The infection biology of pig-associated Salmonella

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Abstract
Through the use of an established line of porcine intestinal epithelial cells, known as IPEC-1, this in vitro work examines the initial adhesion, invasion and persistence abilities of different Salmonella serovars and phage types, including multiresistant and monophasic S. Typhimurium DT193 isolates. The resultant innate immune response of the porcine cells to the isolates is assessed through determination of interleukin (IL)-6 and IL-8 concentrations present in cell culture supernatants. The results are discussed in relation to preliminary work, including growth curve data and basic morphological description of the bacterial isolates. Results show that there is variation between the strains in invasiveness (ANOVA p=<0.001) and in the concentrations of IL-6 and IL-8 elicited (ANOVA p=0.001 and <0.001, respectively). Specifically, an S. Derby isolate investigated here exhibits greatly reduced ability to invade, and a biphasic DT193 isolate stimulates very high levels of IL-8 released from the IPEC-1 cells. The variation between strains shown here emphasises the need for a multivalent vaccination to be developed, as opposed to a monovalent one.

Introduction
The infection of pigs with Salmonella enterica subspecies enterica (hereafter Salmonella) is a continuing animal health problem that has high zoonotic potential. The UK pig industry, under the Zoonoses National Control Programme (ZNCP), is currently working towards its target of reducing the number of pig carcasses at the abattoir testing positive for the ELISA antibody test to below 10%. A missing component of many Salmonella control strategies is the use of vaccination, because only inactivated vaccines are available and the efficacy of these is uncertain. There is therefore a need to investigate the possibility of development of an effective multivalent vaccine for pigs to protect against Salmonella. A multivalent vaccine as opposed to a monovalent one is the most sensible option because it has been shown that pig herds can be exposed to several different phage types and serovars of Salmonella during their time on a particular farm (Wales et al., 2009). S. Typhimurium has consistently been the most frequently isolated serovar in the UK and in the EU as a whole, and S. Derby is often the second most common serovar (EFSA, 2008). The monophasic variants of the Typhimurium serovar are being isolated with increasing frequency, to the extent that they have been more common than S. Derby in the UK in the past year (Veterinary Laboratories Agency, 2010).

Studies investigating host-pathogen interactions of S. Typhimurium and S. Enteritidis in chickens have shown that even within the same phage type there can be marked differences in virulence (Humphrey et al., 1996; Humphrey et al., 1998; Williams et al., 1998). Until now there has been little comparable data for pigs. Existing pig models have looked at the host response to single strains of S. Typhimurium (Arce et al., 2008; Meurens et al., 2009; Collado-Romero et al., 2010) and compared the behaviour of different Salmonella serovars (Osterberg et al., 2009), but little focus has been directed towards populations of Salmonella. The aim of this study is to characterise pig-associated field isolates from the UK in a similar way to that of Bergeron et al. (2009) who examined isolates from the Canadian Salmonella population.

A key attribute of Salmonella pathogenesis following oral infection is the attachment to and invasion of intestinal epithelial cells, both of which are thought to be major steps related to the virulence of the bacterium (Boyen et al., 2006). Following this invasion, the host mounts an innate immunological response through production of several cytokines, including IL-1, IL-6, IL-8 and TNF-α (Volf et al., 2007) by enterocytes and macrophages. This response is elicited both by recognition of the pathogen, primarily **f**agella and lipopolysaccharide, through Toll-like receptors and by secreted virulence factors of the pathogen. For this study, the attachment and invasion abilities of several different Salmonella serovars and phage types were investigated using a porcine intestinal epithelial cell line and the consequent cytokine (IL-6) and chemokine (IL-8) production of these cells was measured.

Materials and Methods

Bacterial strains

Six field isolates of Salmonella kindly provided by the VLA were used in this preliminary study alongside one lab strain, known as S. Typhimurium 474 and which is associated with infection in cattle. Its behaviour in vitro is well characterised. The field isolates include three S. Typhimurium multi-resistant DT193 strains: one with the 4,12:i:- serotype, another with the 4,5,12:i:- serotype and a third with the normal serotype. The other field isolates were one of each of S. Typhimurium DT206 and U288 and one S. Derby. The isolates were grown in LB medium in a shaking incubator at 150rpm and on nutrient agar, both at 37°C. Prior to cell invasion experiments, growth curves were produced for each isolate by measuring optical density (OD600) of a growing culture every hour for 12 hours and then again at 24 hours. Gram staining of each isolate was also done.

