Bovine Respiratory Syncytial Virus and *Histophilus somni*

Interaction at the Alveolar Barrier

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**Running title:** BRSV/H. somni interaction at the alveolar barrier.

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ABSTRACT
Our previous studies showed that *Histophilus somni* and bovine respiratory syncytial virus (BRSV) act synergistically *in vivo* to cause more severe bovine respiratory disease than either agent alone. Since *H. somni* surface and secreted immunoglobulin binding protein A (IbpA) causes retraction of bovine alveolar type 2 (BAT2) cells and invasion between BAT2 cells *in vitro*, we investigated mechanisms of BRSV plus *H. somni* infection at the alveolar barrier. BRSV treatment of BAT2 cells prior to treatment with IbpA-rich *H. somni* concentrated culture supernatant (CCS) resulted in increased BAT2 cell rounding and retraction as compared to either treatment alone. This mimicked the increased alveolar cell thickening in calves experimentally infected with BRSV followed by *H. somni*, as compared to calves infected with BRSV or *H. somni* alone. BRSV plus *H. somni* CCS treatment of BAT2 cells also enhanced paracellular migration. The effect of matrix metalloproteinases (MMPs) was investigated also because microarray analysis revealed that treatment with BRSV plus *H. somni* synergistically upregulated BAT2 cell expression of *mmp1* and *mmp3* compared to cells treated with either agent alone. ELISA confirmed that MMP1 and MMP3 protein levels were similarly upregulated. In collagen I and collagen IV substrate zymography (targets for MMP1 and MMP3), digestion was increased with supernatants from dually treated BAT2 cells as compared with singly treated cells. Enhanced breakdown of collagen IV in the basal lamina and of fibrillar collagen I in the adjacent interstitium in the dual infection may facilitate dissemination of *H. somni* infection.
INTRODUCTION

Respiratory infections are characteristically polymicrobial. We previously investigated the mechanisms of viral and bacterial synergy in bovine respiratory disease (BRD) (1), by infecting calves with bovine respiratory syncytial virus (BRSV) followed by infection with \textit{Histophilus somni} or with either pathogen alone. The calves dually infected with BRSV and \textit{H. somni} had the most severe disease, and the highest serum IgE antibody responses to \textit{H. somni} (1). Duration of pneumonia and persistence of \textit{H. somni} in the lungs was also greatest in dually-infected animals (1). Our earlier immunohistochemistry studies of experimental \textit{H. somni} pneumonia showed that the bacteria are detected primarily in the alveoli at 24 hrs post intrabronchial inoculation (2). Since \textit{H. somni} causes septicemia and its sequelae (3), it is likely that it crosses into the circulation over the alveolar barrier. We also showed that the toxic Fic (Filamentation induced by cAMP) motifs of the direct repeat domains (DR1 and DR2) of the immunoglobulin binding protein A (IbpA) cause bovine alveolar type 2 (BAT2) cells to retract \textit{in vitro}, allowing paracellular migration (4). This cytotoxicity is due to adenylylation of host cell Rho GTPases by the Fic motif (5). Conservation of IbpA and Fic motifs in all tested \textit{H. somni} disease isolates was consistent with the potential role of IbpA in disease (6). Neutralization of cytotoxicity by antibody to IbpA DR2 and protection of calves against experimental \textit{H. somni} pneumonia by active immunization with recombinant IbpA DR2 confirmed its role in cytopathology and disease (6, 7). Since IbpA is shed into the culture supernatant, we utilized concentrated culture supernatant (CCS) as a source of enriched crude native IbpA for studies of BRSV/\textit{H. somni} synergy in breaching the alveolar barrier. BRSV (8) and \textit{H. somni} (4) both infect...
BAT2 cells, so we tested the hypothesis that BRSV enhances *H. somni* invasion at the alveolar barrier by determining the effect of BAT2 cell treatment with either BRSV, *H. somni* CCS, or both BRSV and CCS on retraction of BAT2 cells and on transmigration across a BAT2 cell monolayer. Treated BAT2 cell supernatants were utilized to investigate secreted matrix metalloproteinase (MMP) digestion of collagen, a major component of the alveolar basement membrane. Results indicate that BRSV infection plus CCS treatment of alveolar cells increases cell retraction and *H. somni* paracellular migration. Additionally, dual BRSV and CCS treatment of BAT2 cells results in increased MMP secretion with increased digestion of collagen I and IV.

