Interaction between Host Cells and Septicemic Salmonella enterica Serovar Typhimurium Isolates from Pigs

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Salmonella enterica serovar Typhimurium is an important pathogen in swine and is also a frequently reported zoonotic agent. The objective of this study was to characterize isolates of S. enterica serovar Typhimurium associated with septicemia in swine and to compare them to isolates recovered from clinically healthy pigs. We were particularly interested in comparing the two groups of isolates for their ability to adhere to and invade host cells, to be phagocytized and survive in monocyte cells, to induce apoptosis, and to adhere to intestinal mucus. Their surface properties were also evaluated by interactions with solvents. The isolates recovered from diseased animals were shown to invade intestinal epithelial cell lines at a higher rate (P = 0.003) than isolates from healthy pigs.

Septicemic isolates were phagocytized by human monocytes at a higher rate than isolates from healthy pigs (P = 0.009). The mean percentages of phagocytosis were significantly lower for human monocytes than for porcine monocytes (P = 0.02 and P = 0.008, respectively) for isolates from both diseased and healthy animals. Healthy animal isolates were phagocytized more by porcine monocytes at 15 min (P = 0.02) than septicemic isolates. No difference between isolates from septicemic pigs and isolates from healthy pigs was detected for other tested parameters. These results suggest that septicemic isolates have a particular pattern of invasion.

In pigs, Salmonella strains are related to significant animal infections associated with clinical signs and economic losses but are mainly associated with a carrier state, becoming a reservoir for human infections (3). Infection and/or silent carriage of Salmonella in pigs is an important public health concern. Multiresistance to antibiotics is often associated with Salmonella enterica serovar Typhimurium (14). In order to develop control measures, it is important to characterize these isolates and better understand the pathogenesis of infection.

The pattern of infection by Salmonella is oral ingestion of the bacteria followed by passage through the mucus which covers the epithelial cells to invade mucous membranes and cause disease (35). The intestinal mucus can then serve as the initial binding site for bacteria. Initial adhesion is mainly a physicochemical process founded on nonspecific interactions (van der Waals and coulombic interactions) (17, 33). This type of adhesion can be reversible or not, and surface properties of some bacteria have been show to influence nonspecific interactions with host cells. The first steps of infection are adhesion on the surface, firm attachment, and penetration into intestinal epithelial cells. The invasion of intestinal epithelial cells is believed to be a very important step related to the virulence of Salmonella strains associated with infections (5).

After invasion of the epithelial cells, the bacteria reach the subepithelial lymph tissue and the lamina propria, where Salmonella cells meet host immune cells (28). The overall phagocytic process can be divided into at least two main parts. First, bacteria must adhere to the phagocyte surface in a process called adherence. The second step of phagocytosis involves internalization or ingestion of the adherent particle. Following initial adhesion to phagocytes, special bacterial cell surface structures recognize receptors on the target cell surface (17, 33). The virulence genes of S. enterica serovar Typhimurium located on SPI-1, which encode a type III protein export machinery, are necessary for invasion of either nonphagocytic (5) or phagocytic (28) cells. Salmonella is able to induce cell death in macrophages in two different ways (32). Rapid activation of programmed macrophage cell death depends on SipB and SPI-1, whereas delayed induction of apoptosis in infected macrophages is SPI-1 independent (32). The results reported by van der Velden et al. indicate that ompR and a functional SPI-2-encoded type III protein secretion apparatus are required for delayed induction of apoptosis (32). The survival in phagocytes is an important step to induce septicemia in pigs, causing clinical signs similar to those in humans (16, 31); therefore, this animal model may be used to study human salmonellosis. Salmonella is able to survive and replicate in phagocytic cells, and this is an essential component of the virulence of these bacteria (1). S. enterica serovar Typhimurium cells that have invaded the macrophage by phagocytosis are able to replicate intracellularly (28) and induce apoptosis (32). In the past 10 to 15 years, an increased number of cases of clinical salmonellosis associated with Salmonella serovar Typhimurium were observed in pigs (11).

