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Coinfection of pigs with porcine respiratory coronavirus and *Bordetella bronchiseptica*

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Received 21 February 2007; received in revised form 19 September 2007; accepted 26 September 2007

Abstract

Coinfection with two or more pathogens is a common occurrence in respiratory diseases of most species. The manner in which multiple pathogens interact is not always straightforward, however. *Bordetella bronchiseptica* and porcine respiratory coronavirus (PRCV) are respiratory pathogens of pigs whose relatives, *B. pertussis* and the SARS virus, cause respiratory disease in humans. In an initial experiment, the effect of coinfection of PRCV and *B. bronchiseptica* was examined in thirty, 4-week-old pigs (10 pigs/group) that were infected with either PRCV or *B. bronchiseptica*, or both PRCV and *B. bronchiseptica*. An additional 10 pigs served as sham infected controls. Five pigs from each group were euthanized at 4 and 10 days post-infection. Gross and histopathological lung lesions were more severe in the coinfecting group as compared to the groups infected with *B. bronchiseptica* or PRCV alone. In order to investigate the potential role of proinflammatory cytokines in disease severity after coinfection, a second experiment was performed to examine cytokine transcription in alveolar macrophages from single and dually infected pigs. A total of 48 pigs were divided equally into groups as above, but 4 pigs from each group were euthanized at 1, 4 and 10 days post-infection. Coinfecting pigs showed a greater and more sustained transcription of proinflammatory cytokines, especially IL-6 and MCP-1, than pigs infected with either PRCV or *B. bronchiseptica* alone. Thus, there appears to be a synergistic effect between PRCV and *B. bronchiseptica* with regards to proinflammatory cytokine transcription that may partially explain the increased severity of pneumonia in coinfecting pigs. Published by Elsevier B.V.

Keywords: Porcine respiratory coronavirus; *Bordetella bronchiseptica*; Porcine respiratory disease complex

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1. Introduction

Respiratory disease in swine, as with other species, is generally considered a multifactorial problem caused by a combination of viral and bacterial infectious agents, as well as adverse environmental conditions.

The list of infectious agents that cause respiratory disease in swine is extensive and includes both viral agents, such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV) and porcine respiratory coronavirus (PRCV); and bacterial agents, such as *Mycoplasma hyopneumoniae*, *Bordetella bronchiseptica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*. Although any one of these pathogens can potentially cause disease on its own, more serious and chronic respiratory disease results, and more economic losses are incurred, when infection with multiple pathogens occurs. Although the multifactorial nature of respiratory disease is well accepted, the specific mechanisms by which respiratory pathogens interact with each other or the host to cause more severe disease are poorly understood.

Coronaviruses are enveloped, single-stranded, RNA viruses which cause respiratory and enteric disease in animals, including the common cold and severe acute respiratory syndrome (SARS) in humans. Porcine respiratory coronavirus is a naturally occurring deletion mutant of transmissible gastroenteritis virus (TGE), an enteric coronavirus of swine (Rasschaert et al., 1990; Britton et al., 1991; Wesley et al., 1991). A deletion in the spike gene of TGE has altered the tropism of PRCV from primarily enteric to primarily respiratory in nature. Although experimentally PRCV has been shown to cause bronchointerstitial pneumonia, the clinical relevance of this virus has been debated, since many farms seroconvert without overt clinical signs of disease. What role, if any, PRCV plays in multifactorial respiratory disease is still being investigated. Prior work has shown that exposure to PRCV decreases replication of SIV, *in vivo*, and mixed results have been reported with coinfections of PRCV and PRRSV (Van Reeth and Pensaert, 1994; Van Reeth and Nauwynck, 2000). However, experimental exposure of pigs to bacterial lipopolysaccharide (LPS) following PRCV infection led to more severe respiratory disease and increased tumor necrosis factor-alpha (TNF-alpha) and interleukin (IL)-1 levels than exposure to either LPS or PRCV alone (Van Reeth et al., 2000). Thus, infection with PRCV and gram-negative bacteria may result in a more severe disease.

The species belonging to the genus *Bordetella* are closely related gram-negative bacteria which cause respiratory disease in animals. *Bordetella pertussis* is the obligatory human pathogen that causes whooping

cough, and *B. bronchiseptica* is the more promiscuous pathogen that is the etiologic agent of respiratory disease in a number of animals including swine. Although *B. bronchiseptica* can cause pneumonia in neonatal pigs and rhinitis and bronchitis in older swine on its own, its role in multifactorial disease is also under investigation. Prior infection with *B. bronchiseptica* in swine has led to increased colonization and more severe disease with other bacterial agents such as *P. multocida*, *Streptococcus suis* and *Haemophilus parasuis* (Chanter et al., 1989; Vecht et al., 1992; Brockmeier et al., 2001; Brockmeier, 2004), and coinfection of *B. bronchiseptica* with PRRSV in swine exacerbated clinical disease and induced *B. bronchiseptica* pneumonia (Brockmeier et al., 2000).

The innate immune response of the lung is not only important for initial protection from infectious agents and stimulation of the adaptive immune response, but the nature of the innate response can dictate the character of the pathology observed and the outcome of infection. Proinflammatory cytokines, such as TNF-alpha, IL-1 and IL-6, as well as the chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) are often produced after recognition of pathogen associated molecular patterns for early host protection and recruitment of effector cells. Exposure to multiple infectious agents may result in distinct cytokine patterns and thus alter the severity of disease compared to that observed during infection with individual agents. In this report we determine whether coinfection with PRCV and *B. bronchiseptica* leads to increased severity of respiratory disease, and if so, whether or not the local proinflammatory cytokine response helps to elucidate the mechanism of this interaction. The data reported here demonstrates that coinfection results in a proinflammatory response that is different from each individual infection, and this proinflammatory response likely contributes to the differences in disease pathology observed during infection with PRCV and *B. bronchiseptica*.