**MATERIALS AND METHODS**

*Histophilus somni*, growth condition and CCS preparation. *Histophilus somni* strain 2336 was originally isolated in large numbers from a calf which died of pneumonia. This strain was previously used to induce experimental pneumonia in calves (1, 2, 9, 10). Bacteria were grown on Difco Brain-Heart Infusion (BHI) (BD Diagnostics, Sparks, MD) agar containing 5% bovine blood in Alsevers solution (Colorado Serum Co., Denver, CO) at 37°C in a candle jar. For culture supernatant preparation, bacteria harvested from an 18 h BHI blood agar plate were inoculated at approximately 5 x 10^7 CFU/ml into Bacto BHI broth (BD Diagnostics, Sparks, MD) supplemented with 0.1% Tris-base and 0.01% thiamine monophosphate. Bacteria were grown for 7 h at 37°C with shaking, centrifuged at 5,000 x g for 15 min, and the supernatant was filtered through a 0.22-µm-
diameter filter. CCS was prepared by concentrating supernatant 40 X and washing 2X by Amicon ultrafiltration with a 10-kDa molecular mass cutoff (Millipore, Billerica, MA).

BRSV preparation. A clinical virulent BRSV strain (CA-1) was isolated previously in the Gershwin Lab as previously reported (1, 9). BRSV was propagated in primary bovine turbinate (BT) cells grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (10 U/ml), and streptomycin (100 µg/ml) at 37°C with 5% CO₂. Briefly, 1 ml of frozen virus (3X10⁵ PFU/ml) was added to BT cells (at 90% confluence) in a T75 flask. Viral cytopathic effects (CPEs) usually started to occur on day 3 and reached approximately 30-50% of the cells by day 5, when virus was harvested from cell supernatant and from cells by freeze-thawing. A small amount of the suspended virus preparation was aliquoted and was used to measure its PFU/ml by monolayer plaque assay. The rest was aliquoted and stored at -80°C for infection studies.

Bovine Alveolar Type II (BAT2) Cell culture. Primary BAT2 cells were isolated from newborn calf lung as described previously (4) and were used at a maximum of 13 passages. Cells were grown in DMEM/Keratinocyte medium at 1:1 (Invitrogen, Carlsbad, CA), supplemented with 2% FBS, 5 mM L-glutamine, 0.02% lactalbumin dehydrogenase, and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen), at 37°C in a humidified incubator with 5% CO₂. Culture flasks and plates were pre-coated with 0.1% gelatin and 20% fetal bovine serum (FBS) in water (Gelatin/FBS) by incubating for 1 h at 37°C and air-drying.
Cytotoxicity assay. BAT2 cells were seeded in a 24-well plate at a density of $5 \times 10^4$ cells/well and were grown for 24 h to about 50% confluence, washed once with DMEM, and infected with BRSV at $1 \times 10^4$ PFU/ml (0.5 MOI) in BAT2 cell medium. After 60 h of infection with BRSV, virus-containing medium was removed and replaced with medium that was mixed 1:1 with 40x concentrated H. somni culture supernatant (CCS), which is enriched for crude native IbpA. Wells for virus infection alone were treated with BAT2 cell medium without FBS. After 4 h of treatment, cells were washed twice with PBS and fixed with 4% fresh paraformaldehyde for 20 min at 4°C, permeabilized with 0.1% Triton X-100 for 5 min, and stained with rhodamine-phalloidin (Invitrogen) for 30 min at room temperature. Nuclei were counter-stained with DAPI (Invitrogen). Cells were examined under a 20x objective lens on an Olympus 1X70 inverted microscope (Olympus, Tokyo, Japan) with fluorescent light source model U-LH100 HG (Olympus). A TRITC filter set was used for examining rhodamine phalloidin-stained cells, and a DAPI filter set was used for examining DAPI-stained cells. The microscopist was “blinded” as to the treatments. The number of rounded cells and/or retracted cells were counted and the percentage of the total number of cells was calculated. Ten microscope fields were counted, and experiments were repeated twice.