The aim of this study was to characterize isolates of S. enterica serovar Typhimurium associated with septicemia in
swine and to compare them to isolates recovered from clinically healthy pigs. We were particularly interested in comparing the two groups of isolates in regard to their abilities to adhere to host cells, invade host cells, be phagocytosed, survive in cells, generate apoptosis, adhere to intestinal mucus, and adhere to solvents.

**MATERIALS AND METHODS**

- **Bacterial isolates.** *Salmonella* isolates (n = 33) were recovered from extraintestinal organs or feces of dead pigs with an anamnesis of diarrhea and sudden death and typical lesions associated with salmonellosis and septicaemia at necropsy. A diagnosis of salmonellosis was established following necropsy. These isolates from pigs with clinical signs (CS isolates) were collected at slaughterhouses from apparently healthy animals from farms without a history of clinical signs and without macroscopic lesions observed at this time (24). Sampled animals originated from a total of 55 farms; 7 farms were sampled more than once (4). *S. enterica serovar Typhimurium* strain SL1344, which was previously described as highly invasive in in vitro invasion assays and virulent for mice, was used as a positive control (18, 20), and the noninvasive *Escherichia coli* strain 862B, kindly provided by J. M. Fairbrother (Veterinary Medicine Faculty, Université de Montréal, Saint-Hyacinthe, Québec, Canada). Isolates (n = 33) from healthy pigs without clinical sign (WCS isolates) were collected at slaughterhouses from apparently healthy animals from farms without a history of clinical signs and without macroscopic lesions observed at this time (24). Samples animals originated from a total of 55 farms; 7 farms were sampled more than once (4).

- **Growth and preparation of bacteria for adhesion and invasion assays.** All isolates from a previous study (4) were analyzed for adhesion and invasion assays. LB agar plates were first inoculated with bacteria from frozen glycerol stocks and incubated at 37°C overnight. Late-logarithmic-phase cultures of bacteria (optical density [OD] at 600 nm of ~0.650) were prepared by inoculating LB broth and incubating the bacteria at 37°C for 18 to 20 h. The bacteria were centrifuged at low speed and resuspended in MEM at an OD of 600 nm of ~0.290 (10^9 CFU/ml). Dilutions were prepared to result in a multiplicity of infection (MOI) of 10 bacteria per cell in 1 ml of inoculum.

- **Adhesion and invasion assays.** For adhesion assays, confluent monolayers of Int-407 cells were washed once with prewarmed Dulbecco's phosphate-buffered saline (D-PBS) (pH 7.4) (Invitrogen). The bacterial inoculum was added, and the infected cells were incubated at 37°C for 30 min in a 5% CO2 atmosphere. Cells were rinsed three times with D-PBS to remove nonadherent bacteria. The cell lysate was then diluted and plated on LB agar to determine viable bacterial counts.

- **Evaluation of phagocytosis level.** For logistical reasons, selected isolates were chosen for the remaining experiments according to the results of the invasion assay. From the group of septicaemic pigs, the six most invasive isolates and three isolates with an invasion rate similar to that of the isolates from the group of healthy pigs were chosen. From the group of healthy pigs, six isolates among the eight least invasive isolates were chosen.

**Porcine blood.** Porcine blood was collected at the slaughterhouse, and human blood was collected from volunteer. The institutional review board approved the protocol, and all patients provided written informed consent. Whole blood (porcine or human) was incubated with FITC-labeled *Salmonella* at a phagocyte/bacterium ratio of 30:1, 30:30, and 30:60 for 15, 30, and 60 min at 4°C and 37°C. The reaction was stopped with ice-cold D-PBS, the erythrocytes were lysed with Coulter whole-blood lysing reagents (Beckman Coulter Canada Inc., Ontario, Canada), and the cells were washed and fixed with paraformaldehyde (2%, wt/vol). The phagocytosis level was calculated as the percentage of phagocytosis by monocytes at 37°C minus the percentage of phagocytosis at 4°C (adherent control). The analyses were done by flow cytometry (BD FACSCalibur System; BD Biosciences Pharmagen, Ontario, Canada). Three assays were done with cells from various blood donors.

