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Co-infection of epithelial cells established from the upper and lower bovine respiratory tract with bovine respiratory syncytial virus and bacteria

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ABSTRACT

Bovine respiratory disease complex is a major disease affecting the global cattle industry. Multiple infections by viruses and bacteria increase disease severity. Previously, we reported that bovine respiratory syncytial virus (BRSV) infection increases adherence of *Pasteurella multocida* to human respiratory and bovine kidney epithelial cells. To examine the interaction between the virus and bacteria in bovine respiratory cells, we generated respiratory epithelial cell lines from bovine trachea (bTEC), bronchus (bBEC), and lung (bLEC). Although all established cell lines were infected by BRSV and *P. multocida* susceptibility differed according to site of origin. The cells derived from the lower respiratory tract (bBEC and bLEC) were significantly more susceptible to BRSV than those derived from the upper respiratory tract (bTEC). Pre-infection of bBEC and bLEC with BRSV increased adherence of *P. multocida*; this was not the case for bTEC. These results indicate that BRSV may reproduce better in the lower respiratory tract and encourage adherence of bacteria. Thus, we identify one possible mechanism underlying severe pneumonia.

1. Introduction

Bovine respiratory disease complex (BRDC) is the most common and costly disease in cattle (Larsen et al., 2001; Beaudeau et al., 2010; Klima et al., 2014; Tizioto et al., 2015). BRDC is caused by infection of respiratory epithelial cells by viruses and/or bacteria; interaction between viruses and bacteria can increase disease severity (Härtel et al., 2004; Gershwin et al., 2005). Bovine respiratory syncytial virus (BRSV), a single negative-stranded RNA virus belonging to the *Pneumoviridae* family, shows a close genetic relationship with human respiratory syncytial virus (Larsen et al., 2001; Härtel et al., 2004; Kirchhoff et al., 2014; Blodörn et al., 2015; Gershwin et al., 2015). Indeed, it is a primary agent of BRDC in calves and beef and dairy cattle (Gershwin et al., 2005). BRSV infection in cattle is characterized by atypical interstitial pneumonia with severe bronchitis, bronchiolitis and interstitial pneumonia, viral inclusion bodies, epithelial necrosis, and syncytia formation (Tjønehøj et al., 2003; Spilki et al., 2006; Taylor, 2017); in calves, epithelial necrosis and viral antigens are detected at all levels of the respiratory tract (Taylor, 2017). Initial infection by BRSV facilitates secondary infection of the lower respiratory tract by such as *Pasteurella multocida* (*PM*) and *Mannheimia haemolytica* (Larsen et al., 2001; Gershwin et al., 2005; Beaudeau et al., 2010); this is because it alters the integrity of the epithelial barrier and the associated immune response in BRDC (Härtel et al., 2004; Agnes et al., 2013). The *PM* is most common bacterial can be found in respiratory tract of healthy and sick cattle; and will develop pneumonia in most shipping calve. Although presence of *PM* as a normal flora in upper respiratory tract, it can be predisposing factor to pneumonia with stressor and/or virus infection (Dabo et al., 2007).

Our previous report demonstrated that BRSV increases adherence of *PM* to epithelial cells, human respiratory cell lines (A549 and Hep2), and bovine kidney epithelial cells (Sudaryatma et al., 2018). The viability and infectivity of BRSV is influenced by its natural target cells, which have characteristic features; therefore, some aspects of viral pathogenesis cannot be addressed by experimental infection of immortalized cell lines. Thus, primary bovine epithelial cells are often

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used to examine host-virus interactions (Kalina et al., 2006; Al-Haddawi et al., 2007; Berghuis et al., 2014; Lin et al., 2016). Bovine respiratory epithelial cells (BRECs) used to examine infection by viruses, bacteria, and/or both are expected to show differences according to the organ/site from which they are derived (Kisiela and Czuprynski, 2009; Zekarias et al., 2010; N'jai et al., 2013; Boukahil and Czuprynski, 2016). Critical events in any respiratory disease occur at the initial site of infection; these include pathogen attachment to and invasion of BRECs.

Little is known about the susceptibility of BRECs to co-infection with BRSV and bacteria. The results of the present study suggest that BRSV might infect and propagate in the lower respiratory tract more easily than in the upper respiratory tract, and that infection encourages adherence of potentially pneumonia-causing bacteria. Thus, we identify one possible mechanism by which BRSV causes severe pneumonia in BRDC.