Transmigration assay. BAT2 cells were grown on 24-well polycarbonate Transwell membrane inserts with 3 µM pore size (Corning, Cambridge, MA). The membranes were first coated with gelatin/FBS for one h at 37°C, air-dried, and covered with DMEM for one h at 37°C before seeding cells. Cells were plated at a density of $2 \times 10^4$ cells/well in 100µl BAT2 cell medium, and the bottom chamber was filled with 1 ml BAT2 cell medium. After 24 h cells were washed, and the medium was replaced with BRSV.
containing medium (1 x 10^4 PFU/ml). After 60 h incubation with BRSV, cells were washed three times, and 10^5 CFU of *H. somni* in 80μl cell culture medium (without antibiotics) was added to the Transwell insert (approximately 10 MOI). The lower chamber was filled with 500μl of cell culture medium without antibiotics. After 3 h of incubation, the Tranwell-insert was removed and rinsed in a separate well of a 24 well plate containing 500μl of culture media, which was then pooled with the contents of the lower chamber. The contents of the lower chamber (plus the rinse) were serially diluted and plated in duplicate on BHI blood agar plates for viable bacterial counts. Experiments were done in six duplicate wells, and the mean percentage of transmigrated bacterial CFU recovered from the bottom chamber was calculated.

**Pulmonary alveolar histopathology of calves infected with BRSV, *H. somni*, or BRSV and *H. somni*.** In an earlier study, nine week old calves were infected with BRSV by aerosol at day 0 and/or by inoculation at the tracheal bifurcation with *H. somni* strain 2336 at day 6 (1). After clinical monitoring and sample collection, calves were euthanized at day 28. The entire respiratory tracts were removed, areas of pathology mapped and samples collected for culture and routine histological examination. Histopathological results were previously reported (1). The tissues from these calves were reviewed again in order to examine the effects of viral bacterial interactions at the alveolar membrane *in vivo*.

**Microarray Analysis.** BAT2 cells in 6-well plates were treated as described under “Cytotoxicity” above in three experiments. After supernatant was collected for MMP ELISA, RNA was extracted with the RNeasy Mini Kit (Qiagen, Maryland), according to
the manufacturer’s instructions. RNA amplification and two-cycle labeling was done with the Affymetrix GeneChip 3’IVT Express Kit (Santa Clara, CA), and Affymetrix GeneChip Bovine Genome Arrays were used to profile gene expression. Hybridization and Scan of Affymetrix GeneChip was performed in the UC Davis School of Medicine Microarray Core Facility. Microarray data from the three repeated experiments was analyzed using Web-based software dChip (DNA-Chip Analyzer) and DAVID (The Database for Annotation, Visualization and Integrated Discovery (DAVID), National Institute of Allergy and Infectious Diseases, NIH).

**Matrix metalloproteinase ELISA.** Bovine MMP1 and MMP3 protein levels in BAT2 supernatants were measured using Uscn ELISA kits (E90097Bo for MMP1 and E90097Bo for MMP3) (Uscn, Wuhan, China), according to the manufacturer’s directions.