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**Survival.** In order to evaluate survival rates, the phagocytosis was stopped with ice-cold D-PBS and cells were centrifuged. The pellet was suspended and incubated with D-PBS-colistin (600 µg/ml) for elimination of extracellular bacteria. The cells were washed with ice-cold D-PBS. The pellet was resuspended in D-PBS and incubated at 37°C for 6 and 18 h. Water was added to the cells and left for 10 min, and the cell lysate was then diluted and plated on LB agar for 20 to 24 h at 37°C to determine viable bacterial counts.

**Apoposis.** At 2, 4, and 6 h after the beginning of phagocytosis, the reaction was stopped with ice-cold D-PBS. The erythrocytes were lysed with Coulter whole-blood lysing reagents. The cells were washed with D-PBS, colored with annexin V-allophycocyanin (APC) and 7-amino-actinomycin D (7-AAD) (BD Biosciences) in accordance with the manufacturer's instructions, and analyzed by flow cytometry.

**Mucosal preparations.** The mucus was prepared by using a modified protocol based on previous reports (21, 22). Briefly, the porcine intestinal mucus was isolated from the small intestines (segments of 10 cm) of five pigs from a slaughterhouse. The small intestines were placed in sterile petri dishes containing 10 mmol/liter HEPES-Hanks buffer (IIH) (pH 7.4) and cut into 2- to 3-cm lengths. Any feces and partially digested food were removed from each intestinal section manually. The intestines were shifted to a second set of petri dishes containing HH and split open longitudinally with scissors. The sections were shaken to remove any further debris and transferred to a third set of petri dishes. The layer of mucus gel covering the intestinal lumen was collected by gently scraping the mucosa with a rubber spatula.

**Adhesion assay with mucus.** A modification of the protocol of Laux et al. (21, 22) was used for the adhesion assay with mucus. Bacteria were labeled using the same method as for phagocytosis and apoptosis. Assays, in triplicate, were performed in 15 ml of small petri dishes at room temperature. Samples were taken after 1, 3, and 6 h. Mucosal preparations (10 mg/ml) were immobilized in petri dishes by incubation for 24 h at 4°C. Excess mucus was removed by two washes with ice-cold HH. The fixed mucus was used for binding assays with *S. enterica serovar Typhimurium*. The labeled bacteria were added to each petri dish. After incubation for 1 h at
At 37°C, the petri dishes were rinsed three times with ice-cold HH to remove unattached bacteria. Adherent bacteria were released by adding 5% sodium dodecyl sulfate to each petri dish and incubating for 1 h at 37°C. Samples were removed from each petri dish and fixed with paraformaldehyde (2%, w/vol) before examination by flow cytometry (25). The level of adherence to mucus was calculated as the relative fluorescence of bacteria minus the autofluorescence of the mucus.

Affinity of strains for solvent. In order to study microbial surface properties that can affect interaction with host cells, microbial adhesion to solvents (MATS) was used as described previously (2). This method compares the microbial cell affinities to a monomeric solvent and an apolar solvent. The monomeric solvent can be an electron acceptor or an electron donor, but both solvents should have similar van der Waals’ surface tension components (2). Two pairs of solvents were used, as described by Bellon-Fontaine et al. (2), to determine acidic and basic microbial surface properties: (i) chloroform, an electron acceptor solvent, and hexadecane, a nonpolar solvent; and (ii) ethyl acetate, a strong electron donor solvent, and decane, a nonpolar solvent. All solvents were obtained from Sigma-Aldrich.

The protocol is based on reports by Bellon-Fontaine et al. and Planchon et al. (2, 30). Three successive subcultures were completed for all strains in LB broth, Sigma-Aldrich.

Ability to adhere to Int-407 cells. The adherence of Int-407 cells to bacteria was tested for two groups of isolates. It was not possible to detect any significant difference (P = 0.53) between the two groups of isolates in the ability to adhere to Int-407 cells. The means and standard errors were 16.00% ± 2.48% for septicemic isolates and 18.60% ± 3.13% for isolates from healthy pigs. The results are presented in Fig. 1.

Ability to invade Int-407 cells. We compared the invasion of Int-407 cells by S. enterica serovar Typhimurium isolates from septicemic animals with that by isolates from nonsepticemic pigs. A significant difference (P = 0.003) was observed between the two groups of isolates. Septicemic isolates (42.68% ± 5.54%) invaded epithelial cells at higher rates than isolates from healthy pigs (37.60% ± 6.84%). The results are presented in Fig. 1.

