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

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ABSTRACT

Persistent *Salmonella* Typhimurium infections in pigs are a major concern for food safety and human health. Tonsils play a key role in the persistence of *Salmonella* Typhimurium in pigs. Previous studies indicated that *Salmonella* virulence genes involved in invasion and intracellular survival are of little importance for the colonization of porcine tonsils, suggesting a predominantly extracellular location of the *Salmonella* bacteria. Biofilm formation might promote extracellular persistence of *Salmonella* Typhimurium. The aim of this study was to determine whether the bacterium resides predominantly intra- or extracellularly in tonsils of pigs and to examine the contribution of biofilm-associated genes *csgA*, *csgD* and *adrA* in *Salmonella* persistence in porcine tonsils. Single cell suspensions were prepared from tonsils of orally inoculated pigs (2×10^7 colony forming units (CFU) wild type *Salmonella* Typhimurium) to determine the ratio of extracellular versus intracellular bacteria. Both at 5 and 28 days post-inoculation (pi), the majority of *Salmonella* bacteria was found extracellularly in porcine tonsils. To determine the contribution of biofilm formation in extracellular persistence, pigs were orally inoculated with a mixture of 2×10^7 CFU of the *Salmonella* Typhimurium wild type strain and 2×10^7 CFU of one of the *Salmonella* Typhimurium *csgA*, *csgD* or *adrA* mutants. At 10 days pi, equal numbers of both wild type and mutant *Salmonella* bacteria were found not only in tonsils, but also in ileum, ileum contents, ileocecal lymph nodes and faeces.

In conclusion, we showed that *Salmonella* Typhimurium resides extracellularly in porcine tonsils, using a biofilm independent mechanism.

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1. Introduction

In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the serovar most frequently isolated from slaughter pigs (Anon, 2007, 2008). Porcine carcass contamination with *Salmonella* Typhimurium can largely be attributed to persistently infected pigs (Botteldoorn et al., 2003). In most cases the bacterium will subclinically colonize the palatine tonsils, secondary lymphoid organs situated at the oropharynx. They play a vital role in both innate and adaptive immune responses due to the presence of antimicrobial peptides and a variety of immune cells (Perry and Whyte, 1998; Horter et al., 2003). Even though tonsils are a predilection site for *Salmonella* persistence in pigs (Wood et al., 1989), virulence mechanisms necessary for cell invasion and intracellular survival do not contribute to tonsillar colonization (Boyen et al., 2006b, 2008b), suggesting that *Salmonella* Typhimurium resides mainly extracellularly in porcine tonsils.

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Biofilm formation is a mechanism used by several bacteria to survive in an extracellular context or in hostile environments with for example low pH or limited oxygen (Rosenberg et al., 2008; Boyen et al., 2009). Biofilms are a multicellular behaviour mode, in which bacteria are embedded into a self-produced extracellular matrix. Regulation of biofilm formation in *Salmonella* Typhimurium has been intensively studied in the past years and several genes playing a role in *Salmonella* Typhimurium biofilm formation were identified (Römling, 2005). For example, CsgD stimulates biofilm production indirectly by transcriptional activation of the curli biosynthesis operon *csgDEFG-csgBAC* and *adrA*, a gene crucial for cellulose biosynthesis (Römling, 2005). 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.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Salmonella Typhimurium strain 112910a, phage type 120/ad, was isolated from a pig stool sample and characterized previously (Boyen et al., 2008b). A spontaneous nalidixic acid resistant derivative of the wild type strain (WT_{nal}) was used in the first *in vivo* experiment to minimize irrelevant bacterial growth when plating tonsillar, intestinal and faecal samples. In the second *in vivo* experiment, the WT_{nal} strain was used together with one of the 3 deletion mutants *csgA*, *csgD* or *adrA*. These deletion mutants were constructed according to the one-step inactivation method described by Datsenko and Wanner (2000) and slightly modified for use in *Salmonella* Typhimurium as described before (Boyen et al., 2006a). Primers used to create the gene-specific linear PCR fragments are summarized in Table 1.

