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Vaccination with a Streptomycin-Resistant Strain of *Salmonella enteric serovar Enteritidis* lacking *pefA* and *spvC* Genes Reduces Cecal Colonization and Organ Invasion in *SPF* Chicks

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ABSTRACT

Salmonellosis is one of the most important food-borne diseases and remains an important pathogen of poultry. In this study was evaluated the protection of a vaccine containing a streptomycin-resistant strain of *Salmonella entericas erovar Enteritidis* lacking *pefA* and *spvC* genes with respect to cecal colonization, organ invasion and excretion in SPF chicks and the potential use as a vaccine candidate was tested. Streptomycin-mutant strain was obtained by exposure to high concentration of streptomycin. Parent and resistant strain were evaluated phenotypically by measuring biochemical properties, growth rate and antibiotic resistance, and genetically for expression of twenty-three genes. Mutant strain was tested in SPF chickens by testing excretion, and by challenge using a wild-type isolate three weeks after immunization evaluating cecal colonization and organ invasion. Streptomycin-resistant strain showed lack of expression of *pefA* and s*pvC* when compared to the parent strain. DNA sequencing of a PCR-amplified *gyrA* fragment detected one point of mutation Ser15→Phe. Biochemical properties did not change. Growth rate differences were observed between parent and mutant strains, showing a generation time was increased five-fold in the mutant strain. Excretion of the vaccine strain was reduced 50% at the second week compared to the positive control group and no excretion of the vaccine strain was detected at the third week. Cecal colonization and organ invasion were significantly reduced in the vaccinated group, 80% and 70%, respectively. The vaccine strain was not detected in cecal and organ samples at the end of the trial. Attenuated strains produced by selecting for resistance to streptomycin have been described in mice. This study showed that streptomycin-resistant strain may be an important factor in the attenuation, suggesting that after exposure to streptomycin the parent isolate lost the expression of *pefA* and *spvC* genes and it could be a vaccine candidate to protect chicks against a *Salmonella* Enteritidis challenge.

Key words: Salmonella Enteritidis, streptomycin-resistant strain, potential vaccine strain.

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INTRODUCTION

An alternative approach to the development of bacterial vaccines is the development of attenuated strains for use as live vaccine candidates by exploiting antimicrobial resistance. Bacteria exhibiting resistance to rifampicin typically have reduced virulence (Bhatagar *et al.*, 1994). A direct approach has been tested in mice, demonstrating that *Salmonella* Typhimurium mutants that are resistant to streptomycin, rifampicin, and nalidixic acid are avirulent in this species (Bjorkman *et al.*, 1998). The purpose of the present study was to assess the protective effect a vaccine containing a streptomycin-resistant strain of *Salmonella enterica* serovar Enteritidis against a virulent challenge. We evaluated expression of virulence genes, cecal colonization and organ invasion by this potential vaccine candidate in specific-pathogen-free chicks.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions for DNA Extraction

Field and antibiotic-mutant resistant strains of *Salmonella* Enteritidis were evaluated for temperature sensitivity, generation time, and minimum inhibitory antibiotic concentrations. Polymerase chain reactions used Salmonella Typhimuirum ATCC 14028 as a reference strain. A volume of 200 µL of each pure strain was used for DNA extraction.

PCR Analysis and Sequencing

Twenty-three sets of primer pairs were used to evaluate the expression profiles of wild and antibiotic-mutant resistant *Salmonella* Enteritidis strains. Target genes, oligonucleotide sequences (Invitrogen, Carlsbad, CA, USA), amplification region, PCR conditions and references or accession numbers are described in Table 1. DNA extractions were performed in parent and antibiotic-mutant, as described by Boom *et al.* (1990). All purifications and PCR reactions used ATCC 14028 and E. coli K12 as positive and negative controls, respectively. PCR was performed with 0.75 µL of DNA sample, except for the *agfA* target gene where 1.5 µL of DNA sample was used, 50 nM $MgCl_2$, 200 nMTris pH 8.4, 500 mMKCl, 1.25 nMdNTP mix, 1U *Taq* DNA polymerase (Invitrogen, Carlsbard, CA, USA), and 10 pmol of each primer for a final volume of 25 µL. Primers for *fliC*, *sifA*, *sopB*, *gyrA*, *rpoB*, and *rpsL* were designed based on the GenBank sequence database. Electrophoresis of PCR products was performed on 1.5% agarose gel with a 100-bp ladder (Invitrogen Carlsbad, CA, USA) as the molecular weight marker. Blue Green Loading Dye (LGC Biotecnologia, Cotia, SP, and Brazil) was used to mix the samples and ladder 1:10. DNA sequencing of an amplified PCR gyrA, rpoB and rpsL fragments were evaluated.