2. Materials and methods

2.1. PRCV and *B. bronchiseptica* inocula

The PRCV isolate used was isolated from a pig on a farm in Iowa that was experiencing respiratory

disease. This isolate of PRCV has a 648-nucleotide deletion after base 99 of the spike gene compared to the Purdue strain of TGE (data not shown). PRCV was isolated on swine testicular (ST) cells, passaged two times and frozen at -80°C . Pigs were inoculated intranasally with 4 ml (2 ml/nostril) of cell culture medium containing 10^6 median cell culture infectious dose (CCID_{50})/ml of PRCV. Sham inoculum was prepared from uninfected ST cells.

B. bronchiseptica strain KM22 is a virulent phase I swine isolate cultured from a herd with atrophic rhinitis. To prepare the inoculum, *B. bronchiseptica* was cultured on Bordet-Gengou agar supplemented with 10% sheep's blood at 37°C for 40 h and suspended in phosphate buffered saline (PBS). Pigs were inoculated intranasally with 1 ml of PBS (0.5 ml/nostril) containing 10^6 colony forming units (CFU)/ml of *B. bronchiseptica*.

2.2. Experimental design

Two experiments were performed. At 3 weeks of age, 40 pigs (experiment 1) and 48 pigs (experiment 2) were transferred from herds (a different herd in each experiment), which were negative for antibody to PRCV, PRRSV and SIV, to an animal isolation facility at the National Animal Disease Center. No medications or vaccinations were given to the piglets. Nasal swabs were obtained from all pigs before the start of the experiment, and neither *B. bronchiseptica* nor PRCV was isolated. The pigs were randomly divided into 4 groups with 10 pigs/group (experiment 1) and 12 pigs/group (experiment 2). After 1 week of acclimation, on day 0 of the experiment, the pigs in group 1 were intranasally inoculated with sham PBS and cell culture medium from uninfected ST cells, pigs in group 2 were inoculated intranasally with *B. bronchiseptica* and sham cell culture medium, the pigs in group 3 were intranasally inoculated with sham PBS and PRCV, the pigs in group 4 were inoculated with PRCV followed by *B. bronchiseptica*. In experiment 1, 5 pigs from each group were euthanized on days 4 and 10 of the experiment. In experiment 2, 4 pigs from each group were euthanized on days 1, 4 and 10 of the experiment.

2.3. Pathological evaluation

At necropsy, an estimate of gross lung involvement was assigned based on the percentage of each lung

lobe affected and the percentage of total lung volume each lobe represented. Percentage of total lung volume of each lobe was estimated as 5% for the left cranial, 6% for the left middle, 29% for the left caudal, 11% for the right cranial, 10% for the right middle, 34% for the right caudal and 5% for the intermediate lung lobes (Christensen and Mousing, 1992).

Sections from the lung were taken for microscopic evaluation. All tissues were fixed in 10% neutral buffered formalin for 24 h and then placed in 90% ethanol. All sections were routinely processed and embedded in paraffin, sectioned and stained with hematoxylin and eosin.

2.4. Virus and bacterial isolation

A sample of nasal turbinate, trachea and lung from each pig was weighed and ground individually in PBS to make a 10% weight to volume suspension. The number of CFU of *B. bronchiseptica*/g of tissue was determined by plating serial 10-fold dilutions of homogenates on duplicate selective blood agar plates containing 20 $\mu\text{g/ml}$ penicillin, 10 $\mu\text{g/ml}$ amphotericin B, 10 $\mu\text{g/ml}$ streptomycin and 10 $\mu\text{g/ml}$ spectinomycin. In both experiments, fluid from bronchoalveolar lavage (BALF) was obtained for virus isolation, and in addition, nasal swabs collected in 1 ml of PBS, and samples of ground turbinate, ground trachea and ground lung were obtained from the second experiment for virus isolation and frozen at -80°C . Isolation of PRCV was performed by inoculation of 100 μl of sample onto a monolayer of ST cells in a 24-well plate. The cells were examined for cytopathic effect for 1 week.

2.5. Alveolar macrophage isolation

The lungs were filled with 200 ml of sterile PBS, gently massaged, and following aspiration, approximately 100 ml of the PBS was recovered. This BALF was centrifuged at $450 \times g$ for 15 min and alveolar macrophages (AM) were isolated by plastic adherence. BALF cells were cultured in flat-bottom, 6-well plates for 2-h in cell culture media [RPMI 1640, 2 mM L-glutamine, $1 \times$ antibiotic/antimycotic (all from Invitrogen), 25 mM HEPES, 100 $\mu\text{g/ml}$ Gentamicin and 5% swine sera]. After 2-h the non-adherent cells were washed away. The adherent cell population was

collected in RLT buffer and stored at -80°C for later RNA isolation.