2. Materials and methods

2.1. Virus and bacteria

BRSV wild-type (strain 2205027-1), isolated from a bovine respiratory nasal swab in Kyushu, and *PM* (strain 2368, capsular type B), were used (Sudaryatma et al., 2018). For infection, virus and/or bacteria were diluted in antibiotic- and serum-free Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 GlutaMAX (DMEM/F12; Gibco, NY, USA) to achieve a multiplicity of infection (MOI) of 1 and 100, respectively.

2.2. Collection of bovine respiratory tissue

Bovine respiratory tissue was obtained from three healthy Japanese black cattle freshly slaughtered at Miyazaki slaughterhouse, Japan. A 10 cm section from the larynx and trachea was isolated, and the bronchus was dissected 5 cm below the bifurcation. An edge section of lung was also isolated. Small sections of respiratory tissue were fixed in 98% ethanol; PCR detected no contamination by viruses or bacteria (Kishimoto et al., 2017). All specimens were obtained within 1 h of slaughter and submerged in transfer medium comprising cold DMEM/F12 (Gibco), 500 U/ml penicillin (Wako, Mie, Japan), 500 mg/ml streptomycin (Wako), and 5 μg/ml amphotericin-B (Wako).

2.3. Primary culture of BRECs

Bovine respiratory tissues were washed, scraped, and minced in a sterile culture plate using a scalpel blade. Minced tissues were agitated, the pieces were allowed to settle, and the supernatant was removed carefully. This process was repeated three times. The pellet of minced tissue was digested for 1 h at 37 °C (with shaking) in 0.25% Trypsin-EDTA (Nacalai Tesque, Kyoto, Japan). The trypsin was neutralized by addition of heat-inactivated fetal bovine serum (FBS; Biowest, Nuaillé, France) to a final concentration of 10%. The cells were then filtered through a 70 μm cell strainer (Falcon, NJ, USA) and centrifuged (300 × *g*, 5 min). The pellet was resuspended in isolation medium containing DMEM/F12 supplemented with 15% FBS, 200 U/ml penicillin, 200 mg/ml streptomycin, 2.5 μg/ml amphotericin-B, 15 ng/ml epidermal growth factor, 1% insulin-transferrin-selenium, 1 μg/ml hydrocortisone, 1% non-essential amino acid, and 4 mM L-glutamine (all from Wako). Finally, the cells were seeded (1×10^6 cells/ml) into 6-well plates coated with collagen coating type I (Sumilon, Osaka, Japan) and incubated at 37 °C in 5% CO_2. The culture medium was changed every 3–4 days until the cells reached 100% confluence.

2.4. Continuous culture of BRECs

A combination of techniques was used to purify epithelial cell cultures (first to fourth passages) (García-Posadas et al., 2013) to eliminate contaminating fibroblasts; these techniques involved different trypsinization times and pre-plating. Briefly, primary cells were detached by exposure to 0.25% trypsin-EDTA for 5 min. Detached cells were washed out, and the remaining cells were trypsinized for another 15 min. Cells were pelleted by centrifugation at 300 × *g* for 5 min. Pre-plating purification was performed by seeding cells onto 90 mm culture plates for 2 h; floating cells were collected and cultured in T-25 flasks coated with collagen type I (Sumilon).

After isolation and purification, bovine trachea (bTEC), bronchus (bBEC), and lung (bLEC) epithelial cells were grown and maintained at 37 °C/5% CO_2 in bovine epithelial culture medium comprising DMEM/F12, 2% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 μg/ml amphotericin-B, 10 ng/ml epidermal growth factor, 1% insulin-transferrin-selenium, 1% non-essential amino acid, and 2 mM L-glutamine. To evaluate growth conditions, BRECs (10^5 cells/ml) were seeded in 12-well plates coated with collagen type I (Sumilon) and allowed to adhere for 24 h. Cells were dissociated and counted daily up until Day 7 of culture using a Countess II (Life Technologies, NY, USA).