Antimicrobial Susceptibility Tests

Antimicrobial susceptibility tests were performed using the parent and antibiotic-mutant resistant strains. The disk diffusion method used 15 antibiotics. Two different tests were performed. AST by microdilution and disk diffusion tests were performed in antibiotic-mutant strains obtained after exposure to high concentrations of antibiotics. The first method was the antimicrobial susceptibility test using a microdilution commercial test AviPro® Plate (Lohmann Animal Health GmβH, Cuxhaven, Germany). This microdilution test quantitatively measures the in vitro activity of an antimicrobial agent against a given bacterial culture isolated from poultry.

The round-bottom wells were pre-coated with various concentrations of antibiotics. Each plate contained the following drugs: amoxicillin, ceftiofur, colistin, enrofloxacin, erythromycin, gentamicin, lincomycin, neomycin, oxacilin, penicillin, rifampicyn, spectinomycin, streptomycin, tiamulin, tetracycline, lincomycin/spectinomycin, and trimethoprim/sulfamethoxazole. The test was performed as recommended by the manufacturer and interpreted according the manufacturer guideline. The second test was the disk diffusion method on Mueller-Hinton agar (Difco, Sparks, MD, USA), using fifteen antibiotics (CefarDiagnostica, São Paulo, SP, and Brazil) following standard protocols and interpretative guidelines from CLSI (2008). *E. coli* ATCC 25922 was used as reference strain. The following antibiotic disks were tested: ampicillin (AMP) 10 µg; ciprofloxacin (CIP) 5 µg; chloramphenicol (CLO) 30 µg; colistin (COL) 10 µg; doxycicline (DOX) 30 µg; enrofloxacin (ENRO) 5 µg; erythromycin (ERY) 15 µg; florfenicol (FFN) 30 µg; gentamicin (GEN) 10 µg; kanamycin (KAN) 30 µg; nalidixic acid (NAL) 30 µg; rifampicyn (RIF) 30 µg; streptomycin (STR) 10 µg; trimethoprim/sulfametoxasole (TMP/SMX) 1.25/23.75 µg; and tetracycline (TET) 30 µg. Overnight cultures grown on LB broth were spread on Mueller-Hinton plates and incubated at 37^{o}C for 24 hours.

Evaluation of the Parent and Antibiotic-Mutant Strains in SPF Chicks

A total of 180 one-day-old SPF Ross chicks of the same hatch were used. The birds were housed in floor pen facilities with pine-shaving litter and were provided *ad libitum* with water and a balanced non-medicated diet. The housing environment and paper pads were tested according to ISO 6579:2002/Amd 1:2007. Additionally, house environment and paper pads samples were pre-enriched in tetrathionate broth (Difco, Sparks, MD, USA) and cultured on xylose-lysine-deoxycolate (XLD) agar (Difco, Sparks, MD, USA) and xylose-lysine-tergitol-4 (XLT_4) agar (Difco, Sparks, MD, USA).Groups 1 and 2 contained 60 birds each, and these chicks were vaccinated at one day of age by oral gavage with at least 1×10^8cfu/mL of the parent strain or the antibiotic-mutant resistant strain, respectively. These chicks were challenged three weeks later. Groups 3 and 4 contained 20 birds each, and these chicks were challenged at three weeks of age. Group 5 was maintained as a negative control. Chicks were observed for 21 days. Clinical signs and mortality rates were recorded. After the observation period, birds were killed by CO2 inhalation. Postmortem examination of cecal contents, livers, and spleens was performed.

RESULT

The antibiotic-mutant resistant strain was sensible to ceftiofur, colistin, rifampicin and tetracycline. The biochemical properties of the bacteria did not change. Growth rate differences were observed between the parent and antibiotic-mutant resistant strains, and the generation time was increased five-fold for the mutant strain. The streptomycin-resistant strain showed no expression of the *pefA*and *spvC* genes, which were expressed by the parent strain. Parent (SEO) and antibiotic-mutant resistant (SEAM) strains were not different with respect to the of *gyrA*, *rpoB* and *rpsL* genes, while DNA sequencing of a PCR-amplified *gyrA* fragment showed differences in the antibiotic-mutant (Genbank accession number HQ237456) resistant strain (Phe15) compared to the parent strain (Genbank accession number HQ237457), as shown in Figure 1.