2.6. Cytokine gene expression

RNA was isolated from cells collected in RLT buffer (Qiagen RNeasy Mini kit). Reverse-transcription for cDNA synthesis was done using random primers and SuperScript II Reverse Transcriptase according to the manufacturer's recommendations (Invitrogen). SYBR green based real-time PCR was carried out for various mRNA targets according to manufacturer's recommendations (SYBR Green Master Mix, Applied Biosystems). Briefly, mRNA, SYBR Green Master Mix and primers (final concentration 600 nM) were mixed in a 20 μl reaction and cycled as follows: 95°C for 15 min, 95°C for 15 s followed by 50°C for 1 min (45 cycles) and a final dissociation step. Levels of mRNA were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, which expresses mRNA from the cells of infected pigs relative to the sham infected pigs after normalizing to beta-actin (Livak and Schmittgen, 2001). Primers are listed in Table 1, and PCR products were less than 100 base pairs in size. Primers were generated using Primer Express, purchased from Integrated DNA Technologies (IDT), and primer pair efficiency confirmed using the method described by Livak and Schmittgen (2001).

2.7. Statistics

Statistical analysis was done using Student's *t*-test assuming unequal variance and a significance level of $p \leq 0.05$, comparing the mean percentage of lung affected between single and dual infected groups, the number of CFU's of *B. bronchiseptica* isolated from different tissues between single and dual infected

groups, and the fold change in cytokine message expression between single and dual infected groups.

3. Results

3.1. Macroscopic and microscopic analysis of lesions

Clinical signs were generally mild in all infected groups, and consisted of sneezing, coughing, lethargy and pigs that were quicker to fatigue. There was a general impression that coinfecting pigs were slightly more affected since clinical signs were recorded earlier and there were more of the above mentioned signs recorded for this group in both experiments.

The number of pigs/group with macroscopic lesions of pneumonia, and the mean percentage of lung affected by pneumonia for each group, for both experiments is summarized in Table 2. Gross pneumonic lesions, irrespective of agent given, were characterized by bilateral, multifocal red consolidation which tended to have a more cranial–ventral distribution (Fig. 1). In the first experiment, the mean percentage of lung involvement was greatest for the group infected with both PRCV and *B. bronchiseptica* as compared to either agent alone. This difference was greatest and statistically significant 10 days after challenge, when the mean percentage of lung involvement was 19% for the coinfecting group, but only 1.4 and 5.5% for the groups infected with *B. bronchiseptica* or PRCV, respectively. The mean percentage of lung affected was greater at 10 days than 4 days after challenge for all infected groups. Most pigs in all groups had gross evidence of ascarid migration in the liver.

Table 1
Real-time PCR cytokine primers

Gene	Accession numbers	Forward primer (5' → 3')	Reverse primer (5' → 3')
β -Actin	AJ312193	CTCCTTCCTGGGCATGGA	CGCACTTCATGATCGAGTTGA
TNF- α	M29079	CCACGTTGTAGCCAATGTC	CTGGGAGTAGATGAGGTACAG
IL-6	AF518322	GGCTGTGCAGATTAGTACC	CTGTGACTGCAGCTTATCC
MCP-1	NM_214214	GCTGATGAGCTACAGAAGAG	GCGATGGTCTTGAAGATCAC
IL-8	X61151	CTGGCTGTTGCCCTTCTTG	CACTGGCATCGAAGTTCTG
IL-1 β^a	NM_214055	TTGAATTCGAGTCTGCCCTGT	CCCAGGAAGACGGGCTTT

NCBI accession numbers used to derive primers.

^a Royae et al. (2004).

Table 2

Mean percentage of lung affected by pneumonia (number of pigs with lesions) in groups infected with *Bordetella bronchiseptica* (Bb), porcine respiratory coronavirus (PRCV), or Bb and PRCV (Bb/PRCV) on the various days post-infection

Experiment	Day	Group		
		Bb	PRCV	Bb/PRCV
1	4	0.6 (3/5)	2.2 (5/5)	3.6 (5/5)
	10	1.4 (2/5)	5.5 (5/5)	19 (5/5)
2	1	0 (0/4)	0.25 (1/4)	4.75 (4/4)
	4	0.25 (1/4)	8.25 (3/4)	6.25 (4/4)
	10	3.0 (2/4)	4.5 (3/4)	12.5 (4/4)

Gross lesions for the second experiment were similar in character to the first experiment. The mean percentage of lung involvement for the coinfecting group was again greater than the single agent infected groups at days 1 and 10 post-infection, but at day 4 the mean percentage of lung involvement was greatest for the group infected with PRCV alone. The variances were too great for any of the differences to be statistically significant, however. All 12 pigs in the coinfecting group had pneumonic lesions, whereas only 7 pigs in the PRCV group and 3 pigs in the *B. bronchiseptica* group had gross lesions; the largest difference being at the 1-day time point. Again, lesions were more severe, in general, at 10 days post-infection, except for the group of pigs infected with PRCV alone, in which lesions peaked at 4 days post-infection.