2.5. Immunofluorescence assay

BRECs cultured for 4 days were fixed in 4% paraformaldehyde (Wako), permeabilized with 0.5% Triton X-100 (Nacalai Tesque) in PBS, and blocked with 1% bovine serum albumin. Cells were then incubated with mouse anti-pancytokeratin (1 μg/ml, ab7753, Abcam, Tokyo, Japan) and rabbit anti-vimentin (1 μg/ml, ab45939, Abcam) primary antibodies, followed by Alexa Fluor 488 goat anti-mouse IgG (1:500, ab150113, Abcam) and Alexa Fluor 594 goat anti-rabbit IgG (1:500, Invitrogen, CA, USA) secondary antibodies. Cell nuclei were stained with Hoechst-33342 (2 μg/ml, ab145597, Abcam). Cells were washed three times with PBST (Tween-20; 0.01%), covered with 70% glycerol mounting medium, and examined under a confocal microscope (KEYENCE BZ-900, Keyence, Osaka, Japan). Bovine tracheal fibroblast (EBTr) cells were used as a control. At the second and fifth passages, pancytokeratin-positive BRECs in five randomly chosen fields of 100 cells from each respiratory epithelial source were counted. The presence of BRSV within BRECs was confirmed by immunofluorescence analysis (IFA) as previously described (Sudaryatma et al., 2018).

2.6. Virus and/or bacterial infection of BRECs

Infection of cells with BRSV or with BRSV plus *PM* was described previously (Sudaryatma et al., 2018). Briefly, BRECs from different sites were seeded separately and infected with BRSV (MOI = 1). Supernatant and cell lysates were collected separately at 0, 1, 3, 6, and 12 h post-infection (hpi), and at 1, 3, 5, and 7 days post-infection (dpi). BRSV-infected and uninfected cells were exposed to *PM* (MOI = 100) for 1 h at 37 °C, and the number of adhering bacteria was plated as colony forming unit as previously described (Sudaryatma et al., 2018)

2.7. Quantitative real-time RT-PCR (qRT-PCR) to detect BRSV and cytokine mRNA in BRECs

Viral and cellular RNA was extracted from cells using a NucleoSpin-viral purification kit (Takara, Kyoto, Japan) and a RNeasy Mini Kit (Qiagen, Hilden, Germany), respectively. All quantitative real-time RT-PCR assays from three independent animal experiments were performed in triplicate. Assays were performed in duplicate wells using a LightCycler 96 system (Roche, CT, USA). Quantitative real-time RT-PCR (qRT-PCR) analysis of BRSV was performed using a one-step PrimeScript RT-PCR kit (TaKaRa). Synthetic control DNA (the BRSV N-gene) and primers/probes (Boxus et al., 2005) were purchased from Integrated DNA Technologies (Singapore). The sequence of the forward primer was 5′-GCAATGCTGCAGGACTAGGTATAAT-3′, and that of the reverse primer was 5′-ACACTGTAATTGATGACCCCATTCT-3′. The

sequence of the probe was 5′-FAM/ACCAAGACT/ZEN/TGTATGATGC TGCCAAAGCA/3IABkFQ-3′. To detect mRNA encoding bovine cytokines, qRT-PCR was performed using a one-step TB Green PrimeScript RT-PCR kit II (TaKaRa). The primer sets and amplification conditions used to detect bovine (*b)*IL-1β, *b*IL-6, *b*TNF-α, and *b*GAPDH genes have been published previously (Sobotta et al., 2017). Relative expression of mRNA between infected and uninfected controls was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as -fold change.

2.8. Statistical analysis

All experiments on each respiratory tissue from the three animals were performed in triplicate. Data are expressed as the mean ± standard deviation or standard error of the mean. Statistical analysis was performed using one-way analysis of variance and Tukey's Multiple Comparison Test. A p value < 0.05 was considered significant. Data analysis was performed using RStudio version 1.0.143.