Cell line and culture conditions

The IPEC-1 cell line is an intestinal epithelial cell line derived from the small intestine of a neonatal unsuckled piglet (Gonzalez-Vallina et al., 2006). The cell line was a generous gift from Dr. T. Cogan, University of Bristol, UK. The cells were maintained in 75cm3 plastic cell culture flasks at 37°C in a humidified incubator with a 5% CO2 atmosphere. They were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM/F-12; Invitrogen, UK) supplemented with 5% foetal calf serum, 100 IU/ml penicillin, 100µg/ml streptomycin, 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml selenium (ITS Premix; BD Biosciences, UK) and 1ng/ml human recombinant epidermal growth factor (EGF; BD Biosciences, UK), hereafter referred to as IPEC-1 media. Continuous cultures of IPEC-1 cells were maintained by seeding culture flasks at 1:3 ratios at each passage. For the adhesion and invasion assays cells were grown in antibiotic-free IPEC-1 media in 24-well cell culture plates at 37°C and 5% CO2 atmosphere.

Invasion assay

The number of bacteria that successfully invade and persist in the IPEC-1 cells was determined for each isolate by a cell invasion assay. Bacterial strains were grown overnight in 10ml LB medium in an orbital shaking incubator at 150rpm and 37°C. After 16 hours of growth the optical density was monitored until it reached 1.2 at 600nm, indicative of late-log phase growth. Bacterial cells were then added at a multiplicity of infection of 10 (10 bacteria per cell) to IPEC-1 cells that had reached confluence in a 24-well plate and incubated at 37°C for 1 hour. The media was then removed, fresh IPEC-1 media containing 150µg/ml colistin sulphate was added, and the cells were incubated for 90 minutes to kill any extracellular bacteria. Colistin was chosen because two of the DT193 strains are resistant to the more commonly used gentamicin, and colistin has been shown to be effective at killing extracellular bacteria (Kusters et al., 1993; Bergeron et al., 2009). Cells were washed four times with 1× phosphate-buffered saline (PBS) and overlaid with 0.5% (w/v) Triton-X 100 (Sigma Aldrich, UK) in PBS to lyse the cells and release any internalised bacteria. The cell lysate was then diluted and plated on nutrient agar to determine viable bacterial counts. A second 24-well plate was infected at the same time, but the fresh IPEC-1 media that was added after the incubation period contained a reduced concentration of 30µg/ml colistin. This plate was then incubated for 48 hours before continuing the invasion assay to assess the number of bacteria that persisted within the IPEC-1 cells. Cell culture supernatants (150µl) were collected from each well of this plate at 24h and frozen at -20°C for IL-6 and IL-8 determination.

Adhesion assay

The number of bacteria that adhere to the IPEC-1 cells was determined for each isolate by a cell adhesion assay. Bacterial strains were grown in the same way as for the invasion assay and added to the IPEC-1 cells at the same multiplicity of infection. After the bacteria and cells were incubated together for one hour the media was removed, cells were washed six times with PBS before being lysed and the cell lysate diluted and plated out. The viable counts for this assay gave the total number of bacteria that adhered to and invaded the IPEC-1 cells, so the number of invading bacteria, as determined by the invasion assay was subtracted from this value to give the number of bacteria that adhered.

Enzyme-linked immunosorbance assay (ELISA)

IL-6 and IL-8 concentrations were determined using the porcine IL-6 and IL-8 Quantikine® Immunoassay kits (R&D Systems Europe, Ltd., UK) which employ the quantitative sandwich enzyme immunoassay technique. The assays were performed according to the manufacturer's specifications.

Statistical Analysis

All experiments were performed in triplicate. Mean values and standard deviation were calculated and individual isolates were compared using one-way ANOVA. The Kruskal-Wallis non-parametric test was used on data sets where the standard deviations were not similar enough to satisfy the assumptions of ANOVA. Differences were considered significant at $p<0.05$. MiniTab15 was used.

Results

The data indicate variation between strains in invasiveness (ANOVA $F=5.53$, $p=<0.001$), and in persistence (Kruskal-Wallis $H=13.53$; $p=0.035$) (Figure 1). The S. Derby isolate was significantly less invasive than the following strains: S. Typhimurium 474 ($p=0.0052$), DT208 ($p=0.0081$) and 4,5,12:i:- DT193 ($p=0.006$). The adhesion assays showed that the number of bacteria associated with the cells, which is the total number that adhered and/or invaded, was very similar for all strains: 5.18 ± 0.5 log colony-forming units (CFU).

