Microbial-Resistant *Salmonella enteritidis* Isolated from Poultry Samples

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Abstract

Background: Multidrug resistance in *Salmonella enteritidis* isolates is a public health problem worldwide; the present study, therefore, was designed for antimicrobial-resistance determination in this strain.

Methods: *Salmonella* strains isolated from poultry samples by biochemical positive and negative tests were subjected to PCR and identified as *Salmonella enteritidis*. For detection and identification of *Salmonella enteritidis* isolates, *sdfI* gene-specific primers were used.

Results: We found that 100% of isolates were resistant to ampicillin, 90% were resistant to cephalothin and streptomycin, 70% were resistant to cefotaxime, and 60% were resistant to kanamycin and gentamicin.

***Conclusion*:** *Salmonella enteritidis* isolates had antimicrobial resistance to mentioned antibiotics.

Keywords: Antibiotic Resistance, PCR, Poultry, *Salmonella enteritidis*

Introduction

Food-borne diseases caused by non-typhoid *Salmonella* represent an important public health problem worldwide. Nearly 1.4 million cases of salmonellosis occur each year in the United States, of which 95% are food-borne (1). Poultry, cattle, and dairy products are the major sources of *Salmonella* contamination in foods that cause human salmonellosis (2). Salmonellosis caused by *Salmonella enteritidis* is a disease that affects birds and mammals; therefore, it is considered zoonosis (3). It has been said that the worldwide increase in *S. enteritidis* outbreaks is due to increased virulence. Virulence varies among different strains of *S. enteritidis* (4) and a specific plasmid has been suggested to be the virulence factor (5). It is postulated that *S. enteritidis* colonizes the ovaries and oviducts of chickens, contaminates the content of intact eggs, and is the major egg-associated human pathogen (1, 6-7).

Multidrug-resistant phenotypes have been increasingly described among *Salmonella* species worldwide according to the Infectious Disease Report 2000 released by the World Health Organization (8). A contributing factor in the development of resistance stems from the use of antimicrobials in human and veterinary medicine, animal husbandry, and agricultural and aquacultural practices. In animal husbandry practices, antimicrobial agents are used for treatment and prevention of animal diseases as well as for growth promotion (9). These routine practices are important factors in the emergence of antibiotic-resistant bacteria that can subsequently be transferred from animals to humans through the food chain.

Most antimicrobial-resistant *Salmonella* infections are acquired from eating contaminated foods of animal origin (10). Of recent concern is the increased prevalence and widespread dissemination of *S. enteritidis*. It has been

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reported that *S. typhimurium* resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole increased from 1% to 25%, 1.5% to 25%, and 0% to 25%, respectively, from 1986 to 1993 (11). Currently, data is lacking regarding the prevalence and antimicrobial susceptibility profiles of food-borne pathogens present in imported versus domestic foods in the United States. The objectives of the current study were to determine and establish baseline data of antibiotic-resistant *Salmonella* phenotypes from imported foods isolated in year 2000, and characterize nalidixic acid and integron-mediated antimicrobial resistance mechanisms. For preparation of *S. enteritidis* isolates, we used specific PCR. We describe the identification of a novel *S. enteritidis* locus that serves as a marker for DNA-based identification of this bacterium. In contrast to other tests, this marker is not found in a wide range of closely-related serovars; therefore, this test allows highly-specific detection of *S. enteritidis*. Evidence is presented supporting a chromosomal location for the locus, thus circumventing the potential problems associated with plasmid-borne markers (12).

Materials and Methods
Salmonella enteritidis isolates
S. enteritidis isolates were obtained from poultry samples such as chicken meat, liver, and egg. Five grams of poultry homogenized with 25 ml of Selenite F broth (Enrichment broth) were incubated at 37ºC for 24 h to allow bacterial growth. The bacteria were then cultured in Salmonella-Shigella (SS) agar selective medium to select colorless single colonies. All strains were identified by biochemical standard tests. Fourteen isolates of *S. enteritidis* from egg, meat, and liver origins were prepared for PCR. In this study, the strains were maintained as frozen stocks in Luria Bertani (LB) broth supplemented with 20% glycerol at -70ºC.

