Infection with *Bordetella parapertussis* but Not *Bordetella pertussis* Causes Pertussis-Like Disease in Older Pigs

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The 3 major *Bordetella* species—namely, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*—can be distinguished by their different host ranges. *B. bronchiseptica* infects a wide range of mammals (including humans), whereas *B. pertussis* infects only humans and, under experimental conditions, mice and pigs. In contrast, *B. parapertussis*, also a causative agent of pertussis, displays a unique host specificity with 2 subgroups, one infecting only humans and the other infecting only sheep. Here, we show that both strains of *B. parapertussis* also infect older piglets when delivered intrapulmonarily. Infected piglets displayed mild fever and respiratory symptoms, such as coughing and breathing difficulties. Importantly, transmission was observed between infected and non-infected piglets. In tracheal organ cultures, adherence to ciliated epithelial cells was observed. Furthermore, both strains of *B. parapertussis* displayed higher resistance than *B. pertussis* to neutralization by porcine/H9252-defensin 1 in the respiratory tract, which has been demonstrated to be associated with protection against *B. pertussis* disease in older pigs. The development of this new model will assist us in better understanding the pathogenesis of this disease and in the development of more-effective vaccines against pertussis.

Whooping cough is a serious transmissible human respiratory tract disease caused by gram-negative *Bordetella pertussis* and *Bordetella parapertussis*. Although infection with *B. parapertussis* causes the typical paroxysmal cough, the clinical symptoms are typically less severe than those occurring during infection with *B. pertussis* [1, 2]. Pertussis especially afflicts infants worldwide; however, it is increasingly being recognized as a significant cause of respiratory distress in adults [3–5]. Both *B. pertussis* and *B. parapertussis* may cause complications such as pneumonia and bronchopneumonia in infants, which often have fatal consequences [6, 7].

*B. pertussis* and, more recently, *B. parapertussis* evolved from a common *Bordetella bronchiseptica*-like ancestor [8–10]. Before isolation of *B. parapertussis* from normal and infected ovine respiratory tracts in 1987, this bacterium was considered to be an obligate human pathogen [11, 12]. Later studies demonstrated that *B. parapertussis* strains isolated from humans and sheep were genetically distinct and that transmission of bacteria between humans and sheep did not occur [13, 14]. In contrast, *B. pertussis* is an obligate human pathogen, whereas *B. bronchiseptica* causes disease in a broad spectrum of mammals, from marsupials to ungulates, carnivores, and rodents [13, 15]. Evidence indicates that *B. pertussis* and *B. parapertussis* have adapted to more-restricted host niches, which may have allowed these species to infect humans and sheep more effectively [10, 16].

It has been suggested that the mechanism of disease development after infection with *B. parapertussis* is similar to that after infection with *B. pertussis* [10, 17, 18]. However, pertussis toxin (PT), considered to be the major virulence factor of *B. pertussis* [19, 20], is produced only by *B. pertussis*, whereas the *B. parapertussis* PT gene is transcriptionally silent [21]. This could be a reason for the often milder and shorter clinical disease that follows.
B. parapertussis infection [1, 2, 22, 23]. Several studies have indicated that immunization against pertussis has little or no efficacy against infection with B. parapertussis [24–27]. In contrast, a more-recent study reported that, in mice, a combination of mucosal and Th1 responses may be required for reciprocal protection against B. parapertussis [28]. Thus, the method of immunization and the vaccine formulation might be important for the induction of reciprocal immune responses.

Unfortunately, B. parapertussis has been neglected because of its comparatively lower incidence and milder symptoms and its similarities with B. pertussis. It has been reported that, in the United States and Europe, B. parapertussis is responsible for 5% or less of pertussis cases [29]. However, other studies have suggested that the disease caused by B. parapertussis is not a rarity, indicating that this pathogen indeed causes 20%–30% of cases of whooping cough [25, 29–31] and, in some parts of the world, has a prevalence rate even higher than that of B. pertussis [31]. These observations indicate that B. parapertussis might yet become a serious public health problem in the future. Thus, the cultivation of a more-accurate knowledge of the pathogenesis and protective antigens of B. parapertussis by use of novel animal models will enable us to develop more-appropriate vaccines against this bacterium.

The lack of a suitable animal model has been a major obstacle in the development of novel vaccination strategies [1, 30, 32]. We recently showed that newborn piglets were susceptible to B. pertussis infection [33]. However, older piglets (4–5 weeks) were resistant to challenge with B. pertussis, which was associated with the presence of porcine β-defensin 1 (pBD1) in their respiratory tracts [34]. Here, we show that, in contrast to B. pertussis, both B. parapertussis strains can cause disease in older pigs and adhere in porcine tracheal organ cultures at levels similar to those of B. bronchiseptica. Moreover, B. parapertussis exhibited higher resistance against pBD1 in vitro. The development of this novel model will enable us to better understand the role played by innate and mucosal immunity in disease protection and assist in the development of more-effective vaccines against both B. pertussis and B. parapertussis.