3. Results

3.1. Isolation, purification, and growth characteristics of BRECs

For primary culture, BRECs were seeded at 1×10^6 cells/ml and reached confluence by Day 4 of culture. Cells formed a monolayer showing epithelial- and fibroblast-like morphology and close contact with neighbors. BRECs (bTEC, bBEC and bLEC) showed a consistent, homogeneous cobblestone morphology at passages 4 and 10 (Fig. 1A). For stable growth, BRECs were subcultured at 7–10 day intervals at a ratio of 1:5. Cells were subcultured successfully up until passage 19. Epithelial cells maintained log phase growth up until Day 7 of culture, with a population doubling time of approximately 24 h (Fig. 1B). Cells were purified using differential-time trypsinization and pre-plating, resulting in pure (> 95%) epithelial cell cultures. Double staining with anti-pancytokeratin and anti-vimentin antibodies (markers of epithelial and stromal cells, respectively) was performed when BRECs reached the second and fifth passages (Fig. 1C). The percentage of second and fifth passage cells identified as pancytokeratin-positive was as follows: bTEC, 76.0 ± 0.07 *vs.* 95.3 ± 0.02, respectively; bBEC, 83.0 ± 0.09 *vs.* 96.3 ± 0.02, respectively; and bLEC, 72.7 ± 0.02 *vs.* 95.0 ± 0.02, respectively. By contrast, all EBTr cells expressed vimentin and did not stain for pancytokeratin. Thus, BRECs comprised > 95% epithelial cells. Cells were used for all experiments when they reached passages 8–10.

3.2. Susceptibility of BRECs infected with BRSV

To confirm infection with BRSV, we used qRT-PCR to measure the viral copy number in BRECs culture medium at different time points post-infection. Viral RNA was detected from 6 hpi in bLEC and from 12 hpi in bBEC and bTEC (Fig. 2A). There were significant differences between bLEC/bBEC and bTEC with respect to BRSV replication ($p < 0.05$). From 12 hpi, the viral copy number in bLEC culture medium was higher than that in medium from bBEC and bTEC. The viral copy number in bBEC medium at 5 dpi was equal to that in bLEC medium (8.15 and 8.14 $\log_{10}$/ml, respectively), and significantly higher than that in bTEC medium (7.34 $\log_{10}$/ml). These patterns were maintained up until 7 dpi ($p < 0.05$). Fig. 2B shows typical cytopathic effects (CPEs) in BRSV-infected BRECs at different times post-infection. Syncytium formation was observed in bLEC and bBEC, but not in bTEC. Immunofluorescence staining of BRSV (BRSV-F protein, *red*) at 3 dpi showed that cells from the three different anatomical sites showed differing susceptibility to BRSV infection (Fig. 2C).

Next, we examined expression of mRNA encoding inflammatory cytokines (Fig. 2D). Peak expression of IL-1β mRNA by bBEC, bTEC, and bLEC (an 8-, a 13-, and a 26-fold increase, respectively) was detected at 1 hpi. Peak expression of IL-6 by bTEC was detected at 1 hpi