Extraction and purification of DNA
Chromosomal DNA was isolated by boiling and phenol-chloroform extraction methods. For the boiling method, 10 colonies of bacteria from SS agar were suspended in a 1.5 ml microcentrifuge tube with 300 µl of DNase-RNase-free distilled water by vortexing. After heating the microcentrifuge tube in boiling water for 15 min, the suspension was centrifuged for 5 min at 6,000 x *g*. The supernatant was extracted carefully and was ready for electrophoresis. For the phenol-chloroform method, 1 ml of LB broth containing bacterial cells in a 1.5 ml microcentrifuge tube was centrifuged at 6,000 x *g* for 5 min and the bacterial pellet resuspended in 657 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by vortexing, after which 30 µl of 10% SDS were added and the solution was incubated at 37°C for 1 h. 100 µl NaCl (5 M) and 67 µl CTAB-NaCl were added and the solution was incubated at 65°C for 10 min. Chloroform-isoamyl alcohol (1:24) was added and mixed before centrifuging at 6,000 x *g* for 5 min at 4°C, after which the liquid phase was once more washed with chloroform-isoamyl alcohol and then with phenol-chloroform-isoamyl alcohol (24:24:1). DNA was precipitated by isopropanol, incubated at -20°C for 30 min, and centrifuged at 14,000 x *g* for 5 min at 4°C. Supernatant was then removed and the pellet washed in 1 ml of 70% cold ethanol; the DNA pellet was air-dried and resuspended in 100 µl of double-distilled water. Agarose gels were stained with ethidium bromide and DNA was visualized by UV light illumination.

PCR
The oligonucleotide primer pair used for the specific detection of the *sdfI* gene, *sdfI*_F (5´-TGT GTT TTA TCT GAT GCA AGA GG-3´) and *sdfI*_R (5´-CGT TCT TCT GGT ACT TAC GAT GAC-3´), was designed by Agron *et al*., to give a PCR product of 333 bp (12).

The *sdfI* DNA was amplified by 27 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s) and elongation (72°C, 1 min). Amplification products were visualized by agarose gel electrophoresis.

Antimicrobial-susceptibility testing
Antimicrobial-susceptibility assays were performed by the standard disc-diffusion method on Muller-Hinton agar, as described in the NCCLS guidelines. The following disc antibiotics and their respective concentrations,

purchased from Padtan Teb Co., included: ampicillin (10 μg), cephalothin (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), cefotaxime (30 μg), ceftizoxime (30 μg), cefalexin (30 μg), carbenciline (100 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), gentamycin (10 μg) and trimethoprim-sulfamethoxazole (75 μg).

Results

Optimization of the assay
Two methods of chromosomal DNA preparations were compared; heat-induced bacterial lysis, and lysis using SDS followed by phenol/chloroform extraction. The first option was adopted because it was more rapid and simple than the second.

PCR specificity and sensitivity
The PCR produced a 333 bp amplification product from the *sdfI* gene in *Salmonella* strains isolated by biochemical tests. Six isolates of *S. enteritidis* were identified by PCR. The sensitivity of the PCR was determined for *S. enteritidis* isolates using the non-*Salmonella* pool. The sizes of the PCR products were confirmed by the UVdoc. program.

Antibiotic susceptibility
We found that 100% of the *S.enteritidis* isolates were resistant to ampicillin, 90% were resistant to cephalothin and streptomycin, and 70% were resistant to cefotaxime (Table 1).

All the strains were susceptible to carbenciline, cefalexin, ciprofloxacin, and ceftizoxime. After curing, the antibiotic-sensitive derivatives of the isolates were tested. Changes in the antibiotic susceptibility patterns of the strains upon curing are presented in Table 1. Resistance to antimicrobial agents was changed by curing. Cured isolates were susceptible to many antibiotics to which they were resistant before curing; however, after plasmid curing, resistance to ampicillin was not changed. Antibiotic susceptibility tests proved valuable in differentiating strains of *Salmonella* spp.; however, we did not find any correlation between *S. enteritidis* strains regarding their antibiotic susceptibility, which suggests that the tests cannot be used to distinguish among strains.

