. Materials and methods

2.1. Bacterial Strains

A total of 1276 *Salmonella enterica* isolates from serovars Typhimurium, Enteritidis, Newport, Javiana, Heidelberg, Kentucky, Dublin, Choleraesuis, Infantis and others that had previously undergone antimicrobial susceptibility testing (AST) were evaluated (Foley et al., 2006; Han et al., 2011; Kaldhone et al., 2008; Lynne et al., 2008, 2009a, 2009b; Marrero-Ortiz et al., 2012; Melendez et al., 2010) and those isolates with resistance to at least 5 antimicrobial agents, regardless of drug class, were selected for further characterization. In most of the original studies, the isolates were screened for resistance to 15 different antimicrobials tested by NARMS (Centers for Disease Control and Prevention, 2015). The 251 identified multidrug resistant (MDR) isolates included those belonging to serovars Typhimurium (n = 122), Heidelberg (n = 68), Newport (n = 29), Dublin (n = 19), Kentucky (n = 10), Infantis (n = 2) and subspecies diarizonae (IIIB) (n = 1). These isolates were initially collected from different sources including turkey, chicken, cattle, swine, humans and farm and environmental sources. The isolates were maintained in brain heart infusion broth containing 20% glycerol at −80 °C for the duration of the study. Isolates were removed from the freezer and streaked on tryptic soy agar (TSA) supplemented with 5% sheep's blood (Remel, Lenexa, KS) and incubated at 37 °C for 18 to 24 h before analysis.

2.2. Plasmid replicon (incompatibility) typing

The MDR *Salmonella* isolates were examined for the presence of 18 plasmid replicons (B/O, A/C, T, FIC, P, Y, K/B, FIIA, N, W, FIA, FIB, I1, X, HI2, Frep, HI1, and L/M) using the PCR-based methods described by Johnson et al. (2007). PCR products were separated using 48-well 2% *E*-gels (Invitrogen) and compared to positive controls provided by Dr. Alessandra Carattoli et al. (2005). If an amplicon of the expected size was observed in the sample and positive control, then an isolate was considered positive for that particular gene. In cases of ambiguous multiplex results, PCR reactions were repeated using single pair primers.

2.3. Plasmid profile analysis

Plasmids of IncA/C positive isolates were isolated using the QIAGEN® Plasmid Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. The isolated plasmids were separated in 0.7% SeaKem® LE agarose (Cambrex Bio Science, Rockland, ME) gels prepared with 1× Tris-borate-EDTA (TBE) buffer (Fisher Biochemicals, Baltimore, MD) at 4 V/cm for 6 h at room temperature, and stained with Lonza GelStar® Nucleic Acid Gel Stain (Cambrex). The stained gels were visualized under ultraviolet light and plasmid sizes were analyzed by comparison to a supercoiled DNA standard using the BioNumerics software program (ver. 6.0, Applied Maths, Austin, TX).

2.4. IncA/C plasmid region analysis

To determine approximate core gene structure of IncA/C plasmids, PCR-based methods were used to detect the presence of 12 distinct plasmid backbone regions in the IncA/C positive isolates using primer sets previously described (Welch et al., 2007). Amplicons were visualized on 2% E-gels run according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). If an amplicon of the expected size was observed in the sample, an isolate was considered positive for that particular region. In cases of ambiguous multiplex results, PCR reactions were repeated.

2.5. Plasmid transfer experiment

To evaluate the transferability of the Inc A/C plasmids, conjugation experiments were carried out using methods previously described (Welch et al., 2007) with minor modification. Sodium azide-resistant *E. coli* J53 was used as a recipient and 87 identified IncA/C positive isolates were used as donor strains. Briefly, overnight cultures of 10^8 donor and recipient cells were mixed and spotted onto LB agar plates. After 3 h incubation at 37 °C, the cells were resuspended in 1 ml of phosphate buffered saline (PBS), serially diluted, and plated onto LB agar containing tetracycline (10 μg/mL), LB agar containing sodium azide (350 μg/mL), and LB agar containing both tetracycline (10 μg/mL) and sodium azide (350 μg/mL) for transconjugant selection, respectively. The colonies on the plates were counted after overnight incubation at 37 °C and conjugation efficiency was calculated by dividing the number of transconjugants counted by the number of donors counted. To identify which plasmids were transferred, four transconjugants were randomly picked from each plate and subjected to replicon typing (described above) using the primers for replicons identified in the donor strains.

3. Results

3.1. Plasmid replicon (incompatibility) typing

To identify the MDR resistant isolates for this study, the AST profiles for 1267 *Salmonella* isolates were evaluated and 251 (19.8%) were resistant to at least five antimicrobials. It is important to note that these isolates were originally collected for other studies and the prevalence of MDR isolates may not be representative of the prevalence in the population. Among these 251 MDR isolates, 214 (85.3%) isolates contained plasmids with identified incompatibility (Inc) groups. Approximately 66% (142/214) contained more than one identifiable