In experiment 1, not all pigs infected with *B. bronchiseptica* alone had microscopic lesions. However, in the pigs with microscopic lesions, the lesions consisted of mild multifocal areas of interstitial thickening of the alveoli with mononuclear cells and occasional focal accumulations of neutrophils in the bronchi and alveoli (Fig. 2). Microscopic lesions of the lung of pigs infected with PRCV alone typically consisted of multifocal areas of moderate to severe interstitial thickening of alveoli with mononuclear cells and lymphocytic perivascular and peribronchiolar infiltrates. These changes tended to be more pronounced on day 10 after challenge as compared to day 4, but at day 4 there were mild infiltrates of neutrophils in some of the airways that were not present on day 10 (Fig. 2). Microscopic lesions in coinfecting pigs included changes seen in pigs infected with single agents but also included additional

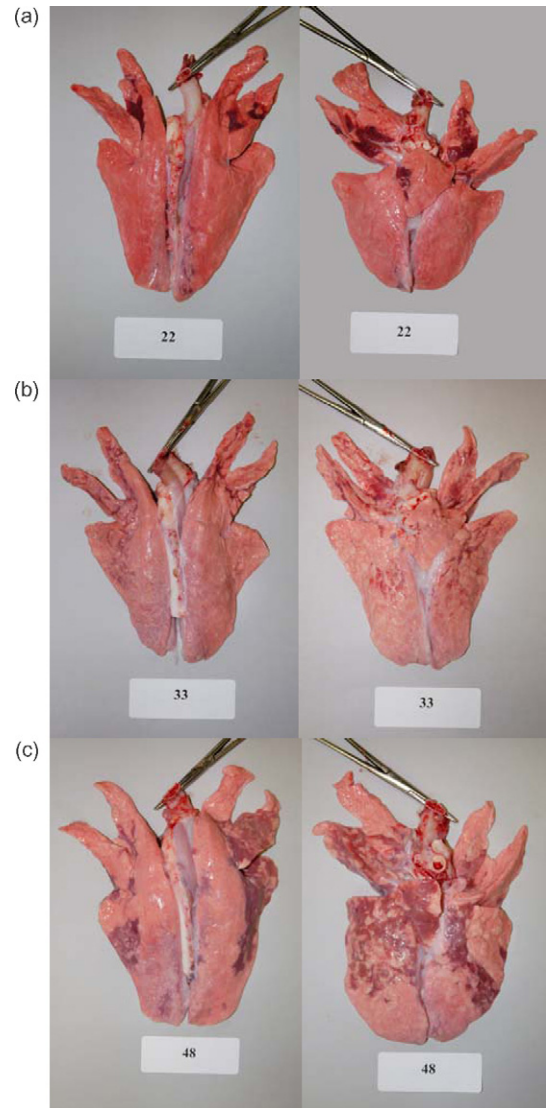


Fig. 1. Macroscopic lesions of the lung from pigs infected with: (a) *B. bronchiseptica* only; (b) PRCV only or (c) coinfecting with both *B. bronchiseptica*. Photos are of the lung of the most severely affected pig of each group 10 days post-infection in the second experiment.

changes and tended to be more severe than pigs infected with either agent alone. Typical microscopic lesions of the lung of pigs infected with both PRCV and *B. bronchiseptica* consisted of multifocal areas of moderate to severe interstitial thickening of the alveoli with mononuclear cells, lymphocytic perivascular and peribronchiolar cuffing, intra-alveolar epithelial cell necrosis with type II pneumocyte hyperplasia, and

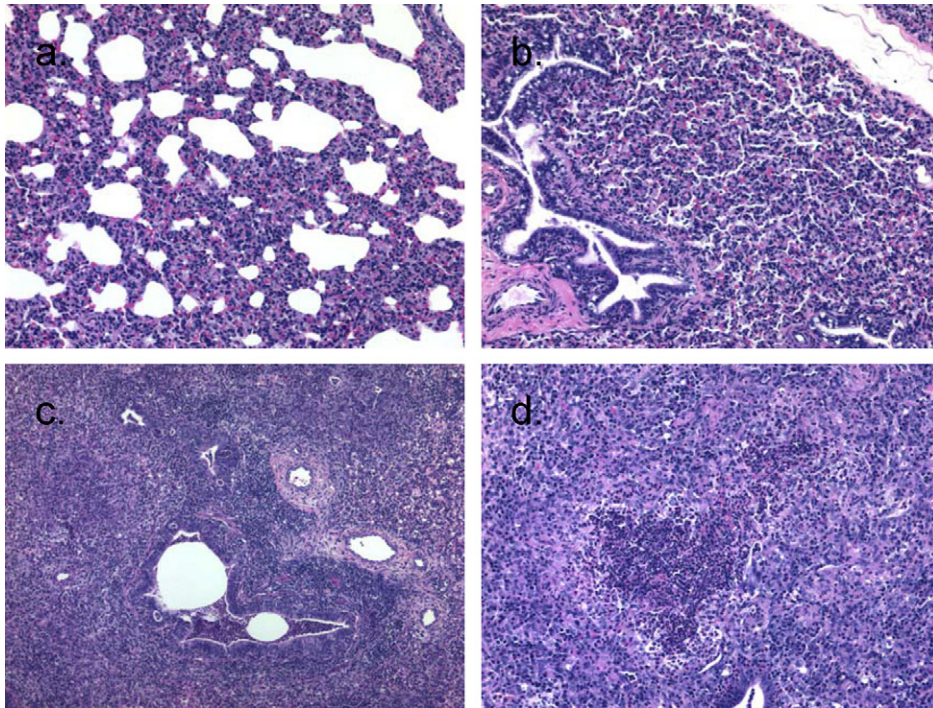


Fig. 2. Microscopic lesions of the lungs from pigs infected with: (a) *B. bronchiseptica* only, showing mild areas of interstitial thickening of the alveoli with mononuclear cells; (b) PRCV only, showing areas of moderate interstitial thickening of alveoli with mononuclear cells or (c and d) coinfecting with both *B. bronchiseptica* and PRCV, showing areas of severe interstitial thickening of the alveoli with mononuclear cells, lymphocytic perivascular and peribronchiolar cuffing, and accumulations of neutrophils in the bronchioles (c); intra-alveolar aggregates of neutrophils, macrophages, epithelial cells, and necrotic debris (d). Sections are from the lungs of pigs shown in Fig. 1.

syncytial cell formation. Intra-alveolar aggregates of neutrophils, macrophages, epithelial cells and necrotic debris were also present in coinfecting pigs, as well as, accumulations of neutrophils in the bronchi and bronchioles, terminal bronchiolar epithelial cell necrosis and flattening, interlobular edema and occasional hemorrhage (Fig. 2). Again, these changes tended to be more pronounced on day 10 post-infection. Three of the control animals had mild microscopic lesions consisting of peribronchial and perivascular infiltrates of lymphocytes, macrophages and eosinophils. Bronchoalveolar lavage was tested by PCR for the presence of *Mycoplasma*, and none was detected (data not shown).