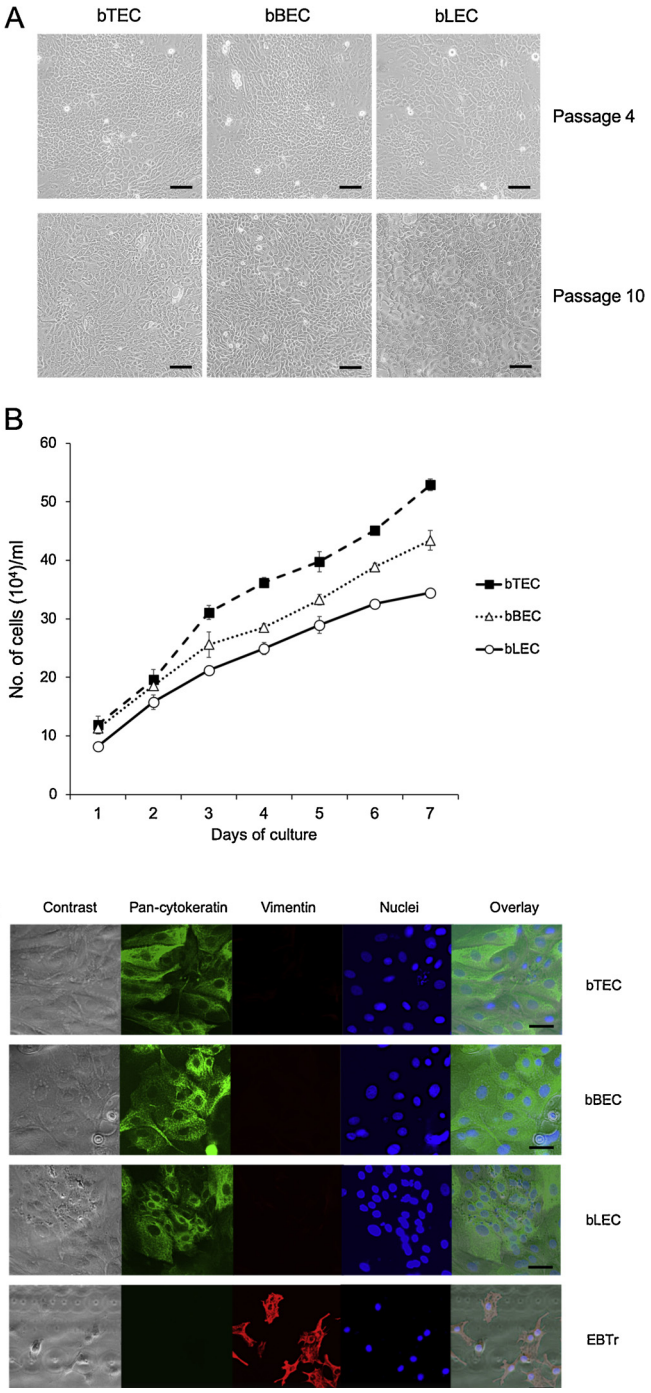

Fig. 1. Bovine respiratory epithelial cells (BRECs) established from trachea (bTEC), bronchus (bBEC), and lung (bLEC). **(A)** Isolated BRECs showed a homogeneous cobblestone morphology after 4 days of culture following the fourth and tenth passages (bar = 100 µm). **(B)** Growth curves. BRECs were seeded (10^5 cells/ml) into 12-well plates coated with collagen and counted every day using a trypan blue dye exclusion assay. Data are expressed as the mean (± SD); n = 3. **(C)** BRECs were cultured and stained with anti-pancytokeratin (*green*) or anti-vimentin (*red*). Nuclei were stained with Hoechst-33342 (*blue*) (bar = 20 µm). Data are derived from triplicate experiments performed on each organ obtained from three different animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(10-fold increase), whereas that by bBEC and bLEC was detected at 3 hpi (an 18- and a 26-fold increase, respectively). TNF expression differed between the three cell types, bTEC and bLEC was detected the

