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Occurrence of *spvA* Virulence Gene and Clinical Significance for Multidrug-Resistant *Salmonella* Strains▽

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Nontyphoidal *Salmonella* strains are important reservoirs of antimicrobial resistance. An important issue that has not been investigated is whether the multiresistant *Salmonella* strains are more virulent than their susceptible counterparts. *Salmonella* isolates collected from clinical human ($n = 888$) and porcine ($n = 2,120$) cases at the same time period and geographic location were investigated. Antimicrobial susceptibility, PCR analysis for the *spvA* virulence gene, and pulsed-field gel electrophoresis (PFGE) genotyping were done. Carriage of *spvA* was associated with multidrug-resistant (MDR) type ACSSuT strains (odds ratio, 7.1; $P < 0.05$), a type often implicated in bacteremic human cases. PFGE revealed that clinical isolates from pigs were more clonally related to those of human origin than the nonclinical porcine isolates. The findings suggest that MDR strains that also carry specific virulence factors are more likely to be of clinical significance.

Multidrug-resistant (MDR) *Salmonella* strains have been among the major public health concerns worldwide, and pigs are known to be important reservoirs of *Salmonella* spp. (4, 9, 11, 12). MDR strains of *Salmonella* exhibit various resistance patterns (R-type), including pentaresistance. MDR with the ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) resistance pattern has been commonly reported. Among several other predominant R-types that were frequently reported for *Salmonella* strains is R-type AKSSuT (ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline); these strains were found to be most commonly in serogroup B, such as *Salmonella enterica* serovar Typhimurium (2, 3, 10, 19). A very important issue that has been debated is whether the MDR strains are associated with virulence determinants and thus have the propensity to be more virulent than their susceptible counterparts. Few studies attempted to address this question. While some of the studies were based on clinical data with various patient-based outcomes, such as length of hospitalization or occurrence of systemic infections (20), others were conducted under experimental conditions more focused on the chromosomally encoded *Salmonella* pathogenicity island (5). However, there is no information on the role of important plasmid-borne virulence factors that also often carry multiresistance.

One important virulence factor located on a plasmid previously shown to be common among predominant nontyphoidal serovars of *Salmonella* spp. is the *spv* operon which contains five genes (*spvRABCD*) (13, 14). One main function of the *spv* operon is to potentiate the systemic spread of the pathogen (7). In the current study, we investigated whether one of the most important genes in this operon, *spvA*, is associated with MDR and whether there is variation in *spvA* carriage among various MDR strains from clinical (human and porcine) and nonclinical (porcine) settings.

A total of 888 *Salmonella* isolates obtained from clinical human cases during the 2000 to 2004 period were retrieved from the North Carolina State Laboratory of Public Health. Additionally, 2,120 isolates that were collected from swine in North Carolina during the same time period were included. A subset of 247 *Salmonella* isolates of porcine origin from clinical ($n = 123$) and nonclinical ($n = 124$) sources and 50 of human origin were further analyzed for carriage of the gene *spvA*.

Salmonellae were isolated using conventional methods described previously (1) and serogrouped using polyvalent and group-specific antisera (Statens Serum Institut, Copenhagen, Denmark) as recommended by the manufacturer. Selected isolates were also serotyped (USDA-NVSL, Ames, IA).

All isolates were tested for susceptibility to 12 antimicrobial agents using the Kirby-Bauer disk diffusion method. Antimicrobials tested and respective disk potencies were as follows: ampicillin (10 mg), amoxicillin-clavulanic acid (30 mg), amikacin (30 mg), ceftriaxone (30 mg), cephalothin (30 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), gentamicin (10 mg), kanamycin (30 mg), streptomycin (10 mg), sulfamethoxazole (250 mg), and tetracycline (30 mg). *Escherichia coli* strains ATCC 25922 and 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were routinely used as quality control organisms, and results were interpreted according to CLSI recommendations (17, 18).