Phagocytosis. The percentage of monocytes that were able to phagocytize FITC-labeled Salmonella in porcine whole blood was measured. The mean values increased with time for CS isolates (16.40% ± 4.43%, 23.66% ± 3.21%, and 37.77% ± 3.11% for 15, 30, and 60 min, respectively) (P < 0.0001) as well as for WCS isolates (31.89% ± 7.81%, 28.65% ± 6.60%, and 36.34% ± 5.49% for 15, 30, and 60 min, respectively) (P = 0.02) but seemed to be more stable. The average was significantly lower for CS isolates than for WCS isolates at 15 min (P = 0.02) (Fig. 2) but not at 30 min (P = 0.70) or 60 min (P =

**RESULTS**

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**Statistical analysis.** All analyses were based on at least three independent replicates. We used SAS (version 9.1; SAS Institute, Cary, NC) for data analysis. For adherence, invasion, phagocytosis, survival, apoptosis, mucus adhesion, and MATS assays, 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 arc sine square root to normalize distributions. For MATS, negative results were considered equal to zero. Linear models with isolates as fixed factors were used to examine differences among isolates. For adherence, invasion, survival, and MATS assays, mixed linear models with the isolate type as a fixed factor and the isolate within a type as a random factor were used to compare types of isolates (CS and WCS). For decane, results were dichotomized (presence versus absence) due to the high prevalence of zero values. In this case, 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 monocyte source (human or swine). Post hoc tests, with the sequential Bonferroni adjustment, were used to compare pairs of means. The statistical significance was set at a P value of <0.05.
0.82), but globally, there was no difference. The percentage of phagocytosis was heterogeneous within CS isolates and within WCS isolates (P/H11005 0.005 and P/H11005 0.004, respectively).

The percentage of monocytes that were able to phagocytize FITC-labeled Salmonella in human whole blood was measured. Septicemic isolates were ingested at a higher level (10.63% ± 3.14%, 16.90% ± 2.19%, and 25.34% ± 3.64% for 15, 30, and 60 min, respectively) by human monocytes than isolates from healthy pigs (4.07% ± 1.39%, 5.84% ± 1.62%, and 11.82% ± 2.07% for 15, 30, and 60 min, respectively) at all times tested (P = 0.009). Figure 2 shows the results for 15 min.

For WCS isolates, the mean values of intracellular bacteria increased with time for human monocytes (P/H11005 0.005). The percentage of phagocytosis was heterogeneous within CS isolates (P/H11005 0.02).

We compared the percentages of bacteria phagocytized by monocytes isolated from porcine blood and human blood. The mean percentages of phagocytosis were significantly lower for the human monocytes than for porcine monocytes for isolates from both diseased and healthy pigs at all times (P = 0.02 and P = 0.008, respectively).

Survival. Since Salmonella is capable of surviving in monocyte cells, we then tested whether there was a difference in the survival rate between the two groups of isolates. Some isolates were able to survive after 6 h and 18 h. Negative control bacteria were not able to survive. It was not possible to detect any significant difference (P = 0.17) between the two groups of isolates in the ability to survive after 6 h (Fig. 3). The means (log transformed) and standard errors were 5.41 ± 0.15 for septicemic isolates and 5.05 ± 0.19 for isolates from healthy pigs. The results at 18 h were similar to those at 6 h.