For the oral inoculation of pigs, the bacteria were grown for 16 h at 37 °C in 5 ml Luria–Bertani broth (LB; Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) on a shaker. The bacteria were washed twice in Hank's buffered salt solution (HBSS; Gibco Life Technologies, Paisley, Scotland) and centrifuged at 2300 × *g* for 10 min at 4 °C and diluted in HBSS to the appropriate concentration of 10^7 colony forming units (CFU) per milliliter. The number of viable *Salmonella* bacteria per milliliter inoculum was determined by plating 10-fold dilutions on brilliant green agar (BGA; Lab M Limited, Lancashire, UK) supplemented with 20 μg/ml nalidixic acid (BGA^{NAL}; Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) for selective growth of the WT_{nal} strain and BGA supplemented with 25 μg/ml chloramphenicol (BGA^{CA}; Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) for selective growth of the mutant strains.

The *in vitro* colony morphology exhibited by the wild type *Salmonella* Typhimurium and the *csgA*, *csgD* and *adrA* mutants on agar at different incubation temperatures was examined by plating the strains on LB without salt, supplemented with 20 μg/ml Coomassie Brilliant Blue (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and 40 μg/ml Congo Red (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) as described by Malcova et al. (2008). The plates were incubated at 28 °C and 37 °C for 7 consecutive days and were then visually examined.

2.2. Experimental animals

The animal experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2007/052 and EC 2008/074).

In both *in vivo* trials, 4-week-old piglets (commercial closed line based on Landrace) from a serologically *Salmonella* negative breeding herd were used. The piglets tested negative for *Salmonella* at faecal sampling. The piglets arrived at the facility 7 days before they were inoculated and were housed in separate isolation units at 25 °C under natural day–night rhythm with ad libitum access to feed and water.

2.3. Localization of Salmonella Typhimurium in porcine tonsils

2.3.1. Experimental inoculation of piglets

One week after their arrival at the facility, 12 individually housed experimental animals were orally inoculated with approximately 2×10^7 CFU of a stationary phase culture of the WT_{nal} in 2 ml HBSS. Five and 28 days post-oral inoculation (pi), 8 and 4 piglets, respectively, were humanely euthanized. Samples of tonsils were collected to prepare 'single cell suspensions' and cryosections.

2.3.2. Localization of Salmonella Typhimurium in porcine tonsils using the single cell suspension method

Adhering muscle and fat tissue was removed from the tonsillar tissue and the tonsil was rinsed once with HBSS. The tissue was subsequently treated with 10 mM dithiothreitol (DTT; Bio-Rad Laboratories, Hercules, CA, USA) for 10 min to remove mucus rests. After tissue samples were cut to small pieces, they were treated twice with 25 ml

Table 1
Primers used in this study to create the *csgA*, *csgD* and *adrA* deletion mutants.

Primers	Sequences
csgA Forward	5′-CAACGCTAATACCGTTACGACTTTTAAATCAATCCGATGGGG GTTTTACCTGTGTAGGCTGGAGCTGCTTC-3′
csgA Reverse	5′-GAAAAAAAACAGGGCTTATGCCCTGTTTTTTTATTAGCGCAG ACGCTAAAC ATATGAATATCCTCCTTAG-3′
csgD Forward	5′-TTTATGGGGGC AGCTGTCAGATGTGCGATTAAAAAAAGTGGA GTTTCATCTGTGTAGGCTGGAGCTGCTTC-3′
csgD Reverse	5′-CGAAC AGTAACTCTGCTGCTACAATCC AGGTC AGATAGCGTT TCATGGCCCATATGAATATCCTCCTTAG-3′
adrA Forward	5′-ATACTTCCTCCATGCGCTCTGTTTCTATAATTTGGGAAAATTG TTTCTAATGTGTAGCTGGAGCTGCTTC-3′
adrA Reverse	5′-TGTATGGAAAAATC AGAGGCGCTC AGTAAATCCTGAAgCCCG GCTGGACGC ATATGAATATCCTCCTTAG-3′

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