Salmonella* Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes *csgA*, *csgD* and *adrA

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INTRODUCTION

In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the serovar most frequently isolated from slaughter pigs¹. Porcine carcass contamination with *Salmonella* Typhimurium can largely be attributed to persistently infected pigs. Even though tonsils are a predilection site for *Salmonella* persistence in pigs, virulence mechanisms necessary for cell invasion and intracellular survival do not contribute to tonsillar colonization², suggesting that *Salmonella* Typhimurium resides mainly extracellularly in porcine tonsils. Biofilm formation is a mechanism used by several bacteria to survive in an extracellular context or in hostile environments³. The role of biofilm formation in *Salmonella* Typhimurium persistence in pigs is still unknown. It was the aim of the present study to determine whether *Salmonella* Typhimurium persists intracellularly or extracellularly in tonsils of pigs. Additionally, the role of biofilm formation in persistence of *Salmonella* Typhimurium in porcine tonsils was determined.

METHODS AND MATERIALS

As wild type (WT), we used a virulent *Salmonella* Typhimurium phage type 120/Ad strain, isolated from a pig stool sample. In the *in vivo* trials, a spontaneous nalidixic acid resistant derivative of the wild type was used. Chloramphenicol-resistant substitution mutants in biofilm-associated genes *csgA*, *csgD* and *adrA* were created as described by Datsenko and Wanner⁴. The difference in colony morphology between the WT and the mutants was examined by growing the strains on LB agar without salt, supplemented

with Coomassie Brilliant Blue and Congo Red (Sigma-Aldrich) at 28 °C for 7 consecutive days. To determine the localization of *Salmonella* Typhimurium in porcine tonsils, 12 piglets were inoculated with 2 x 10⁷ colony forming units (CFU) of the WT. Five and 28 days post inoculation, 8 and 4 pigs were euthanized respectively. Samples of tonsils were collected to prepare single cell suspensions (SCS) and cryosections. To obtain SCS, tonsils were rinsed, cut to small pieces and treated with 100 U/ml collagenase from *Clostridium histolyticum* Type V (Sigma-Aldrich). Half of the SCS was treated with 2 % Triton X-100 (Sigma-Aldrich) to lyse cells. These suspensions were then plated on BGA to assess the total number of intra- and extracellular bacteria. The remainder of SCS was treated with 100 µg/ml gentamicin (Sigma-Aldrich) to kill all extracellular bacteria. Cells were then washed and lysed with 2 % Triton X-100. Ten-fold dilutions were plated on BGA to determine the number of intracellular bacteria. Samples that were negative at direct plating were enriched in tetrathionate broth (Merck). Finally, the percentage of extracellular bacteria was calculated. For cryosections, liquid nitrogen frozen tonsil samples were sectioned at 8 µm with a cryotome, dried and fixed in acetone. Sections were pre-incubated with 30% goat serum to reduce background staining. Sections were subsequently incubated with a primary antibody mixture containing a polyclonal rabbit antibody targeting the *Salmonella* O4 somatic antigen (1:100 dilution; Pro Lab Diagnostics) and a mouse monoclonal antibody targeting CD172α (1:50 dilution; AbD Serotec). After washing, the sections

were incubated with a secondary antibody mixture containing goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (both 1:200 dilution; Molecular Probes). Sections were evaluated using a Leica DMRB fluorescence microscope (Leica Inc). Pictures were taken using a Moticam 2300 camera (Moticam Instruments Inc) and 100 bacteria in each tonsil sample were visually counted.

To examine the role of biofilm formation in *Salmonella* persistence in porcine tonsils, 3 groups of 6 piglets were orally inoculated with 2×10^7 CFU of WT *Salmonella* and 2×10^7 CFU of $\Delta csgA$, $\Delta csgD$ or $\Delta adrA$ *Salmonella*. Ten days after oral inoculation, pigs were euthanized and tonsils, ileum and ileum contents, ileocecal lymph nodes and faeces were collected. Tissue samples were cut to small pieces and stomached. Ten-fold dilutions of tissue samples, ileum contents and faeces were plated on BGA with nalidixic acid for selective growth of the WT and on BGA with chloramphenicol for selective growth of the substitution mutants. When negative at direct plating, samples were enriched in tetrathionate broth and again plated on BGA with the respective antibiotics.

RESULTS

After 7 days of incubation at 28 °C, the WT showed a biofilm-associated rough and dry morphotype, while the mutants exhibited a biofilm deficient smooth and white morphotype. Five and 28 days post inoculation, 97.0 ± 2.0 % and 97.6 ± 3.6 % of the *Salmonella* bacteria respectively was found to reside extracellularly in the tonsils using the single cell suspension method. These results were confirmed using immunohistochemistry. In the mixed infection experiment, in none of the analyzed samples of all three groups the *csgA*, *csgD* or *adrA* *Salmonella* mutants were significantly impaired in colonization and persistence capacity compared to the wild type strain in any of the organs examined (FIGURE 1).

DISCUSSION

Previous work using a SPI-1 deficient strain suggested that *Salmonella* predominantly

resided extracellularly in porcine tonsils². In the present study we confirmed this extracellular localization using single cell suspensions and immunohistochemistry. In a subsequent mixed infection experiment however, we were unable to show a role for the biofilm-associated genes *csgA*, *csgD* or *adrA* in this extracellular persistence⁵.

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TABLES AND FIGURES

FIGURE 1. Recovery of bacteria from various organs of six piglets orally inoculated with a mixture of the wild type *Salmonella* Typhimurium and *csgA*, *csgD* or *adrA* *Salmonella* Typhimurium mutants. The log10 value of the ratio of CFU per gram sample of the wild type and the biofilm-associated gene mutants is given as the mean $\pm$ SD.