Apoptosis. As S. enterica serovar Typhimurium was able to induce apoptosis of monocytes, we compared apoptosis levels in cells infected by CS and WCS isolates. The mean values for living cells infected with all isolates decreased with time for CS isolates (90.72% ± 3.28%, 77.91% ± 2.58%, and 63.69% ± 2.53 for 2, 4, and 6 h, respectively) (P < 0.0001) and WCS isolates (87.11% ± 2.71%, 74.66% ± 2.42%, and 55.55% ± 3.19% for 2, 4, and 6 h, respectively) (P < 0.0001). The results after 6 h of apoptosis are presented in Fig. 4 and 5. The percentage of living cells was heterogeneous within CS isolates (P < 0.0001). The apoptosis level was heterogeneous within CS isolates (P < 0.0001) and within WCS isolates (P = 0.005).
The percentage of necrotic cells varied within CS and WCS isolates (4.52% for CS isolates and 6.69% for WCS isolates). The percentage of apoptotic cells toward necrosis was heterogeneous within CS and WCS isolates (P < 0.0001 and P < 0.0001, respectively). The mean values for necrotic cells infected with all isolates increased with time for CS isolates (4.52% ± 1.59%, 11.74% ± 1.78%, and 26.61% ± 2.82% for 2, 4, and 6 h, respectively) (P < 0.0001) and WCS isolates (6.69% ± 1.22%, 17.15% ± 1.63%, and 31.44% ± 4.29% for 2, 4, and 6 h, respectively) (P < 0.0001). The percentage of necrotic cells varied within CS and WCS isolates (P < 0.0001 and P < 0.0001, respectively).

The averages of the percentages of living cells, apoptotic cells, apoptotic cells toward necrosis, and necrotic cells did not differ between CS isolates and WCS isolates (P = 0.21, P = 0.51, P = 0.91, and P = 0.91, respectively).

Adhesion with mucus. Isolates were tested for their ability to adhere to the porcine intestinal mucus. It was not possible to detect any significant difference (P = 0.89) between the two groups of isolates in the ability to adhere to mucus. The means and standard errors were 35.36% ± 5.19% for septicemic isolates and 34.21% ± 6.36% for isolates from healthy pigs. The results are presented in Fig. 6.

MATS. Four different solvents were used to study the microbial surface properties of all isolates. Two monopolar solvents, chloroform and ethyl acetate, were chosen for the estimation of the Lewis acid/base character (i.e., electron donor/acceptor), while two apolar solvents, hexadecane and decane, were employed to estimate the hydrophobic/hydrophilic cell surface properties of isolates. All isolates of S. enterica serovar Typhimurium had maximal affinity with chloroform, the acidic solvent. The lowest affinity was for ethyl acetate, the basic solvent. These results showed that the cell surfaces of both groups of isolates were strong electron donors and weak electron acceptors. For all isolates, adhesion to the nonpolar solvent was near zero. Adhesion to chloroform was higher than that to hexadecane, the apolar solvent; the two solvents have comparable van der Waals properties. These differences showed electron donor and basic characters of S. enterica serovar Typhimurium. It was thus not possible to detect any difference between the groups of isolates in MATS (P = 0.10, P = 0.54, P = 0.97, and P = 0.75, respectively, for chloroform, hexadecane, ethyl acetate, and decane). The results are presented in Fig. 7.

DISCUSSION

In this study, different procedures were used to discriminate between S. enterica serovar Typhimurium isolates recovered from diseased and clinically healthy pigs. Gianella et al. (15) have proposed an in vitro model for Salmonella invasion, and they found that only strains that penetrated HeLa cells were able to invade the mucosa of the rabbit ileum. Many parameters can influence the results and interpretation of this type of assay, such as the MOI. Kusters et al. (20) observed that the MOI has an impact on the fraction of the inoculum that adheres to or invades cells. Other authors enhanced the invasion by use of a low-speed centrifugation step (8, 19).

In our study, we used the same control strain (S. enterica serovar Typhimurium SL1344) and similar parameters (MOI, cell line, and growth conditions) as Galan and Curtiss (13) and found significant differences among septicemic and nonsypticemic isolates in invasion rates but not in adhesion rates. Septicemic isolates invaded epithelial cells at higher rates than isolates from healthy pigs. However, some strains isolated from clinically healthy animals also possessed a high invasiveness rate, 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 possible that some septicemic isolates could have been recovered from clinically healthy animals. Another possibility is contamination during transport or lairage.

The greater morbidity and mortality caused by S. enterica serovar Typhimurium DT104 has led to the proposal that these strains may be more virulent (34). In our study, the prevalence of DT104 was 36.4% (12/33) for CS isolates and 51.5% (17/33) for WCS isolates (4). When we used invasion assays, we did not observe any increase in invasiveness for DT104. This is in accordance with the study by Allen et al. (1), who failed to show that S. enterica serovar Typhimurium DT104 was more virulent than S. enterica serovar Typhimurium ATCC 14028 using in vitro and in vivo experiments.