The microscopic lesions seen in the second experiment were similar to the first experiment at days 4 and 10 post-infection except that there were fewer epithelial cell changes seen. At day 1 post-infection, the lung of 1 pig infected with PRCV and all

the coinfecting pigs had interstitial thickening, alveoli containing macrophages, neutrophils and fibrin, and mild peribronchiolar and perivascular lymphocytic infiltrates.

3.2. *B. bronchiseptica* and PRCV isolation

B. bronchiseptica was isolated from the turbinate and/or trachea of all pigs from both experiments that were challenged with the bacterium in numbers that were similar between the two groups (Table 3). In the first experiment, *B. bronchiseptica* was isolated from the lung of 8 of 10 challenged with the bacteria alone and 6 of 10 coinfecting pigs (Table 3) and in similar numbers. *B. bronchiseptica* was isolated from the lung of more coinfecting pigs than single infected pigs in the second experiment. Eight of 12 coinfecting pigs had *B. bronchiseptica* in their lungs, whereas only 3 of 12 pigs challenged with *B. bronchiseptica* alone did (Table 3).

Table 3
Number of pigs from which *Bordetella bronchiseptica* (geometric mean log₁₀ titer) was isolated on the various days post-infection

Experiment	Day	Sample	Group	
			Bb	Bb/PRCV
1	4	Turbinates	4/5 (5.77)	5/5 (4.66)
		Trachea	5/5 (5.73)	5/5 (5.48)
		Lung	3/5 (4.84)	3/5 (4.89)
	10	Turbinates	5/5 (6.85)	4/5 (5.86)
		Trachea	5/5 (5.21)	5/5 (5.73)
		Lung	5/5 (6.29)	3/5 (3.79)
2	1	Turbinates	4/4 (5.03)	4/4 (5.27)
		Trachea	4/4 (4.26)	4/4 (3.52)
		Lung	0/4 (<2)	2/4 (3.37)
	4	Turbinates	4/4 (7.26)	4/4 (7.30)
		Trachea	4/4 (7.18)	4/4 (5.87)
		Lung	1/4 (2.83)	3/4 (5.03)
	10	Turbinates	4/4 (6.99)	4/4 (7.38)
		Trachea	4/4 (6.53)	4/4 (7.05)
		Lung	2/4 (3.94)	3/4 (3.64)

In the first experiment, PRCV was isolated from the BALF on day 4 post-infection from 9 of 10 pigs given the virus, but the virus had been cleared from all pigs euthanized on day 10 (Table 4). PRCV was isolated from the BALF or lung of more coinfecting pigs than

Table 4
Number of pigs from which porcine respiratory coronavirus (PRCV) was isolated on the various days post-infection

Experiment	Day	Sample	Group	
			PRCV	Bb/PRCV
1	4	BAL	5/5	4/5
	10	BAL	0/5	0/5
2	1	Nasal swab	2/4	3/4
		Turbinates	2/4	4/4
		Trachea	1/4	0/4
		Lung	1/4	2/4
		BAL	2/4	3/4
	4	Nasal swab	4/4	3/4
		Turbinates	4/4	4/4
		Trachea	2/4	0/4
		Lung	0/4	2/4
		BAL	0/4	3/4
	10	Nasal swab	0/4	0/4
		Turbinates	0/4	0/4
		Trachea	0/4	0/4
		Lung	0/4	0/4
		BAL	0/4	1/4

single infected pigs in the second experiment. Seven of 12 coinfecting pigs had PRCV in their lung or BALF, whereas only 2 of 12 pigs that were challenged with PRCV alone did (Table 4). PRCV was isolated from the nasal swab, turbinates or trachea of all pigs challenged with the virus that were euthanized on days 1 and 4 post-infection, indicating that all pigs were indeed successfully infected (Table 4). Again, the virus was largely cleared by day 10 post-infection, as PRCV was not detected in any of the samples tested except the BALF from 1 coinfecting pig.

3.3. Cytokine gene expression

Cytokine production after infection likely plays a role in pathological changes observed during infection. Alveolar macrophages reside in the airway space of the lungs and are one of the first cells encountered during respiratory disease. Thus, during the second experiment we investigated changes in cytokine and chemokine mRNA levels in AM isolated from infected pigs. Fig. 3 summarizes changes in mRNA levels of proinflammatory cytokines of infected pigs relative to sham treated. In general, pigs challenged with *B. bronchiseptica* alone had increased mRNA levels of all the proinflammatory cytokines genes measured (TNF-alpha, IL-6, MCP-1, IL-8 and IL-1beta) at day 1 post-infection, with the majority of pigs responding with 4-fold or greater increases in transcript. *B. bronchiseptica* was not isolated from the lung of any of these pigs nor did any of the pigs have lesions either macroscopically or microscopically. By day 4 post-infection, cytokine mRNA levels in alveolar macrophages collected from pigs infected with *B. bronchiseptica* alone were near or below levels observed in uninfected animals. Fifty percent or more of the pigs had a greater than 2-fold reduction in transcript detected for IL-6, MCP-1, IL-8 and IL-1beta. The cytokine mRNA levels in *B. bronchiseptica* infected pigs were essentially equal to sham infected pigs by 10 days post-infection with one exception. One pig infected with *B. bronchiseptica* alone, which had lung lesions involving a significant portion of the lung (see Fig. 1a), had a 2-fold or greater increase in transcription of TNF-alpha, IL-6 and MCP-1.