**Substrate Zymography.** Matrix metalloproteinase activity in BAT2 supernatants was measured using collagen I and collagen IV substrate zymography (11, 12). The BAT2 supernatants were activated with 1 mM 4-aminophenyl mercuric acetate (APMA) (Sigma, St. Louis, MO) at 37°C for 30 minutes (13). Supernatants were separated by electrophoresis on 10% acrylamide gels that were co-polymerized with either 0.02% collagen I from bovine skin (Sigma) or 0.02% collagen IV from human placenta (Sigma). Gels were sequentially incubated at room temperature for 20 min each in the following buffers: 1.) 2.5% Triton X-100 for 20 minutes, 2.) 2.5% Triton X-100 + 50 mM Tris-HCl pH 7.5 for 20 minutes, 3.) 2.5% Triton X-100, 50 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 1 uM ZnCl₂ for 20 minutes, and 4.) 50 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 1 uM ZnCl₂ for 20 minutes. The gels were then incubated in 50 mM Tris-HCl pH 7.5, 5 mM CaCl₂, 1
uM ZnCl₂ for 18 hours. Since divalent cations are required for MMP activity (14), negative controls included separate identical gels incubated in the above buffers plus 20 mM EDTA to chelate divalent cations as well as in buffers without CaCl₂ or ZnCl₂. After incubation, gels were stained in 0.1% Coomassie Brilliant Blue G-250 for 2 h and were destained in 20% methanol/7.5% acetic acid until clear bands corresponding to degraded collagen appeared on a blue background. Densitometry analysis to quantify band intensity was done with imageJ software (version 1.64, http://imagej.nih.gov/ij/) (15).

Data analysis. Data for transmigration studies were analyzed for significance with an unpaired Student's one-tailed t-test. Data for cytotoxicity assays, microarray studies, ELISA, and zymography densitometry were analyzed for significance by one-way ANOVA, followed by a Tukey-Kramer multiple comparison test.

RESULTS

BRSV/H. somni cytotoxicity. We previously showed that live H. somni or IbpA enriched CCS caused BAT2 cells to retract and round up (4). Since BRSV and H. somni together cause more severe disease than either agent alone (1), we tested the hypothesis that BRSV and H. somni synergistically induce cytotoxic effects at the alveolar barrier. Cytotoxicity assays showed that BAT2 cells infected with 0.5 MOI BRSV prior to CCS treatment retracted and rounded more than those treated with either agent alone (Fig. 1A). When retracted/rounded cells were counted, treatment of BAT2 cells with both BRSV and CCS resulted in a significantly higher (p<0.01) percentage of cells exhibiting cytotoxic effects as compared to cells that were treated with CCS or...
BRSV alone (Fig 1B). Furthermore, we previously showed that IbpA-producing *H. somni* strain 2336 transmigrates between BAT2 cells in a monolayer (4). Since BRSV plus *H. somni* CCS treatment enhances BAT2 cytotoxicity, as shown above, we determined whether pretreatment of BAT2 cells with BRSV would enhance the paracellular migration of *H. somni* across a confluent BAT2 cell monolayer. Results show that 61% of the *H. somni* inoculum crossed the BRSV infected monolayer as compared with 41% of the inoculum crossing the non-BRSV infected monolayer (p=0.03) (Fig. 2).

**Pulmonary alveolar histopathology of calves infected with BRSV, *H. somni*, or BRSV and *H. somni***. In sections of lung of dually infected calves examined by light microscopy, evidence of focal alveolar exudation and septal thickening with enlarged cuboidal alveolar type 2 epithelial was detected, especially in areas with chronic bronchiolitis (Fig 3a). Alveolar septa in the singly BRSV infected calves (Fig 3b) or solely infected *H. somni* calves (Fig 3c) were unremarkable.