METHODS

Bacterial cultures. Bacterial suspensions of B. parapertussis (both a human clinical isolate [strain 12822; provided by A. Preston, University of Bristol, United Kingdom] and an ovine isolate [strain H1, Scotland isolate; provided by J. Miller, University of California, Los Angeles]), B. pertussis (strain Tohama I), and B. bronchiseptica (strain RB50) were cultured as described elsewhere [33].

In vitro growth inhibition assays. The sensitivity of B. parapertussis (both the human and the ovine strain) to synthetically derived pBD1 was compared with that of B. pertussis and B. bronchiseptica by coculturing appropriate concentrations of this peptide in 20 mmol/L PBS (280 μL) with 5 × 10^6–7 × 10^6 cfu (10 μL) of bacteria at 37°C for 1.5 h, followed by plate count dilution assays [34].

Adherence of bacteria in porcine tracheal organ cultures. The approach for determination of bacterial adherence was modified from methods described by Funnell and Robinson [35]. Tracheas were cut into rings and held in place in 12-well tissue culture plates (Nunc) with molten 1% (wt/vol) agar (37°C). Approximately 5 × 10^6–7 × 10^6 cfu of bacteria was added to the inside of each ring. The tracheal rings were incubated for 2 h at 37°C in 5% CO₂, after which they were washed and homogenized for determination of the amount of adherent bacteria.

Piglets. Weaned piglets were purchased from the Prairie Swine Centre, University of Saskatchewan. At the time of challenge, piglets were 30 days old. All experiments were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

Housing conditions. Piglets were housed under biocontainment conditions on slatted flooring (18 × 15 × 9 feet) equipped with air exchange of 15–20 cycles/h. The room was cleaned every day by pressure hosing, and animals were divided into 2 separate holding pens (10 × 6 feet). All animals were fed commercial feed (Whole Earth Pork-Gro; Co-Op) without antimicrobial additives.

Intrapulmonary challenge of piglets. Piglets were anesthetized with isoflurane and then challenged intrapulmonarily with 5 × 10^18–7 × 10^10 cfu of bacteria as described elsewhere [33]. Animals were monitored daily; clinical symptoms such as fever, cough, and respiratory problems were noted, and the pattern of weight gain and/or loss was recorded. Nasal swab samples were collected before challenge and every other day thereafter. Triangular foam-tipped swabs (PVA surgical spears; IVALON Surgical) were inserted as far into the nasal cavity as possible and rotated. Swabs were then stored in Eppendorf tubes until being streaked onto charcoal agar plates and rinsed vigorously in 100 μL of physiological solution (100 mmol/L potassium acetate, 20 mmol/L HEPES, and 2.5 mmol/L magnesium acetate).

Polymerase chain reaction (PCR). The swab rinses were boiled for 10 min, and 5 μL of the lysate was used as a template for PCR to detect IS1001, which is unique to B. parapertussis, with primers BPPA and BPPZ [36] as well as primers specific for the fim2 gene found in ovine B. parapertussis (Bpp4) [37]. RED-Taq ReadyMix PCR Reaction Mix (Sigma-Aldrich) was used for amplification. PCR conditions were 1 min of denaturation at 95°C; followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 64°C, and extension for 30 s at 72°C; followed by a final extension for 5 min at 72°C. PCR products were visualized by electrophoresis on 2% agarose gel containing 0.5 μg/mL ethidium bromide.

Postmortem investigation. Piglets were killed at different time points over a 20-day period, and the extent of pathological changes was monitored macroscopically. The number of bacte-
Results were determined by plate counts. Results are expressed as mean ± SE values for at least 3 independent experiments. Values for B. parapertussis and for the human strain of B. pertussis (h) were significantly different from those for their respective controls (white bars) (P < .0001 and P < .003, respectively). o, ovine strain of B. parapertussis.

Statistical analysis. All outcome data from this study followed nonnormal distributions. To account for this, all outcome data were ranked, and then analysis of variance or Student’s t test was used to detect differences among the experimental group. The means of the ranks were compared using Tukey’s test. The Mann-Whitney U test was used to reconfirm differences. P ≤ .05 was considered to indicate a significant difference.