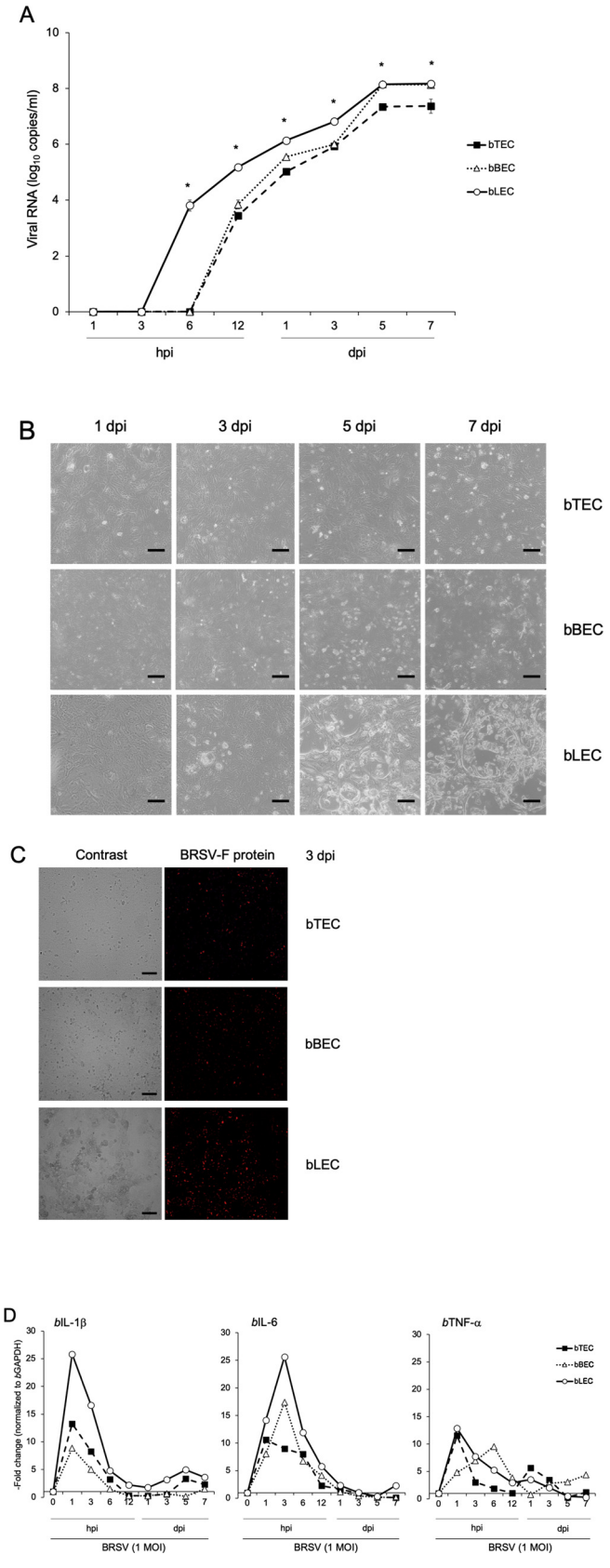

Fig. 2. Bovine respiratory syncytial virus (BRSV) infection of bovine respiratory epithelial cells (BRECs). BRECs were infected with BRSV at a multiplicity of infection = 1. Culture medium and cell lysates were collected at different hours or days post-infection (hpi or dpi, respectively). **(A)** BRSV RNA isolated from culture medium was analyzed by qRT-PCR of a N-protein target gene. The graph shows the mean viral copy number (± SEM); n = 3; * $p <$ 0.05 as BRSV-infected BRECs origin-dependent). **(B)** Syncytia-induced cytopathic effects in epithelial cells from the lung (bLEC) and bronchus (bBEC), but not from the trachea (bTEC) (bar = 100 μm). **(C)** The results confirm expression of BRSV (*red*) at 3 dpi by BRECs of origin-dependent (bar = 150 μm). **(D)** Time-dependent expression of mRNA encoding bovine (*b*)IL-1β, *b*IL-6, and *b*TNF-α by BRSV-infected BRECs. Data represent the mean (± SEM) total mRNA expression, normalized to that of *b*GAPDH; n = 6. Data are derived from triplicate experiments performed on each organ derived from three different animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peak at 1 hpi (an 11- and a 12-fold increase, respectively), whereas bBEC was detected an 9-fold increase at 6 hpi.

3.3. BRSV-infected BRECs influences response of PM adherences

Colony forming unit (CFU)-based adherence assays revealed differences between BRSV-infected and uninfected BRECs with respect to the number of adhered *PM* (Fig. 3A). The number of bacterial cells adhered to BRSV-infected bLEC at 1, 2, and 3 dpi was significantly higher than that adhered to uninfected cells (51.1, 61.1, and 74.3 *vs.* 35.3, respectively, $p < 0.05$). Similarly, the number of *PM* adhered to BRSV-infected bBEC at 3 dpi was significantly higher than that adhered to uninfected cells (60.4 *vs.* 32.1, respectively, $p < 0.05$). By contrast, the number of bacteria adhering to BRSV-infected bTEC at 2 and 3 dpi was significantly lower than that adhered to uninfected cells (37.0 and 31.3 *vs.* 46.6, respectively, $p < 0.05$). Interestingly, *PM* adherence to uninfected bTEC was significantly higher than that to uninfected bBEC and bLEC. These results indicate that BRSV-infected BRECs show site of origin-dependent susceptibility to *PM*.

Finally, we measured expression of mRNA encoding IL-1β, IL-6, and TNF-α by BRECs co-infected with BRSV and *PM* (Fig. 3B). Co-infection of bLEC upregulated expression of IL-1β, IL-6, and TNF-α mRNA ($p <$ 0.05). Furthermore, co-infection of all BRECs with BRSV plus *PM* led to marked upregulation of IL-6 mRNA expression; this was not observed after infection of cells with *PM* alone. BRSV infection of bTEC and bBEC suppressed *PM*-induced expression of IL-1β and TNF-α mRNA.