With the exception of MCP-1, mRNA levels of the cytokines examined on day 1 post-infection in animals infected with PRCV alone were the same as sham

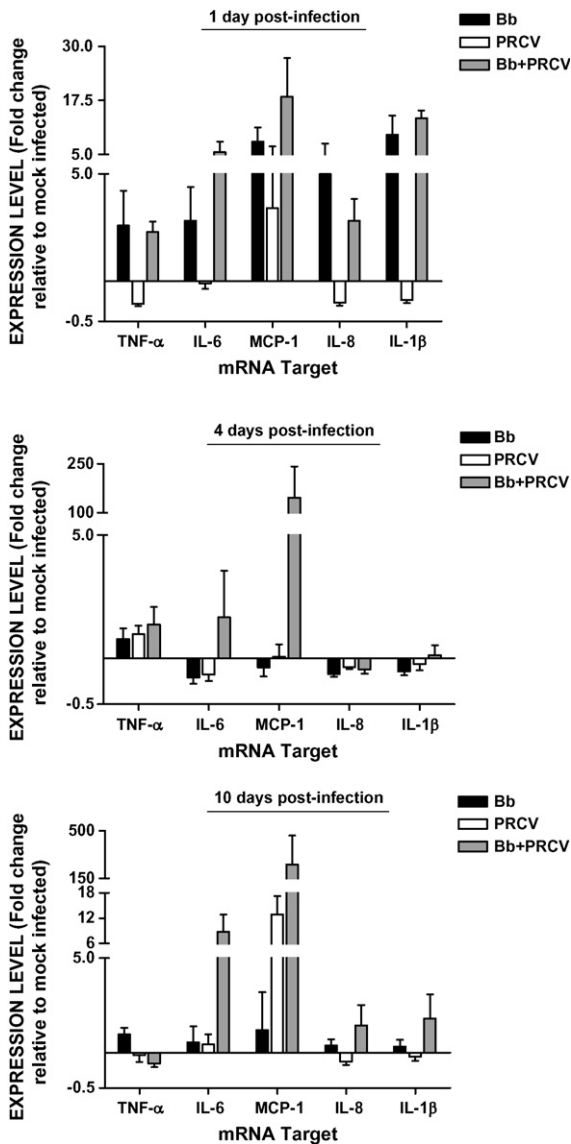


Fig. 3. Alveolar macrophage (AM) cytokine response during infection with *Bordetella bronchiseptica* (Bb), porcine respiratory coronavirus (PRCV), or Bb and PRCV. AM's were isolated from infected animals, RNA isolated, and mRNA levels were assessed by real-time PCR. Data is expressed as the mean \pm S.E.M. fold change in gene expression relative to mock infected animals.

infected pigs (IL-6) or were 2-fold or more below levels examined for sham infected pigs (TNF-alpha, IL-8 and IL-1beta). The one pig that had increased MCP-1 transcript levels (13-fold) in this group at this time was the pig with the microscopic lesions

described above on day 1. This trend continued in the pigs infected with PRCV alone, as mRNA levels of IL-6, MCP-1, IL-8 and IL-1beta were at or below the levels measured in sham treated animals on day 4. MCP-1 mRNA levels in PRCV infected pigs showed marked elevation on day 10 post-infection with an average 12-fold increase. The level of MCP-1 was negatively correlated ($R = -0.904$) with the percentage of lung macroscopically affected with lesions. Three of the pigs at this time point had increases in MCP-1 transcript of 12- to 20-fold and had gross lesions affecting 0–3% of their lung, while the pig with 14% affected lung had no increase in MCP-1 transcript compared to sham treated pigs. TNF-alpha, IL-6, IL-8 and IL-1beta mRNA levels remained at levels observed in control animals in this group 10 days post-infection.

Cytokine changes observed on day 1 post-infection in coinfecting pigs were similar to those observed in pigs infected with *B. bronchiseptica* alone. Coinfecting pigs exhibited at least a 2-fold increase in mRNA levels of TNF-alpha and IL-8, a 5-fold increase of IL-6 and IL-1beta, and nearly 15-fold increase of MCP-1. Although the increase in mRNA levels were similar to that of pigs infected with *B. bronchiseptica* alone, the mRNA levels of TNF-alpha, IL-8, IL-6 and IL-1beta were significantly greater in coinfecting pigs when compared to PRCV infected pigs. While none of the AM collected from pigs receiving PRCV or *B. bronchiseptica* alone showed an increase in MCP-1 mRNA levels 4 days post-infection, 3 of the 4 pigs in the coinfecting group had a 55-fold or greater increase of MCP-1 mRNA, which made the coinfecting group's mRNA levels of this cytokine statistically greater than for either of the single infected groups. These three pigs had the largest percentage of lung affected by macroscopic lesions, more severe microscopic lesions, and both *B. bronchiseptica* and PRCV were isolated from the lung, while the one pig that did not have an increase in MCP-1 transcript had the fewest lesions and no *B. bronchiseptica* or PRCV isolated from its lungs. At 10 days post-infection, the majority of coinfecting pigs had increased mRNA levels for all the proinflammatory cytokines tested except TNF-alpha, for which all 4 pigs had a greater than 2-fold reduction of mRNA. The coinfecting group had significantly less TNF-alpha transcript and significantly more MCP-1 transcript, than the group infected with *B. bronchi-*