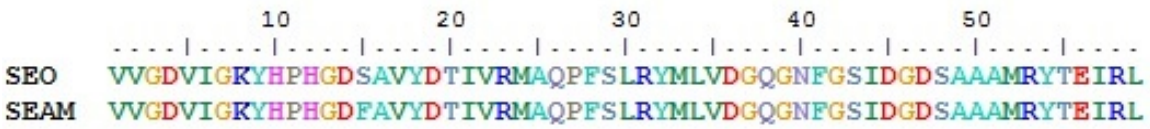

Figure 1. Sequencing of a PCR-amplified *gyrA* fragment

Before inoculation, *Salmonella* was not detected in the following areas: paper pads, pine-shaving litter, house environment, equipment, balanced diet food, feeders, water, or water containers. Cecal colonization showed significant differences ($P \leq 0.05$) in groups vaccinated when compared to unvaccinated group challenged orally. The group challenged subcutaneously did not show any evidence of cecal colonization. Liver and spleen invasion showed a significant ($P \leq 0.05$) reduction in birds vaccinated and challenged orally when compared to the group vaccinated orally and challenged subcutaneously. Positive control groups showed at least 90% of organ invasion. No clinical signs or mortality was recorded during the trial in the groups inoculated with the parent and antibiotic-mutant SE strain.

As shown in Figure 2, evaluation of the cecal colonization revealed a significant ($P<0.05$) reduction in recovery of the challenge strain in the vaccinated groups compared to the positive control group that was challenged orally. Liver and spleen invasion was significantly ($P<0.05$) reduced in the vaccinated groups as compared to the groups challenged orally and subcutaneously.

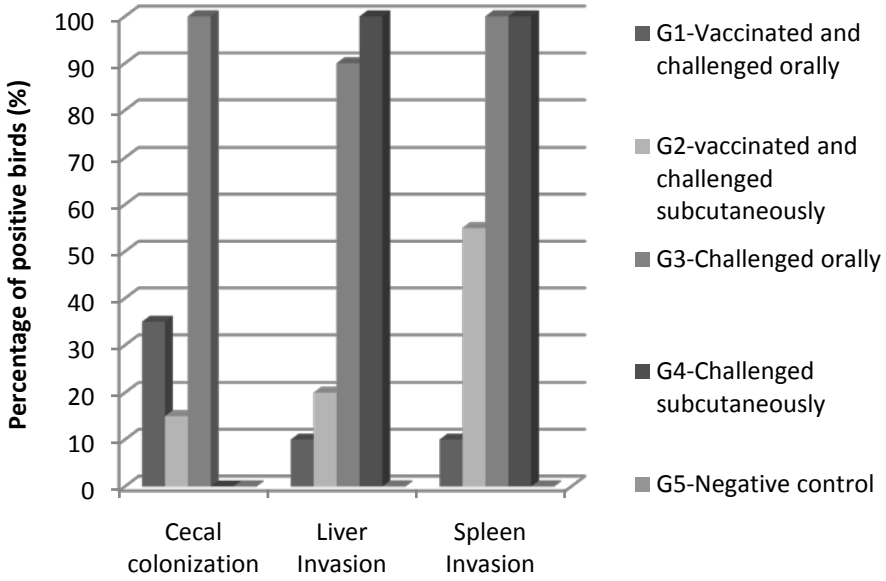

Figure 2. Recovery of challenge strain

DISCUSSION

Bjorkman *et al.* (1998) demonstrated that restrictive mutations and resistance to streptomycin are associated with a loss of virulence in the mouse model system. Streptomycin resistance is often attributable to aminoglycoside modifying enzymes (Vakulenko and Mobashery, 2003). The

stability of antibiotic mutation should be guaranteed by no less than 2 markers, which can provide vaccine strains with an optimal attenuation for a particular animal species, conferring full protection after a single vaccination (Linde *et al.*, 1990). p*efA*and *spvC*mutations were observed after exposure of a field isolate of SE to high levels of streptomycin. Nontyphoidserovars that lack the *spv*genes are less able to proliferate beyond the superficial epithelial layer (Fluit 1995), because *spv* genes are required for the systemic phase of the disease. This fact could explain the reduced organ invasion obtained in groups vaccinated because mutation in this gene causes various defects in *Salmonella* virulence (Rotger e Cadesús, 1999). Our results suggest that streptomycin may have a negative effect on the expression of the *pefA*and *spvC*genes in SE field strains, inducing a mutation that has a positive effect in vivo and thereby reducing cecal colonization and organ invasion by the resistant strain. Further research can be done to determine if differences in sequence of the *gyrA* fragment are related to reduction in virulence.

CONCLUSION

- Vaccination with a streptomycin-resistant strain of *Salmonella entericaserovar Enteritidis* that lacks *pefA* and *spvC* genes protected SPF chicks against a challenge with a virulent strain of wild-type *Salmonella*.
- DNA sequencing of a PCR-amplified *gyrA* fragment showed differences in the antibiotic-mutant resistant strain compared to the parent strain.
- The antibiotic-mutant resistant SE had diminished cecal colonization and organ invasion abilities in SPF chicks, and this strain could be a potential vaccine candidate.

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