RESULTS

Inhibitory effect of pBD1 against B. pertussis, B. parapertussis, and B. bronchiseptica. We recently demonstrated that pBD1 is associated with disease protection against B. pertussis in older piglets [34]. To determine the bactericidal activity of pBD1 against B. parapertussis (both the human and the ovine strain), inhibition assays were performed, and results were compared with those for B. pertussis and B. bronchiseptica. As shown in figure 1, B. pertussis was highly susceptible to 5 μg/mL pBD1 after a 1.5-h incubation (P < .0001). In contrast, B. parapertussis (human strain 12822 and ovine strain H1) and B. bronchiseptica showed greater resistance to killing by this peptide; both the human and the ovine strain of B. parapertussis displayed higher resistance to pBD1 than did B. pertussis. Interestingly, the human strain was more susceptible than the ovine strain.

Adherence of bacteria in porcine tracheal organ cultures. Tracheal organ cultures were inoculated with 5 × 10⁶–7 × 10⁶ cfu of bacteria and incubated for 2 h, after which adherent bacteria were quantified in homogenized tracheal tissues. Although B. pertussis was not able to adhere to the tracheal tissue of 4–5-week-old piglets, both strains of B. parapertussis as well as B. bronchiseptica adhered to porcine tracheal cells (figure 2). The number of adhered B. parapertussis (both the ovine and the human strain) was significantly greater than that of B. pertussis (P < .0001). Interestingly, there was no significant difference between B. parapertussis (both strains) and B. bronchiseptica.

Infection of 4–5-week-old piglets with B. parapertussis. Piglets (30 days old) were challenged in 2 separate experiments with either 5 × 10¹⁰–7 × 10¹⁰ cfu of the B. parapertussis ovine or human strain (table 1). The challenge dose was based on the results of previous studies, in which piglets of this age were resistant to infection with 5 × 10¹⁰ cfu of B. pertussis [33]. Control piglets were divided into 2 separate groups; one group was challenged with 5 × 10¹⁰ cfu of heat-inactivated B. parapertussis, and the other group was treated with PBS. Only piglets infected with live B. parapertussis (either the human or the ovine strain) displayed clinical manifestations, including fever and mild respiratory symptoms such as nonparoxysmal cough and breathing difficulties (table 1). Symptoms were observed as early as 4 days after challenge and were found to last for up to 7 and 10 days in piglets.
challenged with the ovine or human strain, respectively. In contrast, control piglets showed no clinical symptoms during the course of infection. Furthermore, there was a pronounced retardation in weight gain in at least 40% of *B. parapertussis*–infected piglets, which also displayed more-severe clinical symptoms. Piglets lost weight for 4 days and later gained weight at the same rate as the control piglets. Postmortem investigation and macroscopical examination indicated pathological alterations (such as fibrinous pleuritis) and hemorrhagic and necrotizing pneumonia in the lungs of *B. parapertussis*–infected piglets (figure 3). The extent of the lung lesions varied from 5% to 40% of the infected lungs, depending on the duration of infection. The extent of lung lesions was similar in piglets challenged with the human or ovine strain (figure 4). In contrast, control piglets did not show any pathological alterations in the lungs. Mortality was not seen in any group.

**Quantification of bacteria in BAL fluid and lung lesions.** Lungs were removed after piglets were killed, and BAL fluid was obtained by filling the lungs with 15 mL of Stainer-Scholte medium. The number of bacteria in BAL fluid (figure 5A) and lung lesions (figure 5B) was determined over a period of 20 days. The human strain of *B. parapertussis* was found in BAL fluid at levels between $1 \times 10^{10}$ and $1 \times 10^{12}$ cfu/mL for up to 15 days after infection. However, bacteria could still be isolated ($<1 \times 10^2$ cfu/mL) af-

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* Two control piglets displayed peribronchiolar lymphoid infiltration and interstitial thickening due to infiltration of mixed inflammatory cells and mild fibrosis.

**Figure 3.** Gross pathology of infected lung tissues. Shown are lungs from piglets infected with $5 \times 10^{10}$ cfu of the human (A) or ovine (B) strain of *Bordetella parapertussis* on day 2, the human (C) or ovine (D) strain on day 4, and the human (F) or ovine (G) strain on day 15 after challenge. Also shown is severe fibrinous pleuritis in a piglet infected with the ovine strain on day 4 after challenge (E) and lungs from a piglet infected with $5 \times 10^{10}$ cfu of heat-killed *B. parapertussis* on day 2 after challenge (H).
from day 7 until day 13, at a range of 13 to 42 cfu. The identity of isolated colonies was confirmed by PCR (data not shown).