The PCR amplification reaction for the gene *spvA* was carried out with 1 μl of purified DNA using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA), 300 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 50 pmol of primers, and 0.5 U of

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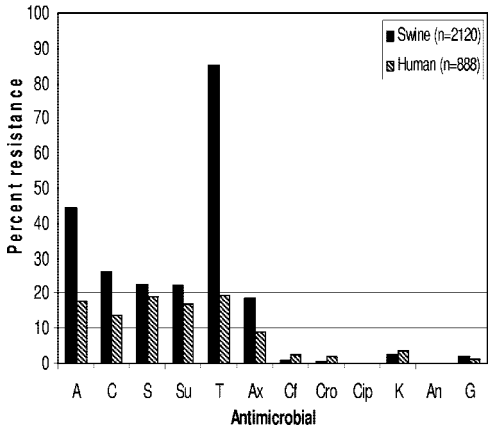

FIG. 1. Frequency of antimicrobial resistance among *Salmonella* isolates of porcine (swine) and human origin collected in North Carolina between 2000 and 2004. A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; Ax, amoxicillin-clavulanic acid; Cf, cephalothin; Cro, ceftriaxone; Cip, ciprofloxacin; K, kanamycin; An, amikacin; and G, sulfamethoxazole.

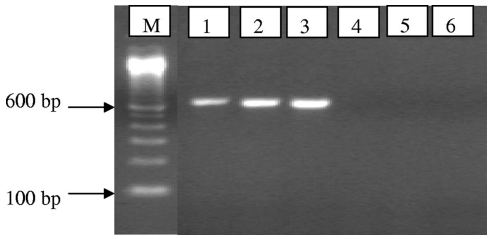

FIG. 2. PCR amplification of a 641-bp product of the gene *spvA*. Lane M, 100-bp ladder; lane 1, S300 (swine/clinical origin; R-type ACSSuT); lane 2, S669 (human/clinical origin; R-type ACSSuT); lane 3, UAE19 (swine/nonclinical origin; R-type ACSSuT); lane 4, UBD2 (swine/nonclinical origin; R-type AKSSuT); lane 5, S3536 (human/clinical origin; R-type ASTK); and lane 6, (no-template control).

Gold *Taq* polymerase (Perkin-Elmer, Foster City, CA), with a final volume of 20 μl. The primers used included *spvA*-F (GTC AGA CCC GTA AAC AGT) and *spvA*-R (GCA CGC AGA GTA CCC GCA). The PCR cycle included initial denaturation at 95°C for 5 min, 30 cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 54°C, and extension for 1 min at 72°C. Amplicons with an expected band size of 641 bp were considered positive for the gene *spvA*.

Pulsed-field gel electrophoresis (PFGE) genotyping was performed as recommended by the CDC (19) to determine the genotypic diversity of isolates from clinical and nonclinical sources of porcine and human origins. Briefly, 200 μl of overnight culture cells was lysed and intact genomic DNA was digested in agarose-embedded plugs with XbaI restriction enzyme. The digested DNA was then separated using a contour-clamped homogeneous electric field-DRIII (Bio-Rad Laboratories, Hercules, CA). *Salmonella enterica* serovar Braenderup Universal Marker was used as the reference marker. Analysis of PFGE data was performed using BioNumerics software (Applied Maths, Kortrijk, Belgium).

Chi-square (χ^2) statistics, odds ratio (OR), and 95% confidence interval (CI) were calculated to determine the association between MDR, the origin of isolates (independent variables), and carriage of *spvA* (outcome). A P value of 0.05 or lower was considered statistically significant. All analysis was done using Egret software (version 2.0.3; Cytel Corp., Cambridge, MA).

Overall, resistance to 11 of the 12 antimicrobials (except amikacin) was detected (Fig. 1). The *Salmonella* isolates of porcine origin (both clinical and nonclinical) showed resistance to eight antimicrobials. The frequency of resistance, particularly to tetracycline (83.6% of isolates from swine and 19.5% from humans), ampicillin (40.7% of isolates from swine and 17.7% from humans), and kanamycin (21.9% of isolates from swine and 3.5% from humans), was significantly higher in isolates of porcine origin than in those of human origin ($P < 0.001$). Resistance to cephalothin (2.25%), ceftriaxone (2%), and ciprofloxacin (0.11%) was detected only among isolates of human origin.