**BAT2 cell matrix metalloproteinases**. Microarray analysis demonstrated synergistic up-regulation in the expression of *mmp1* and *mmp3* mRNA transcripts when BAT2 cells were dually treated with BRSV and CCS as compared with either agent alone (Fig. 4A and B). Expression of other *mmps* was not up-regulated (data not shown). The full results of the microarray analysis will be reported separately (Shao M et al., manuscript in preparation). Collagen, a critical component of the basement membrane and the lung parenchyma, is a major substrate for MMPs. Since the basement membrane presents...
a second barrier to *H. somni* invasion of the pulmonary parenchyma and the microvasculature, we investigated the role of collagen degrading matrix metalloproteinases from BAT2 cells. MMP1 and MMP3 protein levels in BAT2 supernatants were determined by ELISA, because mRNA transcript levels do not necessarily correlate with protein levels (16, 17). Results showed that levels of MMP1 and MMP3 in BAT2 cell supernatants after treatment with both BRSV and CCS increased more than the sum of the levels in BRSV and CCS treated cells (Table 1 and Fig. 5), demonstrating synergism. The MMP function of BAT2 supernatants was investigated by collagen I and collagen IV zymography because those collagens are targets of MMP1 and MMP3. The control consists of BAT2 cells treated with media alone. BAT2 cells secrete a base line level of MMPs without treatment with BRSV or *H. somni* CCS, as demonstrated by digested bands in zymography (Fig. 6 A and B).

Digestion of both substrates was much greater for BAT2 supernatants treated with both BRSV and CCS as compared with either agent alone as shown by staining of digested gels (Fig. 6A and B) and by densitometry (Fig.6C and D). No protease activity was detected in the presence of EDTA or in the gel incubated in buffers without zinc and calcium, indicating that collagenase activity is due to MMPs present in the BAT2 supernatants, since divalent cations are required for activity (Fig. 6A and B).

**DISCUSSION**

This study shows that dual treatment with BRSV and *H. somni* enhanced cytotoxicity for alveolar epithelial cells, transmigration of *H. somni* across the alveolar cell barrier and...
mmp1 and mmp3 expression, protein production and activity. This enhanced effect at the alveolar barrier is likely to contribute to the increased severity of disease in calves dually infected with BRSV and H. somni, which we previously reported (1). That previous study showed that dually infected calves had higher IgE antibody levels to H. somni, partially accounting for the increased clinical scores, inflammation and prolonged infection. It is not surprising that there was little inflammation in lungs of the singly infected calves at 28 days post BRSV infection or 22 days post H. somni infection, because pneumonia is most pronounced in the first week or so with single infections. Histopathological examination of the areas of pulmonary inflammation in those calves showed greater thickening of alveolar septae in the dually infected calves than those given either agent alone. The alveolar septal thickening associated with type 2 alveolar epithelial hyperplasia is a typical reparative response to alveolar epithelial damage which can occur as a consequence of alveolar exudation associated with bronchopneumonia. However, the in vitro data showing enhanced retraction/rounding up of alveolar cells after treatment with BRSV and H. somni concentrated culture supernatant (CCS) suggests that the alveolar cell changes may be a direct synergistic effect of these two pathogens on alveolar epithelial cell retraction as well.

The first step in crossing the alveolar barrier is migration of H. somni across the epithelial cell monolayer. We previously showed that the H. somni IbpA DR2 Fic cytotoxin causes retraction of BAT2 cells by adenyllyating Rho GTPases, resulting in impairment of the host cell cytoskeleton (4, 5). Here we have shown that cytotoxicity is synergistically increased when BAT2 cells are infected with BRSV for 60 hours prior to treatment with crude native IbpA. This could be due to BRSV enhancement of the
IbpA-induced cytotoxicity or a direct effect of BRSV on the BAT2 cells or both. It has been shown that BRSV replicates in and causes apoptosis of alveolar type 1 and type 2 cells during experimental infection of calves (8) which may partially explain the synergistic cytotoxicity observed for our studies. We previously showed that *H. somni* does not invade bovine epithelial cells, but IbpA is internalized (4). Internalization of IbpA could potentially be enhanced by BRSV infection, providing another mechanism for enhanced cytotoxicity. Regardless of the mechanism, enhanced cellular cytotoxicity may partly explain the increased disease severity observed for calves dually infected with BRSV and *H. somni*. Increased cell retraction was followed by increased *H. somni* invasion, since treatment of cells with BRSV prior to the addition of *H. somni* significantly increased paracellular migration of *H. somni* between BAT2 cells in a monolayer. BRSV may increase transmigration by increasing IbpA uptake by BAT2 cells, as suggested above, or BRSV may have a direct effect on the alveolar cell cytoskeleton, since rearrangement of actin filaments in human RSV infected epithelial cells increases paracellular permeability (18, 19). Thus *H. somni* IbpA and BRSV induced cell retraction may partially explain the increased disease severity for animals dually infected with BRSV and *H. somni* (1).