4. Discussion

Here, we generated BREC cultures derived from the trachea, bronchus, and lung of Japanese black cattle. We then used these BRECs to demonstrate that cells lining different sites within the respiratory tract are not equally susceptibility to infection by BRSV. The results suggest that BRSV infects and propagates more easily in cells lining the lower respiratory tract, which increases adherence of *PM*. Thus, we have identified a potential mechanism underlying severe pneumonia associated with BRDC.

Primary cultures of BRECs from adult Japanese black cattle contained a mixture of epithelial-like and fibroblast-like cells. However, serial passage using a differential trypsinization and plating technique led to BREC cultures of 95% purity after the fourth passages (confirmed by IFA with a pancytokeratin antibody). These cells were passaged successfully for 19 generations, with high viability and maintenance of the epithelial structure. To the best of our knowledge, this represents the first long-term subculture of a cell line derived from the respiratory organs of adult cattle. Although other groups succeeded in establishing primary bovine epithelial cells from intestinal (Kaushik et al., 2008), tracheal (Mitchell et al., 2007), and bronchial (Goris et al., 2009; Cozens et al., 2018) tissues, the subcultured cells did not survive long-

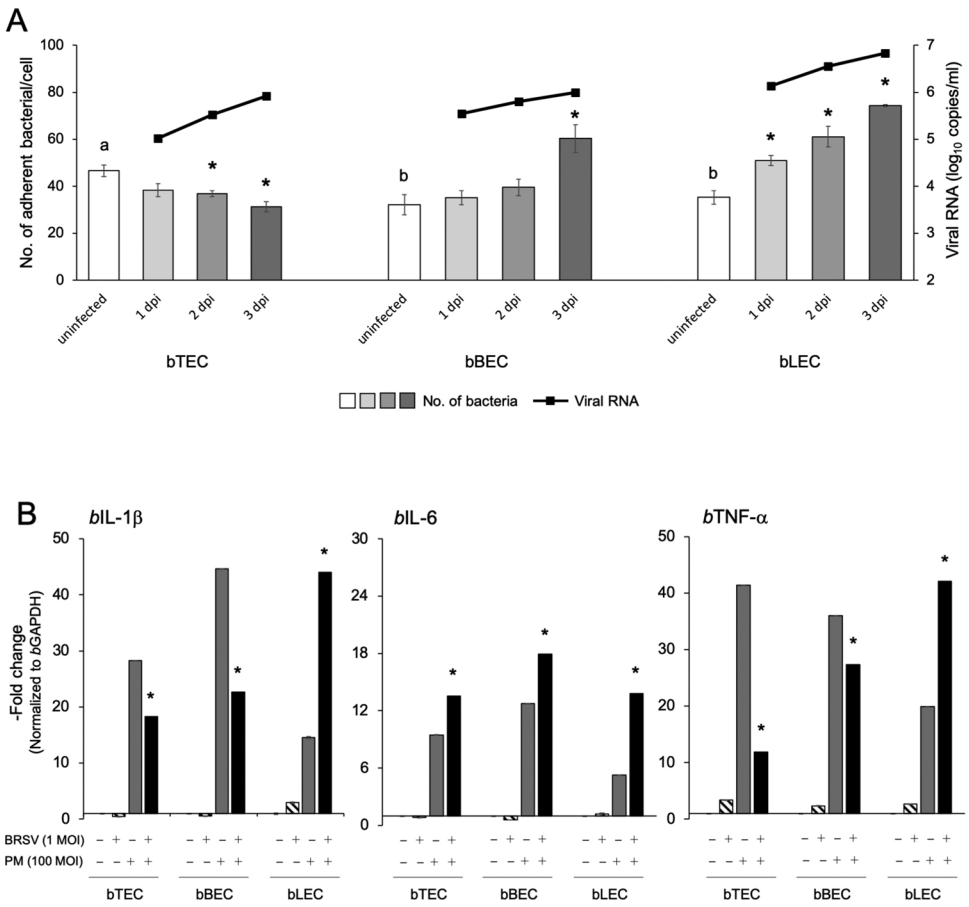

Fig. 3. Bovine respiratory epithelial cells (BRECs) were co-infected with bovine respiratory syncytial virus (BRSV) and *Pasteurella multocida* (*PM*). BRECs pre-infected for 3 days with BRSV at a multiplicity of infection (MOI) = 1 were exposed to *PM* (MOI = 100) for 1 h. **(A)** Expression of BRSV was measured by quantitative RT-PCR (black *line*), and the number of *PM* adhering to BRECs (*bar*) on Brucella agar was counted. Data are expressed as the mean (± SD); n = 3; * $p < 0.05$ as *PM* adherent from BRSV-infected to uninfected cell; $^{a, b}$ $p < 0.05$, *PM* adherent to BREC-uninfected cells. **(B)** Expression of bovine (*b*)IL-1β, *b*IL-6, and *b*TNF-α mRNA by BRECs infected with BRSV alone (MOI = 1) at 3 dpi, *PM* (100 MOI) alone at 1 hpi, or BRSV 3 dpi plus *PM* 1 hpi. Data are expressed as the mean (± SEM) total mRNA expression, normalized to that of *b*GAPDH; n = 3; * $p < 0.05$ (co-infection with BRSV and *PM versus* single infection with *PM*). Experiments were performed in triplicate on each organ derived from three different animals.

term passage.

BRECs are the primary target of BRDC-related pathogens and play an important role in initiating immune responses. Here, we found that BRECs from different respiratory tissues showed differences in susceptibility to infection by BRSV. Replication of BRSV in bBEC and bLEC (representative of the lower respiratory tract) was significantly higher than that in bTEC (upper respiratory tract). This suggests that BRSV infection may cause more severe damage