Analysis of the presence of *spvA* in the 247 isolates of porcine origin from clinical ($n = 123$) and nonclinical ($n = 124$) sources with various antimicrobial resistance patterns was conducted as shown in Fig. 2. Carriage of *spvA*, the virulence gene, was found to be associated with an MDR pattern, ACSSuT, with an OR of 7.1 (95% CI, 2.5 to 21.3). On the other hand, none of the isolates with R-type AKSSuT ($n = 14$), a very common MDR pattern in apparently healthy pigs, were found to carry *spvA*. The findings show that there is variation of *spvA* carriage among MDR *Salmonella* strains. In previous reports, strains with an ACSSuT resistance pattern have commonly been associated with clinical salmonellosis and *Salmonella* outbreaks (8).

Isolates with *spvA* were more than three times as likely to be of clinical origin (OR, 3.1; 95% CI, 1.6 to 6.2; $P < 0.05$). We detected this virulence gene only in isolates that belonged to serogroup B, a group that includes the most common serovars of clinical significance, such as serovar Typhimurium and *Salmonella enterica* serovar Heidelberg, which also often exhibits MDR. A multivariate analysis to determine interaction among the various contributing factors showed that the occurrence of *spvA* is associated with clinical occurrence at an OR of 3.5. In addition, more importantly, the occurrence of *spvA* with specifically R-type ACSSuT and its association with clinical occurrence was even higher at an OR of 4.13 ($P < 0.05$). This finding supports the hypothesis that the occurrence of virulence factor *spvA* within a strain exhibiting specific MDR phenotypes may make strains clinically more relevant.

In order to determine the clonality of isolates of human and porcine origins and isolates of clinical and nonclinical origins, further analysis using PFGE and carriage of *spvA* was carried out on a limited number of serovar Typhimurium (serogroup B) isolates (28 from pigs and 7 from humans). The swine isolates genotyped originated from clinical ($n = 13$) and nonclinical ($n = 15$) sources. As shown in Fig. 3, two genotypic clusters were identified. Six of the seven human isolates were clustered together with clinical isolates of porcine origin. The second cluster (cluster type B) was formed entirely of isolates from nonclinical isolates of porcine origin. The finding suggests that specific strains of *Salmonella enterica* serovar Typhimurium that are of clinical significance in swine can also be important causes of salmonellosis in humans (particularly