septica alone. The coinfecting group had close to significantly greater ($0.5 \leq p \leq 0.1$) IL-6, MCP-1, IL-8 and IL-1beta transcripts, than the group infected with PRCV alone. Overall, at 10 days post-infection more coinfecting pigs responded with at least a 2-fold increase of any cytokine compared to pigs infected with a single pathogen. For all timepoints, coinfecting pigs exhibited enhanced mRNA levels of proinflammatory cytokines 60% of the time as compared to 23% for pigs infected with *B. bronchiseptica* alone and 13% for pigs infected with PRCV alone. With the exception of TNF-alpha at 10 days post-infection, coinfecting pigs also had no apparent down regulation of examined cytokines.

4. Discussion

Because of its unique role in gas exchange and constant exposure to the environment, the respiratory tract is one of the most common sights for pathogen entry. Although any pathogen by itself may cause disease, it is not uncommon for multiple agents to be involved in respiratory disease. Interactions among multiple pathogens often appear to generate a more severe or chronic outcome than is observed with the individual pathogens by themselves, but little is known about the specific interactions. In both experiments performed in this study, coinfection with PRCV and *B. bronchiseptica* resulted in a higher percentage of the lung, on average, affected by pneumonia and an increased severity of microscopic lesions of pneumonia as compared to pigs infected with either pathogen by itself. The increased severity of gross and microscopic lesions at day 1 post-infection indicate that lesions developed earlier in coinfecting pigs than in pigs infected with either agent alone. In addition, the increased severity of lesions in coinfecting pigs at day 10 post-infection, a time when lesions should be resolving, suggests there was also a delay in resolution of the lesions compared to pigs infected with virus or bacteria alone. It is unlikely that lesions observed in coinfecting animals were solely an additive effect, since the percentage of lung affected in the coinfecting group was greater than the combined effect of the single infected groups. Furthermore, microscopic lesions in coinfecting pigs were not just a combination of those induced by PRCV or *B. bronchiseptica* alone, as new lesions not

observed in pigs infected with either pathogen alone were observed.

The isolation of both *B. bronchiseptica* and PRCV from the lungs of coinfecting pigs was greater, as compared to singly infected pigs, in the second experiment. These results may help explain the increased severity of lesions seen in coinfecting pigs. This was not the case in the first experiment, where the isolation rate was roughly the same for both single and dual infected pigs. However, there were only minor, if any, gross or microscopic lesions that accompanied the isolation of *B. bronchiseptica* from the pigs infected with bacteria alone in the first experiment. Although isolation of *B. bronchiseptica* from the turbinates and trachea is common in the age of pig used for these experiments, isolation from the lung in pigs challenged with *B. bronchiseptica* alone is not typical of prior experiments with this isolate (Brockmeier et al., 2000).

There was evidence of ascarid infection and migration through the liver and lung of the pigs in the first experiment. Previous studies have indicated that parasitic infections can alter the response to bacterial and viral infections. For example, aerosol exposure to *P. multocida* in mice with migrating *Ascaris suum* larvae developed more severe pneumonia than *Ascaris* free mice (Tjornehoj et al., 1992), and coinfection with *Schistosoma mansoni* and vaccinia virus resulted in a decreased antiviral Th1 response and delayed viral clearance in mice (Actor et al., 1993). *A. suum*, the probable cause of the lesions seen in the pigs in the first experiment, has also been shown to skew the adaptive immune response to a Th2 type response (Dawson et al., 2005). Thus, the increased isolation of *B. bronchiseptica* and PRCV, the appearance of mild microscopic lesions in control pigs, and the increased severity of microscopic lesions in coinfecting pigs that were all seen in the first experiment as compared to the second, may be attributed to the resolving ascarid migration that was evident in these pigs. These results illustrate the difficulties in reproducing the effects of experimental combined infections in out bred conventionally raised animals such as pigs.

Alveolar macrophages are first-responders during pulmonary disease and take action against invading pathogens through the production of proinflammatory cytokines and chemokines. In addition, alveolar macrophages can recruit effector cells to the site of

infection and act as antigen-presenting cells. As well as pathogen burden, another explanation for the increased disease severity observed in coinfecting pigs may be, in part, the result of enhanced proinflammatory cytokine expression in alveolar macrophages. Although cytokines were only examined at the transcriptional level, and enhanced transcription does not assure the cytokines are secreted and functional, an increase in transcription of at least one proinflammatory cytokine was detected in 100% of the coinfecting pigs at each of the various time points, whereas only 50% of the pigs from each of the groups infected with a single agent responded. Quantitatively, IL-6 and MCP-1 showed the largest transcriptional changes in coinfecting pigs when compared to pigs infected with either agent alone.