to cells lining the lower respiratory tract. These findings support those in BRSV-infected calves (Jordan et al., 2015). Furthermore, virus shedding in cattle is highest in the bronchus and alveolae and is related to virus entry (Viuff et al., 2002; Tjønehøj et al., 2003; Jordan et al., 2015; Taylor, 2017); fewer virus antigens are found in the trachea (Blodörn et al., 2015). Syncytia formation is an early CPE; we observed this phenomenon in bLEC and bBEC but not in bTEC (Fig. 2B). Differences in CPE due to differences in replication of BRSV were again site of origin-specific and correlated with expression of inflammatory cytokines (IL-1β and IL-6). BRSV-related pneumonia is characterized by syncytium formation and epithelial hyperplasia, resulting in plugging of bronchioles and alveoli (Tjønehøj et al., 2003). The differences lesion of BRSV-infected tissue were indicated target cell of infection (Larsen et al., 2001; Tjønehøj et al., 2003; Kalina et al., 2006; Blodörn et al., 2015; Jordan et al., 2015). Established of BRSV infected with BRECs in our study have been reproduce BRSV infection in respiratory.

Co-infection of the bovine respiratory tract by multiple viruses or by viruses and bacteria causes more severe pneumonia (Al-Haddawi et al., 2007; Agnes et al., 2013; N'jai et al., 2013; Kirchhoff et al., 2014; McGill et al., 2016; Sudaryatma et al., 2018). Previously, we showed that BRSV increases adhesion of *PM* to human respiratory cells (Sudaryatma et al., 2018). This phenomenon was observed in BRSV-infected bLEC and bBEC, but not in bTEC. Adherence of *PM* to BRSV-infected bBEC and bLEC was significantly higher than that to bTEC. However, adherence of *PM* to uninfected bTEC was significantly higher than that to uninfected bBEC and bLEC. The bTEC plays as a "gateway" of respiratory tract to protective role against the bacterial respiratory pathogens. Dysfunction of *PM* binding activity of bTEC by BRSV infection might permit to access bacteria for infection in the lower respiratory tract. Regardless of the underlying mechanism, dysregulation of the upper airway by BRSV infection might induce severe lower respiratory disease upon co-infection with bacteria such as *PM*.

In conclusion, we established epithelial cell lines from different parts of respiratory tract of adult Japanese black cattle and found differences in susceptibility to BRDC-related pathogens. Infection of lower respiratory epithelial cells with BRSV increased adherence of *PM*, thereby triggering an immune response. In addition, *PM* adherence to BRSV-infected and uninfected upper epithelial airway cells might contribute to the more severe lower respiratory tract symptoms in BRDC. Taken together, the data shed new light upon the mechanism by which BRDC-related infection of BRECs induces severe pneumonia and may help us to develop new therapeutic strategies for BRDC.

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