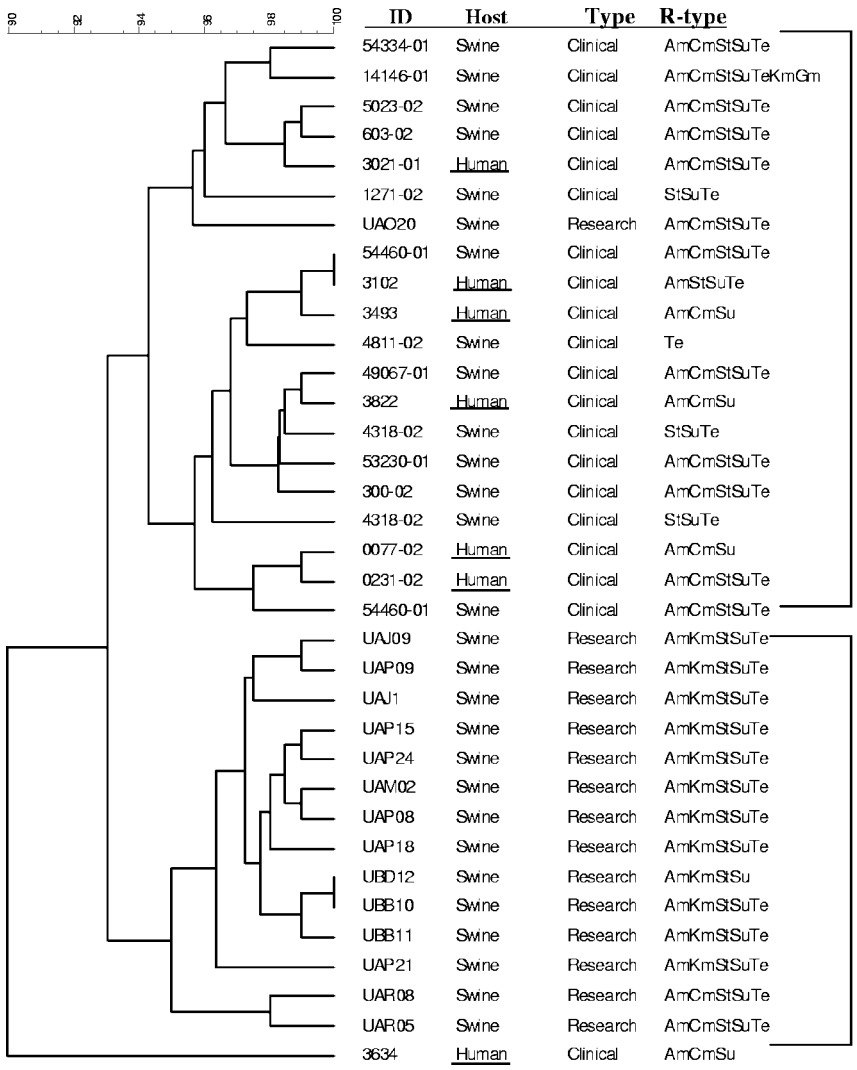

FIG. 3. A dendrogram generated from PFGE analysis of 35 MDR *Salmonella* serovar Typhimurium isolates of human and porcine origins. Two predominant genotypic clusters (A and B) are indicated. Cluster A is composed predominantly of isolates of clinical origin, and cluster B is composed entirely of nonclinical isolates. One isolate from a human was found to be distinctly different from all the remaining isolates and also had a unique phenotype based on antibiogram (AmCmSu).

strains with R-type ACSSuT). In addition, these strains were distinctly different from the strains that were commonly detected in healthy pigs which were not associated with clinical illnesses (various R-types that are predominantly AKSSuT).

The findings described above underscore the fact that there may be a clinically significant distinction among MDR *Salmonella* strains of clinical and nonclinical origins and that carriage of the gene *spvA* could be associated with important phenotypic characteristics. The higher proportion of resistance to some classes of antimicrobials in isolates of porcine origin could be associated with the routine use of antimicrobials in animal production settings at growth-promoting levels (11, 12, 15). Though low in frequency, the occurrence of resistance to ciprofloxacin and third-generation cephalosporin among isolates of human origin is of high significance since these two antimicrobials are the primary agents used against invasive salmonellosis cases in humans.

As reported previously, the two most common MDR patterns detected in humans and pigs were ACSSuT (in both humans and pigs) and AKSSuT (primarily in pigs). The absence of an association between pentaresistance R-types (ACSSuT and AKSSuT) and the origin of isolates is consistent with previous reports that MDR strains of *Salmonella* may also be found very commonly in pigs with no apparent clinical symptoms (1, 12). On the other hand, the MDR pattern of AKSSuT was most often detected from nonclinical specimens of porcine origin but was extremely rare among clinical samples both from pigs and from humans. Previous reports from the National Antimicrobial Resistance Monitoring System reported occurrence of isolates with the MDR pattern of AKSSuT from humans, but the frequency is relatively lower than that of isolates with the ACSSuT type (6).

The association between *spvA* and R-type ACSSuT may explain the common occurrence of this MDR strain in isolates of clinical origin and isolates from both pigs and humans. The invasiveness of strains with this pattern, such as serovar Typhi-

murium phage type DT104 (R-type ACSSuT), has been documented previously (16, 20). The higher invasiveness of these MDR strains, unlike that of other MDR strains such as those with R-type AKSSuT, can be explained by the carriage of *spv*. A previous study that did not find any genetic difference in *Salmonella* genomic island-associated virulence genes between serovar Typhimurium DT104 and pansusceptible strains may also have implied that differences in virulence may also be harbored on plasmids (5). The *Salmonella* virulence plasmid may also be self-transferrable, and that could be a very important factor for the evolution of virulence as well as resistance in various strains. The findings imply that carriage of *spvA* together with the MDR phenotype enables these strains to be of primary clinical importance compared to those MDR strains lacking the *spvA* gene or part of the operon.

We further investigated the genotypic diversity of MDR serovar Typhimurium isolates using PFGE genotyping. The clustering of isolates based on their clinical status regardless of host origin could have a significant implication that not all *Salmonella* strains in food animals are of clinical relevance. While serovar Typhimurium is commonly reported in clinical human cases, the findings in this study imply that distinct subsets of this serovar are what prevail clinically.

Overall, despite the limitations in the external validity of the data (limited geographic origin), the findings have important implications; not all members of *Salmonella* strains may be equally important as a cause of clinical salmonellosis. The carriage of *spvA* among MDR strains may increase the propensity of such strains to be of major clinical relevance.

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