**Pulmonary inflammation after infection.** Histopathological examination of lung tissues from piglets infected with live *B. parapertussis* (either the human or the ovine strain) revealed a severe subacute necrotizing pneumonia with mild fibrinous pleuritis and necrotizing bronchiolitis. Cellular infiltrations (neutrophils, macrophages, and plasma cells) were noted in the alveolar spaces and lumen of bronchioles (figure 6B and 6C). This pattern was associ-

**Colonization of the upper respiratory tract and transmission.** Piglets were swabbed every other day after challenge, and swabs were streaked onto charcoal agar plates. *B. parapertussis* (both the ovine and human strain) were isolated from the nasal swab samples on day 7 and thereafter. Although the ovine strain of *B. parapertussis* was recovered from the nasal swab only until day 11 after challenge, the human strain of *B. parapertussis* was isolated at higher numbers (≥1 × 10^4 cfu/g of tissue) and for a longer period of time, until day 20.

**Transmission between infected and control piglets.** Colonization of bacteria in the upper respiratory tract facilitates bacterial transmission to other animals. Shedding of both the human and the ovine strain of *B. parapertussis* was confirmed by positive bacterial cultures of nasal swab samples obtained from control piglets on various days. The control piglets were housed in the same pen, allowing direct contact among animals. The ovine strain of *B. parapertussis* was isolated from the nasal mucosa of 3 of 6 control piglets on day 7 and continued to be isolated until day 11 after challenge, at a range of 4 to 23 cfu. In contrast, the human strain of *B. parapertussis* was isolated from the nasal mucosa of 4 of 6 control piglets.
ated with severe alveolar hemorrhage, congestion, edema, and necrosis. There were also numerous bacteria and necrotic neutrophils in some of these locations. In addition, focal arteriolar degeneration as well as bronchiolar necrosis and evidence of proliferation of type 2 pneumocytes were seen by day 2. On days 4 and 7, severe subacute hemorrhagic and necrotizing pneumonia with mild pleuritis and necrotizing bronchiolitis was noted. This was characterized by severe infiltration of inflammatory cells, which were predominantly neutrophils. Furthermore, there was evidence of vasculitis and thickening of the alveolar walls by congestion and infiltration of lymphocytes and macrophages. On day 20, a mild bronchointerstitial pneumonia was noted in piglets infected with the human strain, characterized by mild interstitial thickening due to congestion and infiltration of lymphocytes, macrophages, and occasional neutro-

**Figure 6.** Histopathology of the lungs of piglets. A, Photomicrograph of lungs from a noninfected piglet (×10 objective plus digital zoom). B and C, Photomicrographs of lungs from piglets infected with $5 \times 10^{10}$ cfu of either the human (B) or the ovine (C) strain of *Bordetella parapertussis* 7 days after challenge (×20 objective plus digital zoom). D, Photomicrograph of ciliated tracheal cells from a noninfected piglet (×100 objective plus digital zoom). E and F, Photomicrographs of ciliated tracheal cells from piglets infected with $5 \times 10^{10}$ cfu of either the human (E) or the ovine (F) strain of *B. parapertussis* 10 days after challenge (×100 objective plus digital zoom).
phil in the alveolar spaces and walls. In contrast, after infection with the ovine strain, there was no evidence of pneumonia but there was a moderate, locally extensive fibrosis in the parenchyma and surrounding small bronchioles and blood vessels. This pattern was associated with the infiltration of lymphocytes, macrophages, and neutrophils in the alveolar walls. In addition, there was mild peri-bronchiolar and perivascular lymphoid infiltration. Interestingly, we also observed peribronchiolar lymphoid infiltration and interstitial thickening due to infiltration of mixed inflammatory cells and mild fibrosis in some control piglets (PBS treated). There was no evidence of arteriolar hypertrophy in the lungs of any of the piglets in the study.

**Tracheal damage after infection.** Histopathological examination of the trachea from infected piglets with live *B. parapertussis* (both strains) showed very mild lymphocyte and plasma cell infiltration into the submucosa, locally extensive flattening of the mucosa, and loss of the ciliated cells from the surface (figure 6E and 6F). In addition, a few neutrophils infiltrated into the lumen, and traces of debris were observed near the adjacent surface.