Table 1. Resistance patterns of strains

Strains	Before curing	After curing
E1	AM, S, AN, CF, CTX, STX	AM, CF
E3	AM, CF, CTX, NA, CT, FM	AM, CF, NA
E8	AM, K, GM, S, CF, NOR	AM, K, GM, CF
M2	AM, K, GM, S	AM, K, GM
L1	AM, K, GM, S,CTX, STX, FM	AM, K, GM
L2	AM,S, AN,CF,CTX,CP,STX,FM	AM, CF, FM

AM: ampicillin, AN: amikacin, CB: carbenciline, CF: cephalothin, CN: cefalexin, CP: ciprofloxacin, CT: ceftizoxime, CTX: cefotaxime, GM: gentamicin, FM: nitrofurantoin, K: kanamycin, N: nalidixic acid, NOR: norfloxacin, S: streptomycin, STX: trimethoprim-sulfamethoxazole.

Discussion
Biochemical typing of *S. enteritidis* isolates can be considered the first step in their classification (13). The application of PCR techniques has had a revolutionary impact on the diagnosis of infectious diseases. Because these techniques have the ability to detect or allow analysis of minute amounts of microbial DNA, they have emerged as highly sensitive and specific methods for identifying pathogens.

Brown *et al*. detected a very low percentage (3.7% and 1%) of antibiotic resistance among the *S. enteritidis* strains they tested. The drug resistance tests on all of the *S. enteritidis* strains showed resistance to the various antibiotics examined; however, all strains that harbored only the 36 MDa plasmid were drug sensitive (14).

In the present study, our findings indicate that most strains of *Salmonella* are resistant to a wide range of antimicrobial agents. Considering that 100% resistance to ampicillin was found in *S. enteritidis* strains containing 1 to 6 plasmids before and after curing, it was not possible to correlate the presence of these plasmids with the antibiotic resistance detected in some strains; however, the isolates were susceptible to a wide range of antibiotics after curing with SDS.

Although generally resistance in *Salmonella* is not considered to be dependent on plasmids, resistance to antimicrobial agents is considered to be relatively unstable, because most bacterial resistance factors are encoded by plasmids that are often transferable between strains and may be dependent on selection pressure to be maintained (15). Some examples of plasmid-encoded resistances have been described (16-17). The function of low-molecular weight plasmids in *S. enteritidis* is usually unknown, although some indications on their contribution to phage typing results have been described (18); however, plasmids of higher molecular weight have been shown to affect the phenotype of *S. enteritidis* considerably, including their virulence (19), phage type (20), and/or antibiotic resistance (17).

Besides chromosomal mutations, DNA mobile elements such as transposons and integrons have greatly contributed to the rapid dissemination of resistance. These elements often spread by incorporation into plasmids and may move from plasmid to chromosome where they are inherited by daughter cells. Integrons are mobile DNA elements primarily found in Gram-negative bacteria. Four classes of integrons have been identified to date based on the homology of integrase genes; however, the majority of integrons identified among clinical isolates belong to class 1 type (21). To date, more than 60 distinct antibiotic-resistant gene cassettes have been identified (22). Interestingly, class 1 integrons often contain the *sul1* gene, which encodes resistance to sulfonamides (23). With increasing international travel and trade over the past several years, there has been an accelerated dissemination of resistant pathogenic organisms from one geographic location to another (8). A further complication is that the extent of antimicrobial drug-resistant food-borne pathogens in developing countries, where inappropriate antimicrobial usage may be more common than in developed countries, is unknown. It is therefore critical to establish effective food safety barriers on an international scale to reduce the prevalence of zoonotic food-borne pathogens.

Acknowledgements

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