Microarray and ELISA results show that *mmp1* and *mmp3* expression, protein production and activity are upregulated synergistically in dually infected BAT2 cells. MMP1 degrades several matrix proteins including collagen I and entactin (20). Entactin is noted to be a basement membrane protein (21), while collagen I comprises 50-60% of the lung extracellular matrix (22). Therefore, increased MMP1 levels should increase both basement membrane and pulmonary matrix destruction. We demonstrated an
increase in matrix collagen I degradation from BAT2 supernatants treated with both BRSV and *H. somni* CCS as compared to cells treated with either agent alone. This is consistent with our previously reported studies of BRSV/*H. somni* synergy in calves where, even at necropsy, 28 days post BRSV and 22 days post *H. somni* infection, the pulmonary interstitium of dually infected calves was fibrotic and infiltrated with inflammatory cells, and *H. somni* cultures were positive (1). Lungs of calves infected with BRSV or *H. somni* alone had minimal to mild inflammation, no interstitial fibrosis and negative *H. somni* cultures. MMP1 may be important in the ongoing remodeling of fibrosis. MMP3, on the other hand, degrades collagen IV fibers in the basement membrane. We found that BAT2 supernatants from cells dually-treated with BRSV and *H. somni* CCS showed an increased level of collagen IV degradation as compared to BAT2 cells treated with either CCS or BRSV alone, consistent with MMP3 mRNA and protein levels. The basement membrane is a barrier against pathogen invasion (23, 24), so degradation of the basement membrane proteins would allow *H. somni* to cross the inner alveolar barrier. Since *H. somni* causes septicemia in the natural host, crossing of the alveolar barrier to invade the blood stream is critical to pathogenesis. MMP3 also cleaves and activates the pro-forms of other MMPs, including MMP1, which would result in further matrix protein destruction in the host (20, 25). In addition to matrix proteins, the pro-form of TNF-α is cleaved to the active form by MMP1 and MMP3 (26). Increased active TNF-α due to increased MMP production may partly explain the increased inflammation observed in animals dually infected with BRSV and *H. somni* (1, 27).
In conclusion, BRSV and *H. somni* IbpA increase BAT2 cell retraction and paracellular migration, which enhances *H. somni* crossing the first barrier, the alveolar epithelial cell layer. Dual treatment of BAT2 cells with BRSV and *H. somni* CCS also increases host cell matrix metalloproteinase expression and activity. MMPs degrade components of the basement membrane allowing *H. somni* to cross the second barrier, the basement membrane, to invade the tissue and the microvasculature resulting in enhanced disease, as previously described (1). These investigations have identified two mechanisms to account for the increase in alveolar invasion by *H. somni* during BRSV/*H. somni* synergism: alveolar cell retraction and increased degradation of collagen. Both mechanisms would be expected to facilitate lung damage, subsequent pneumonia, and bacterial dissemination.