**DISCUSSION**

*B. parapertussis* represents an interesting model of host specificity, because its 2 strains are isolated from only 2 hosts, one only from humans and the other only from sheep. Although the human strain causes whooping cough in humans and an acute infection of the lower respiratory tract in mice [38], the ovine strain can cause respiratory disease and pneumonia in lambs [29, 39] and mice [40]. However, there have been no reports of human infection by the ovine strain or ovine infection by the human strain. Here, we demonstrate that 4–5-week-old pigs can be infected by both the human and the ovine strain of *B. parapertussis* via intrapulmonary inoculation with $5 \times 10^{10} - 7 \times 10^{10}$ cfu. This challenge dose was chosen because piglets of the same age had been found to be fully resistant to infection with *B. pertussis* [33]. We previously demonstrated that this resistance is associated with the expression of pBD1 [34]. In the present study, we compared the susceptibility of the 3 major *Bordetella* species and demonstrated that *B. bronchiseptica* (RB-50) and the 2 strains of *B. parapertussis* displayed much higher resistance against pBD1 than *B. pertussis*. This might explain why older pigs were susceptible to infection with both the human and the ovine strain of *B. parapertussis*. However, in the present study, only the specified strains of *B. parapertussis* and *B. bronchiseptica* were used, and other strains need to be included in future analyses.

The infected piglets displayed a wide range of clinical symptoms similar to those observed in human adults, including mild upper respiratory tract symptoms, cough, and mild fever. In piglets, a dramatic influx of neutrophils, macrophages, and plasma cells into airways and interstitial tissues was observed, resulting in severe subacute hemorrhagic and necrotizing bronchopneu-}

monia. This is consistent with the observed association between *B. parapertussis* infection and bronchopneumonia in mice, lambs, and humans [7, 39, 40]. Interestingly, we also observed peribronchiolar lymphoid infiltration and interstitial thickening due to infiltration of mixed inflammatory cells and mild fibrosis in some control piglets (PBS treated). We suspect that this was the result of animal-to-animal transmission, given that we isolated bacteria from the lungs of these control piglets.

After challenge, the ovine and human strains were isolated for up to 15 and 20 days, respectively, from BAL fluid and lung tissues from infected piglets. Given the size of piglet lungs, this amount may even be higher than the $1 \times 10^{6} - 1 \times 10^{7}$ cfu routinely isolated from the whole lungs of infected mice [38]. Although the mouse model is superior in terms of lower cost, size, space, challenge dose, and availability of immunological tools, it does not mimic human disease. The lack of clinical symptoms, pathological alterations such as pneumonia, and transmission are some of its limitations. Therefore, this newly developed porcine model can complement the existing murine models to improve our understanding of the disease.

Pertussis is a contagious disease, and transmission of bacteria occurs directly from infected patients to healthy individuals. Adherence of bacteria to the mucosal surfaces of the upper respiratory tract is therefore a hallmark of disease transmission. Adherence of bacteria typically results in marked lesions of the ciliated epithelium in the trachea and larger airways. Several in vitro and in vivo studies have shown that bordetellae are capable of adhering to the tracheobronchial airways [41, 42]. Using tracheal organ cultures, we have demonstrated that both *B. parapertussis* and *B. bronchiseptica* adhere in large quantities to porcine tracheal cells in vitro. In contrast, *B. pertussis* was unable to adhere to tracheal cells from older animals. This correlates with the observation that *B. parapertussis* and *B. bronchiseptica* caused disease in older pigs, whereas *B. pertussis* induced disease only in newborn piglets. Interestingly, the human strain of *B. parapertussis* colonized the nasal mucosa more effectively and for a longer period than did the ovine strain in vivo. However, the reason for this difference is not clear. Whether adherence of bacteria is required for disease is the subject of current investigations in our laboratory. Damage to the epithelium is caused by the secretion of inducible NO synthase by secretory cells, which is induced by the bacterial tracheal cytotoxin and endotoxin of *B. pertussis* [43, 44]. Whether *B. parapertussis* has this ability is not known. Furthermore, we showed bacterial transmission between infected piglets and noninfected control piglets. Modeling the transmission of bacteria is not only critical for mimicking the disease but is essential for evaluating novel vaccines against pertussis in outbred populations.

Current commercial vaccines against pertussis contain either whole-cell inactivated bacteria or filamentous hemagglutinin, PT, pertactin, and/or fimbriae as protective antigens. With the exception of PT, *B. parapertussis* also expresses these antigens
However, it has been reported that pertussis vaccines induce little or no protective immunity against infection with B. parapertussis in murine models and field studies [1, 2, 27, 31]. On the basis of several clinical trials, Mastrantonio et al. [47] suggested that current pertussis vaccination might even be irrelevant in preventing infection with B. parapertussis. This, therefore, could lead to speculation that infection with B. parapertussis will increase if infection with B. pertussis decreases.

In summary, we have developed a new model of parapertussis in older pigs, one that complements our existing newborn model of pertussis. This newly developed model can be used to validate and optimize new generations of vaccines that protect against infection with both B. pertussis and B. parapertussis.

References

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