Overall, isolates were phagocytosed more by porcine monocytes than by human monocytes. CS isolates were phagocytosed less by porcine monocytes in 15 min than WCS isolates. One hypothesis would be that the phagocytosis is delayed with septicemic isolates. Although the mean values of phagocytosis...
were similar, it appeared that CS isolates are more heterogeneous than WCS isolates. On the other hand, when in contact with human monocytes, CS isolates are phagocytosed more than WCS isolates. *S. enterica* serovar Typhimurium survival in macrophages is essential for virulence (10). Some authors, using cultured macrophages, demonstrated that invasive strains of *S. enterica* serovar Typhimurium invaded macrophages 10 times more than strains that have a noninvasive phenotype (28). Invasive *S. enterica* serovar Typhimurium strains can induce apoptosis, whereas noninvasive mutant strains did not (28). Interestingly, we did not observe any significant difference between the two groups of isolates in their ability to induce apoptosis. These results suggest that the apoptosis process is not associated with virulence in *S. enterica* serovar Typhimurium isolates that induce disease in pigs. We can speculate that these isolates take advantage of intracellular replication and use this mechanism to spread through the host.

In this study, we also examined the binding of *S. enterica* serovar Typhimurium to pig intestinal mucus, since this was described as an important step leading to colonization. We observed that all isolates of *S. enterica* serovar Typhimurium can bind to crude mucus, with no significant difference between CS and WCS isolates. One study had indicated that both virulent and avirulent *S. enterica* serovar Typhimurium strains were able to bind to rat intestinal mucus; however, in this animal species, the binding of virulent strains was six times greater than that of avirulent *S. enterica* serovar Typhimurium (35). Those authors proposed that virulent *Salmonella* strains take advantage of a specific interaction with the mucus and possibly favor the colonization of the epithelial cells. Other studies reported that virulent strains of various bacterial species can bind to intestinal mucus from different animal species with a higher affinity than avirulent strains (21, 22, 26), in contrast to our findings and those of McCormick et al. (27).

There are probably other virulence factors which were not examined in this study that could be implied. The examination of virulence-related genes by use of a microarray could be used to complete this study.

The MATS method was used for the characterization of the electron donor/electron acceptor properties of bacteria (2). In addition, this method confirmed that adhesion between the bacterial and cell surfaces is the result of an association between van der Waals, Lewis acid-base, and electrostatic interactions (2). According to our results, we can conclude that all isolates possess a higher affinity for chloroform. This high affinity for chloroform, an acidic solvent, it is due to the basic or electron donor character of the bacteria, while the weak affinity found in bacteria for a basic solvent is due to the weak acidic or electron acceptor property (2). This basic character can be attributed to the presence of carboxylic groups on the microbial surface (2) or to the negatively charged surface of microorganisms (30). Furthermore, most bacteria are negatively charged in the pH range frequently found in most food (pH of <7) (30). The fact that both types of isolates were found to possess similar surface properties suggests that virulence of *S. enterica* serovar Typhimurium in swine is linked not to a variation of their surface properties but probably to specific adhesins.

Whether the isolates (no. 42, 43, and 46) whose invasion is weaker are included or excluded, there is no difference in the results, indicating that the difference could be at the level of the invasion in epithelial cells.

In this study, we characterized isolates from septicemic animals in order to identify putative virulence factors. However, host factors may also affect this issue of infection by septicemic isolates, since a range of clinical signs can be observed during outbreaks in affected herds. One cannot rule out that host susceptibility may also influence the status of isolates. The influence of host factors in the outcome of infection by virulent isolates should be further studied.

In summary, 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 phagocytized at a lower level at early steps than isolates from healthy animals. However, most other classical virulence features of isolates related to survival within host cells, such as induction of apoptosis or survival within monocytes, were found to be identical, suggesting that early steps in the establishment of infection and spread within host phagocytes are most important for the outcome of the infection in animals. Further studies at the molecular level will be necessary to better elucidate the pathology of this infection in swine.

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