Interaction between host cells and *Salmonella* Typhimurium isolates from septicemic pigs

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Abstract

In this study, we characterized isolates (n=33) from septicemia outbreaks in swine herds as well as isolates (n=33) recovered from healthy animals at slaughter. At first, the rates of adhesion and invasion on intestinal epithelial cell lines were measured for all isolates. The isolates recovered from diseased animals have been shown to invade at a higher rate ($P < 0.05$) than isolates from healthy pigs. There was no significant difference for adhesion between 2 groups of isolates ($P > 0.05$). Some isolates were chosen for the remaining experiments according to invasion assay results. No difference between isolates from septicemic pigs and isolates from healthy pigs was detected for other phenotypic methods such as phagocytosis rates and survival in monocytes, apoptosis, adhesion to intestinal mucus and microbial surface properties. However, for the phagocytosis rates, a significant difference between the 2 groups of isolates was observed at 15 minutes ($P < 0.05$). These results suggest that early steps in the infection and spread within host phagocytes can be important in the outcome of the disease caused by these virulent isolates.

Introduction

Infections caused by septicemic strains of *Salmonella* Typhimurium can be associated with significant mortality in mature pigs. Since previous studies showed persistence of strains in various tissues for many days following the infection (Côté *et al.*, 2004), the presence of these strains represent as well a food safety concern. Thus, it is important to better characterize these isolates in order to understand pathogenesis of infection and develop appropriate control measures. The aim of this study was to characterize isolates of *S.* Typhimurium associated with septicemia in swine and to compare them to isolates recovered from clinically healthy pigs.

Materials and methods

Bacterial strains. *Salmonella* isolates (n = 33) recovered from extra-intestinal organs and/or feces of dead pigs and submitted for necropsy were obtained from Dr. S. Messier (Faculty of Veterinary Medicine, Université de Montréal). Isolates (n = 33) from healthy pigs without clinical sign (WCS) were collected at slaughterhouse from apparently healthy animals without macroscopic lesions observed at this time (Letellier *et al.*, 1999). *S.* Typhimurium strain SL1344 was previously described as highly invasive, was used as positive control (Kusters *et al.*, 1993) and a noninvasive *Escherichia coli* strain 862B, kindly provided by Dr. J. M. Fairbrother (Faculty of Veterinary Medicine, Université de Montréal) was used as negative control.

Adhesion and invasion assays. The human embryonic intestinal epithelial cell line (Int-407) was used for adhesion and invasion assays and cells were maintained as Kusters *et al* (Kusters *et al.*, 1993). All isolates were analysed in late logarithmic-phase cultures of bacteria. Dilutions were prepared for resulting in a multiplicity of infection of 10 bacteria per cell. Adhesion and invasion assays were made based on the protocol of Kusters *et al* (Kusters *et al.*, 1993). Finally, the cell lysate was diluted and plated on LB agar to determine viable bacterial counts. The adhesion and invasion rate were calculated according to the

following formula: percent adhesion or invasion = (number of bacteria recovered/total number of bacteria added) x 100.

Phagocytosis level. Selected isolates were chosen for the remaining experiments according to the results of invasion. In the group of septicemic pigs, the 6 most invasive isolates were chosen and 3 isolates were chosen with an invasion rate similar to the invasion rate of the isolates of the group of healthy pigs. In the group of healthy pigs, 6 isolates among the 8 least invasive isolates were chosen. The protocol for the phagocytosis were inspired from Busque *et al* (Busque *et al.*, 1998). The phagocytosis level was calculated as the percentage of phagocytosis by monocytes at 37°C substracted by the percentage of phagocytosis at 4°C (adherent control). The analysis were done by flow cytometry (BD FACSCalibur™ system, BD Biosciences Pharmagen, Ontario, Canada).
Survival. In order to evaluate survival rates, the phagocytosis was stopped after 60 minutes. The pellet was suspended and incubated with D-PBS-colistin (600 µg/ml) for elimination of extracellular bacteria. The cells were washed, and incubated at 37°C for 6 and 18 hours. Water was added to the cells for 10 minutes and the cell lysate was then diluted and plated on LB agar for 20-24 hours at 37°C to determine viable bacterial counts.
Apoptosis. Two, 4 and 6 hours after the beginning of phagocytosis, the reaction was stopped. The cells were stained with Annexin V-APC and 7-AAD (7-Amino-Actinomycin D) (BD Biosciences) in accordance with the manufacturer's instructions and analyzed by flow cytometry within one hour.

Mucosal preparations. The mucus was prepared by using a modified protocol based on works of many authors (Laux *et al.*, 1984; Laux, 1986). Mucus was stored at -70°C after measurement of the protein concentrations (Protein Assay, Dye Reagent Concentrate, Bio-Rad Laboratories inc, Ontario, Canada) and used for binding studies. A culture was made to verify the presence of *Salmonella* in the mucus gel.