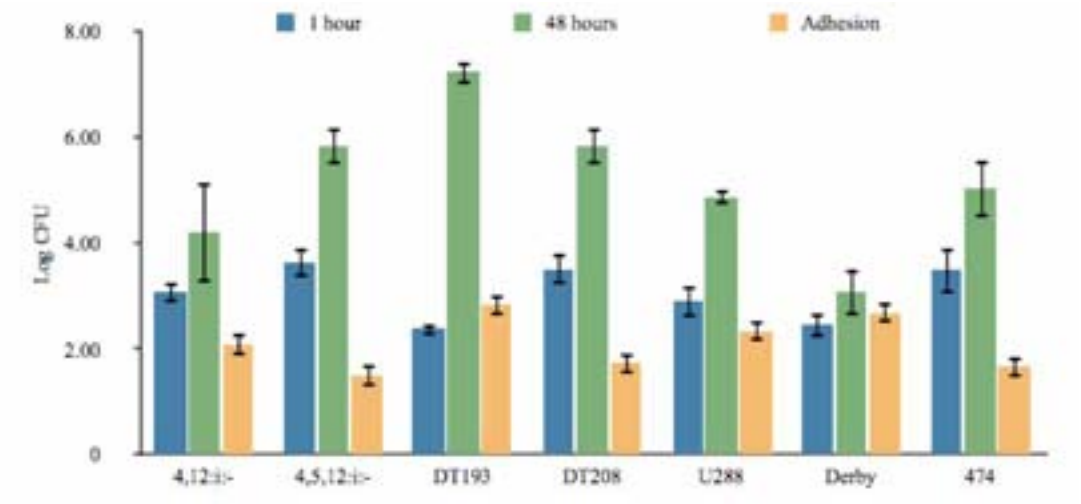

Figure 1 Mean and standard error of log number of intracellular Salmonella after 1 hour and 48 hour culture periods with IPEC-1 cells, and log number of Salmonella adhered to IPEC-1 cells after 1 hour. The number internalised after 1 hour indicates invasive ability and number internalised after 48 hours indicates ability to persist within the cells.

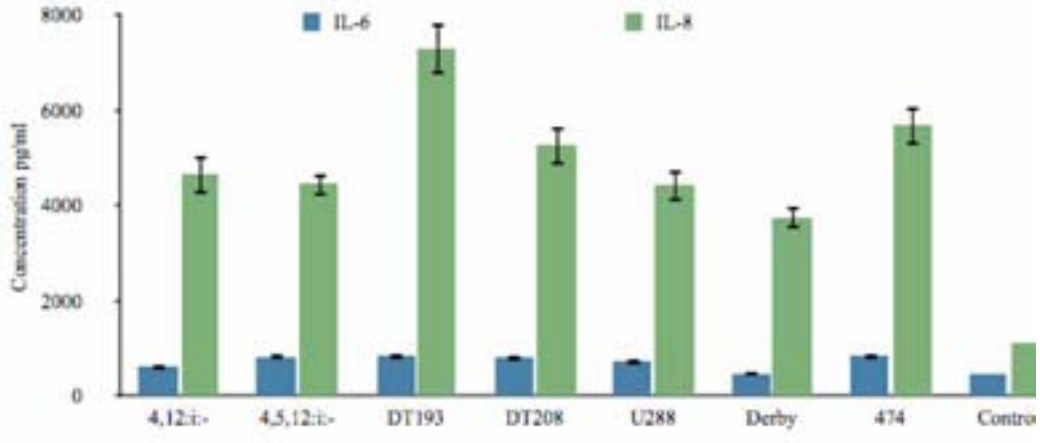

Figure 2 Mean and standard error of concentrations of porcine IL-6 and IL-8 detected by ELISA in IPEC-1 cell culture supernatants after 24 hour incubation with different Salmonella isolates. Uncontaminated LB broth served as a negative control.

After the same incubation time, the concentration of IL-8 released by the IPEC-1 cells was much higher than that of IL-6 (Figure 2). Variation between isolates in production levels of both IL-6 (F=4.64; p=0.001) and IL-8 (F=6.49; p=<0.001) was found. For example, the S. Derby isolate elicited significantly less IL-6 production than S. Typhimurium 474 (p=0.0027), DT193 (p=0.0204), DT208 (p=0.0465) and 4,5,12:i:- DT193 (p=0.0277). Similarly, infection with the multi-resistant DT193 isolate resulted in a significantly higher release of IL-8 by IPEC-1 cells than U288 (p=0.0006), S. Derby (p=0.0002), and both of the monophasic DT193 strains (p=0.0040 and 0.0027).