In general, pigs infected with *B. bronchiseptica* alone had an increase of mRNA of all cytokines tested at day 1 post-infection, even though no lesions were seen nor was bacteria recovered from the samples of lung tested. This was likely an acute response to *B. bronchiseptica* LPS in the inoculum. Little increase in cytokine transcriptional response was noted after day 1 in these pigs. In fact, at day 4 there appeared to be a down regulation of most of the cytokines examined. It is likely that the bacteria were largely cleared from the lungs of these pigs and few, if any, lesions were seen. The one exception was a pig on day 10 that had significant lung lesions affecting an estimated 11% of the lung and had increased TNF-alpha, IL-6, and MCP-1 mRNA. In this regard, at days 4 and 10 post-infection, lesions correlated to increased cytokine responses. Previous research in mice shows that IL-6 and TNF-alpha are induced in bone marrow derived macrophages 24 h after exposure, as well as in lung homogenates 12 h after exposure, to *B. bronchiseptica* in a toll-like receptor-4 (TLR4) dependent manner (Mann et al., 2004a). Furthermore, this early TNF-alpha response appears vital for limiting the extent of bacterial growth and pneumonia, permitting recovery (Mann et al., 2004b).

Pigs infected with PRCV alone displayed a limited cytokine response, that was basically restricted to an increase in MCP-1. If anything, there appeared to largely be a down regulation of most of the cytokines examined. These results agree with those of a previous study which found no increase in the bioactive levels of TNF-alpha or IL-1 in the BALF of PRCV infected pigs up to 4 days after infection (Van Reeth et al., 1999). The results reported here are also similar to a

recent report describing responses to SARS coronavirus in humans (Law et al., 2005). Human dendritic cells responded to SARS coronavirus with low expression of the antiviral cytokines (IFN-alpha, IFN-beta, IFN-gamma and IL-12) and proinflammatory cytokines (IL-6 and TNF-alpha), but large increases in CXC chemokines that preferentially act on mononuclear cells (MIP-1 α , RANTES, IP-10 and MCP-1). Of these CXC chemokines, induction was strongest for MCP-1 and IP-10. Additionally, MCP-1 was also found to be elevated in SARS patients as well (Huang et al., 2005). MCP-1 is important for the recruitment and activation of macrophages and has been shown to influence viral load in an influenza model (Dawson et al., 2000). The increase in MCP-1 in 1 pig at day 1 seemed to be positively correlated with microscopic lesions seen in that pig, while at 10 days post-infection the pig with the largest percentage of lung affected had no increase in MCP-1, and the other 3 pigs all had significant increases in this cytokine and little to no lung lesions. Thus, lesions at 10 days post-infection seemed to be inversely correlated with the level of MCP-1 produced.

On day 1 post-infection, the cytokine response in coinfecting pigs was most like that of pigs infected with *B. bronchiseptica* alone, in that there was a general increase in the transcriptional response to all cytokines tested. Again, this may largely be due to the response to bacterial LPS. However, unlike the pigs that were challenged with bacteria alone, bacteria were isolated from some of the coinfecting pigs and lesions were seen in all of the coinfecting pigs as compared to pigs infected with bacteria or virus alone. The coinfecting pigs responded with an increase to more cytokines per pig and quantitatively greater, on average, to IL-6 and MCP-1, than did pigs infected with the bacteria alone. This might explain the increase in the severity of lesions observed early in the coinfecting pigs compared to the pigs infected with bacteria or virus alone. Whether or not this early cytokine response in coinfecting pigs, which was not typical of pigs infected with the virus alone, might have altered the host response to PRCV resulting in the decreased clearance of the virus observed in the second experiment, warrants further investigation.

The increased transcriptional response of MCP-1 on day 4, and IL-6, MCP-1, IL-8 and IL-1beta on day 10 post-infection in coinfecting pigs demonstrates an

enhanced response of proinflammatory cytokines compared to what is seen with either of the pathogens alone. A previously proposed hypothesis suggested that proinflammatory cytokines, particularly TNF- α , produced during exposure to PRCV and bacterial LPS might be responsible for more severe clinical respiratory signs in pigs (Van Reeth et al., 2000). Although the results reported here do not point specifically to TNF- α , the lung lesions in coinfecting pigs support this hypothesis. In another study, exposing human macrophages to SARS coronavirus and subsequent suboptimal LPS led to a dramatic increase in IL-6 over what was observed with cells exposed to either virus or LPS alone (Tseng et al., 2005). Overall, the data presented here does not implicate a single cytokine or chemokine in causing more severe lung disease in coinfecting animals than in PRCV or *B. bronchiseptica* infection alone.

Ultimately, the early interaction of pathogens with the innate immune system and its subsequent influence on the adaptive immune response is critical in determining the outcome of infection. The host has evolved specific mechanisms to deal with diverse classes of pathogens in order to induce the most effective response. In addition to the innate mechanisms, such as epithelial barriers, muco-ciliary clearance, and antimicrobial substances, that are ubiquitously and constitutively present, innate responses induced through TLR receptors, which recognize conserved molecular patterns present in microorganisms, can stimulate specific responses to different pathogens. For example, neutrophils usually dominate the initial inflammatory infiltrate subsequent to bacterial infections, while viral infection is often characterized by primarily mononuclear infiltration. In this study, the cytokine response in coinfecting pigs was unique compared to the cytokine response in pigs infected with either agent by itself, suggesting that the alteration in the innate response, which has developed over time to deal with these pathogens, may be one mechanism through which coinfection alters the disease outcome.

Acknowledgements

The authors wish to thank William Mengeling and Ann Vorwald for providing the PRCV isolate and Kim

Driftmier, Theresa Waters, Michael Mullins and Don Hackbarth for their excellent technical assistance.

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