Adhesion assay with mucus. A modified protocol from Laux *et al* (Laux *et al.*, 1984; Laux, 1986) was used for the adhesion assay with mucus. Bacteria were labelled using the same method than for phagocytosis and apoptosis. Assays, in triplicate, were performed in small petris dishes (Tissue culture dish, Sarstedt inc, Québec, Canada). Samples were removed from each petri and fixed with paraformaldehyde 2% (wt/vol), before examination by flow cytometry (Logan *et al.*, 1998). The adherence level to mucus was calculated as the percentage of relative fluorescence by bacteria subtracted by the autofluorescence of the mucus.
Affinity of strains for solvent. In order to study the microbial surface properties that can affect interaction with host cells, microbial adhesion to solvents (MATS) was used following protocol previously described (Bellon-Fontaine et al., 1996; Planchon et al., 2007).

Statistical analysis. All analysis were based on at least three independent replicates. We used SAS (version 9.1; SAS Institute, Cary, NC) for data analysis. For adhesion, invasion, phagocytosis, survival, apoptosis, mucus and MATS, the mean and the standard deviation were used for descriptive statistics. Prior to analysis, survival data were log-transformed and phagocytosis and apoptosis data were transformed with the arcsine square root. For MATS, the negative results were considered equal to zero. Linear models with isolates as fixed factors were used to examine differences among isolates. For invasion, adhesion, MATS and survival, linear models with isolate type as a fixed factor and isolate within a type as a random factor were used to compare types of isolates (CS and WCS). For decane, in the MATS assays, exact chi-square tests were used to compare the prevalence of positive results among isolates and to compare isolate types. For phagocytosis and apoptosis data, repeated-measures linear models, with time as a within-subject factor, were used to examine the effect of time, isolate, isolate type and monocytes source. Sequential Bonferroni post-hoc tests were used to compare pairs of means. The statistical significance was set at a value of $P < 0.05$.

Results

Ability to adhere and invade. It was not possible to detect any significant difference ($P > 0.05$) between both groups of isolates in the ability to adhere to Int-407 cells. A significant difference ($P < 0.05$) was observed between two groups of isolates in the invasion assays. Septicemic isolates invaded epithelial cells at higher rates than isolates from healthy pigs.

Phagocytosis. The mean values increased with time for CS and WCS isolates ($P < 0.05$). The average was significantly lower for isolates from CS than for isolates from WCS at 15 min ($P < 0.05$), but not at 30 min ($P > 0.05$) nor after 60 min ($P > 0.05$).

Survival. Some isolates were able to survive after 6 hours and 18 hours. It was not possible to detect any significant difference ($P > 0.05$) between both groups of isolates in the ability to survive after 6 hours.

Apoptosis. The average of the percentages of living cells, apoptotic cells, apoptotic cells toward the necrosis and necrotic cells did not differ between isolates from CS versus isolates from WCS ($P > 0.05$).

Adhesion with mucus. It was not possible to detect any significant difference ($P > 0.05$) between both groups of isolates in the ability to adhere to mucus.

MATS. All isolates of S. Typhimurium had the maximal affinity with chloroform, the acidic solvent. The lowest affinity was viewed for ethyl acetate, the basic solvent. It was thus not possible to detect any difference between groups of isolates for MATS ($P > 0.05$) for all sovents.

Discussion

In this study, different procedures were used in order to discriminate *S.* Typhimurium isolates recovered from diseased and clinically healthy pigs. In our study, we found significant differences in invasion rates among septicemic and non septicemic isolates but not in adhesion rates. Septicemic isolates invaded epithelial cells at higher rate than isolates from healthy pigs. However, some strains isolated from clinically healthy animals also possessed a high invasiveness rates, suggesting that healthy animals may carry potentially pathogenic strains. Since pigs may carry *Salmonella* for prolonged period after onset of diarrhea and disease, it is thus likely that some septicemic isolates could have been recovered from clinically healthy animals. Another possibility is a contamination during transport or lairage period.

Isolates from CS are less phagocyted by porcine monocytes in 15 min than isolates from WCS. One hypothesis would be that the phagocytosis is delayed with septicemic isolates. Although the means value of phagocytosis was similar, it appeared that CS isolates seemed to be more heterogenous than WCS isolates. *S.* Typhimurium survival in the macrophages is essential for virulence (Foley *et al.*, 2008). Some authors, using cultured macrophages, demonstrated that invasive strains of *S.* Typhimurium invaded macrophages 10 times more than strains that have a non-invasive phenotype (Monack *et al.*, 1996). Some invasive *S.* Typhimurium strains can induce apoptosis, whereas noninvasive mutant strains did not (Monack *et al.*, 1996). Interestingly, we did not observe any significant difference between the 2 groups of isolates in their ability to induce apoptosis, suggesting that the apoptosis process is not linked to virulence in these virulent *S.* Typhimurium isolates for pig.

Conclusion

In this study, using various methods to assess virulence of isolates from healthy or diseased animals, we were able to demonstrate that isolates from diseased animals possess an increased capacity to invade intestinal cells and were phagocyted at a lower level at early step than isolates from healthy animals.

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