The growth curves produced showed that there is apparent variation in the growth patterns of the different isolates. The multi-resistant S. Typhimurium strains with normal serotype and 4,5,12:i:- serotype both appeared to exhibit reduced growth rates. Gram staining of these two isolates revealed that both have a different phenotype to the characteristic rod-shape of Salmonella, with smaller barrel-shaped morphology being evident.

Discussion

The results presented here indicate that there is variation between certain Salmonella isolates associated with infection in pigs in: phenotype, ability to invade and persist within intestinal epithelial cells and in the resultant chemokine and cytokine expression. Finding that the number of bacteria associated with the cells during incubation together was the same across all strains suggests that all have similar attachment abilities, and that variation in invasiveness is the key difference. This is an important finding because it indicates that for a potential vaccination to be multivalent, it would need to prevent the initial adhesion of the bacteria to the enterocytes. Of potential importance is the evidence from the invasion data and the cytokine data is that S. Derby might be of less concern to public health because it has significantly reduced invasive ability and fails to amount an IL-6 driven inflammatory response from the IPEC-1 cells. S. Derby, however, might be capable of causing systemic disease in pigs through this evasion of host immune responses. However the current study is limited to one isolate of the serovar and analysis with a greater number of isolates is needed before any conclusion can be reached. IL-8 is well described as a neutrophil chemoattractant that induces neutrophil degranulation and also promotes CD4+ and CD8+ T cell migration. The high concentration of IL-8 produced by IPEC-1 cells following infection with the biphasic S. Typhimurium DT193, and the consequent gut inflammation that would be expected to occur in vivo, suggests that this strain is likely to cause enteritis in pigs. It is interesting that the monophasic variants of this phage type do not elicit as strong chemokine response, and possibly suggests that they have different mechanisms of invasion.

References

1. ARCE, C., et al. 2010. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from Salmonella Typhimurium. Comparative Immunology, Microbiology and Infectious Diseases, 33, 161-174.
2. BERGERON, N., et al. 2009. Interaction between host cells and septicemic Salmonella enterica serovar typhimurium isolates from pigs. Journal of Clinical Microbiology, 47, 3413-3419.
3. BOYEN, F., et al. 2006. Salmonella Typhimurium SPI-1 genes promote intestinal but not tonsillar colonization in pigs. Microbes and Infection, 8, 2899-2907.
4. COLLADO-ROMERO, M., et al. 2010. Quantitative analysis of the immune response upon Salmonella Typhimurium infection along the porcine intestinal gut. Veterinary research, 41, 23.
5. EFSA, 2008. Report of the Task Force on Zoonoses Data Collection on the analysis of the baseline survey on the prevalence of Salmonella in slaughter pigs in the EU, 2005-2007, Part A: Salmonella prevalence estimates. The EFSA Journal, 135, 1-111.
6. GONZALEZ-VALLINA, R., et al. 1996. Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). Am J Physiol, 271, G249-259.
7. KUSTERS, J. G., et al. 1993. Effects of multiplicity of infection, bacterial protein synthesis, and growth phase on adhesion to and invasion of human cell lines by Salmonella Typhimurium. Infection and Immunity, 61, 5013-5020.
8. MEURENS, F., et al. 2009. Early immune response following Salmonella enterica subspecies enterica serovar Typhimurium infection in porcine jejunal gut loops. Veterinary Research, 40.
9. ÖSTERBERG, J., et al. 2009. Patterns of excretion and antibody responses of pigs inoculated with Salmonella Derby and Salmonella Cubana. Veterinary Record, 165, 404-408.
10. VETERINARY LABORATORIES AGENCY, 2010. EFIG Salmonella summary, Quarterly update January-June 2010, 1-30.
11. VOLF, J., et al. 2007. Cytokine response of porcine cell lines to Salmonella enterica serovar Typhimurium and its hilA

and ssrA mutants. Zoonoses and Public Health, 54, 286-293.
12. WALES, A. D., et al. 2009. Longitudinal survey of the occurrence of Salmonella in pigs and the environment of nucleus breeder and multiplier pig herds in England. Veterinary Record, 165, 648-657.
13. WRAY, C. 2001. Review of research into Salmonella infections in pigs., Meat and Livestock Commission. 1-62.