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FIGURE LEGENDS

Figure 1. BAT2 cell rounding and retraction caused by treatment with lbpA-enriched concentrated culture supernatant (20xCCS) and/or 60 hour treatment with BRSV. (A) Morphology of BAT2 cells treated with media as a control, BRSV alone, 20x CCS alone, or with both BRSV and 20x CCS. Cells were stained with rhodamine phalloidin and DAPI, which stain F-actin fibers and cell nuclei, respectively. White arrowheads indicate
examples of rounded cells and white arrows indicate examples of retracted cells. (B)

Percentage of rounded and retracted BAT2 cells after treatment as in (A). Percent of retracted cells in ten microscope fields were calculated. Bars represent average values with error bars of SEM. (* P<0.01 between groups)

Figure 2. Transmigration of *H. somni* across a monolayer of BAT2 cells, with or without BRSV infection. BAT2 cells were allowed to grow into a monolayer on transwell membranes and were treated with BRSV or not. Live *H. somni* bacteria were then added to the top of the transwell and were allowed to migrate across the monolayer into the media in the lower transwell chamber. The viable bacterial colony forming units (CFUs) in the lower chamber were counted. Bars show the average number of transmigrated bacterial CFU. The percentage of *H. somni* bacteria that transmigrated through the transwell membrane as compared to the total number of added bacteria is indicated above each bar. Error bars represent the standard deviation for three replicates. *P=0.03

Figure 3. Alveolar histology 28 days after experimental respiratory infection of calves with BRSV at day 0, *H. somni* at day 6 or both. Note alveolar septal thickening associated with cuboidal type 2 alveolar epithelia with alveolar exudation after the dual infection (a) but minimal septal thickening or exudation after either BRSV (b) or *H. somni* (c) treatment alone. 20X magnification.
Figure 4. Up-regulation of msp1 (A) and msp3 transcripts (B) following treatment with BRSV and/or CCS. Fold increase was calculated as the mean number of transcripts for treated cells over that of control cells treated with media alone. Error bars indicate the SEM for three independent experiments. * P<0.001

Figure 5. Increased production of MMP1(A) and MMP3(B) protein by BAT2 cells following treatment with BRSV and/or CCS. MMP1 and MMP3 were measured in BAT2 supernatants by ELISA and increases were determined by subtracting the basal level of MMP1 or MMP3 in supernatants from media treated BAT2 cells from amounts in supernatants from pathogen treated cells. Bars represent the average increased protein expression for three independent experiments. Error bars indicate the SEM for three independent experiments. * P<0.05 between indicated groups.

Figure 6. Digestion of collagen I and IV by BRSV and/or CCS-treated BAT2 supernatants. Supernatants from treated BAT2 cells were tested for MMP activity by collagen I zymography (A) and collagen IV zymography (B). Collagen zymography was performed with the calcium and zinc ions required for matrix metalloproteinase activity (top gel). Parallel zymography with the addition of 20 mM EDTA to chelate divalent cations (middle gel) or with incubation buffers not including zinc or calcium (bottom gel) showed no digestion, confirming the activity was due to MMPs. Band densities were measured by densitometry, and the value for media-treated BAT2 supernatants was subtracted from the values for BRSV-treated, CSS-treated, and CCS+BRSV-treated BAT2 supernatant demonstrating increases in band intensity (C). Gel images are
representative of two experiments, and bar graphs show densitometry data from the gel images shown in A and B.

**TABLES**

**TABLE 1.** Increased production of MMP1 and MMP3 by BAT2 cells after treatment with BRSV and *H. somni* CCS as compared to either agent alone.

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<th>MMP3 (ng/mL)</th>
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<td><strong>0.60</strong></td>
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<td>BRSV + CCS</td>
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<td><strong>8.60</strong></td>
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*a* Increase in protein levels found in BAT2 supernatant

*b* "Sum" refers to the arithmetic sum of MMP1 or MMP3 levels induced by separate treatment of BRSV or *H. somni* CCS.
C  Collagen I digestion

D  Collagen IV digestion

* Media-treated BAT2 control densitometry values were subtracted from the total value obtained for each treatment group.
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