Klebsiella pneumoniae isolated from bovine mastitis is cytopathogenic for bovine mammary epithelial cells

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ABSTRACT

Klebsiella pneumoniae, a common cause of clinical mastitis (CM) in dairy cows, can cause severe clinical symptoms. However, its pathogenicity in the bovine mammary gland is not well understood. Our objectives were to establish an in vitro infection model of *K. pneumoniae* on bovine mammary epithelial cells (bMEC) to assess (1) cytopathogenicity (adhesive and invasive ability, damage and apoptosis, pro-inflammatory effects) of *K. pneumoniae* on bMEC and (2) the role of hypermucoviscous (HMV) phenotype on cytopathogenicity. Two *K. pneumoniae* isolates from CM cows, 1 HMV and 1 non-HMV, were used to infect bMEC. Adhesion and invasion ability, release of lactate dehydrogenase (LDH), ultrastructural morphology, apoptosis, transcriptional expression of pro-inflammatory genes and production of pro-inflammatory cytokines were characterized at various intervals. Both *K. pneumoniae* isolates rapidly adhered to and invaded bMEC within 1 h post infection (pi), causing ultrastructural damage (swelling of mitochondria and vesicle formation on cell surface) after 3 h pi and apoptotic death after 9 h pi. In addition, *K. pneumoniae* promoted transcriptional expression of pro-inflammatory genes IL-6, IL-8, IL-1 β , and tumor necrosis factor (TNF)- α and production of IL-8, IL-1 β , and TNF- α cytokines. Compared with non-HMV *K. pneumoniae*, the HMV isolate had lower adhesive and invasive abilities but caused more serious cellular damage. In conclusion, *K. pneumoniae* was cytopathogenic on bMEC and induced a pro-inflammatory response; however, the HMV phenotype did not have a key role in pathogenicity. Therefore, more attention should be

paid to milk loss, and targeted prevention and treatment strategies should be implemented in *Klebsiella* mastitis episodes.

Key words: bovine mastitis, *Klebsiella pneumoniae*, bovine mammary epithelial cells (bMEC), apoptosis, inflammation

INTRODUCTION

Bovine clinical mastitis (CM) is a common and costly disease in the dairy industry worldwide, which can be caused by many pathogens, with *Klebsiella pneumoniae* considered a major pathogen (Roberson et al., 2004; Olde Riekerink et al., 2008; Oliveira et al., 2013). *Klebsiella* CM is often severe and does not respond well to antimicrobial treatment, resulting in prolonged duration of intramammary infection and substantial loss of milk yield (Schukken et al., 2012). Virulence factors may be a crucial reason for severity of intramammary infection with *K. pneumoniae*. Capsular serotypes, iron-scavenging systems, and fimbriae have widely been reported to be involved in virulence of *K. pneumoniae* from human origin (Holt et al., 2015). Recently, a new hypervirulent *K. pneumoniae* with a hypermucoviscous (HMV) phenotype has emerged and attracted worldwide attention (Shon et al., 2013). *Klebsiella pneumoniae*, when hypermucoid, can cause invasive disease in humans and in several animal species (Twenhafel et al., 2008; Jang et al., 2010; Holt et al., 2015; Davies et al., 2016). However, whether HMV *K. pneumoniae* can cause CM with severe clinical symptoms in cattle has apparently not been reported.

Bovine mammary epithelial cells (bMEC) are the udder's functional, milk-producing cells. After pathogenic infection, bMEC can be damaged and undergo apoptosis, decreasing milk production. The ability of an intracellular pathogen to adhere to and invade bMEC could confer protection from the immune system and from antibacterial agents, predisposing to development

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of a chronic infection (Almeida et al., 2011). In addition, because bMEC are front-line cells, they may act as sentinels by producing mediators of inflammation and local defenses against pathogens (Gilbert et al., 2013).

Distribution of virulence factors implicated *rmpA*, *kfu*, *uge*, *magA*, *aerobactin*, and K1 and K2 in pathogenicity of *K. pneumoniae* isolated from bovine mastitis (Osman et al., 2014). Distribution of potential virulence factors in *K. pneumoniae* from cows with CM on Chinese dairy farms has also been determined (Gao et al., 2019). Therefore, it is very important to establish a bacterial infection model with bMEC to facilitate studying virulence of *K. pneumoniae*. Objectives of the present study were (1) to determine cytopathogenicity (adhesive and invasive ability, damage and apoptosis, and pro-inflammatory effect) of *K. pneumoniae* on bMEC, and (2) to explore whether HMV phenotype increases cytopathogenicity of *K. pneumoniae* on bMEC. Results of the present study would be useful to determine whether more effort should be given to minimizing milk loss during and after episodes of mastitis caused by *K. pneumoniae*, especially concerning HMV isolates.

MATERIALS AND METHODS

Statement of Ethics

Approval was granted by the Ethical Committee of the College of Veterinary Medicine, China Agricultural University (CAU), Beijing, and the study was conducted according to the standard ethical guidelines of CAU. Milk samples from cows with CM were collected according to National Mastitis Council (NMC, 2017) standard procedures, with appropriate consultation with farm owners and management.

Klebsiella pneumoniae Isolates

Cases of CM were detected at milking and confirmed by herd veterinarians, who collected milk samples aseptically from quarters with visible signs of CM (abnormal milk, swelling) before antibiotic treatment, following standardized protocols (NMC, 2017). Milk samples were initially stored in a freezer (approximately -20°C) and then placed on ice and sent to the Mastitis Diagnostic Laboratory, China Agriculture University (MDL-CAU; Beijing, P.R. China) for culture. A total of 124 CM isolates obtained from March 2014 to May 2017 were preliminarily identified as *K. pneumoniae*, as previously described (Gao et al., 2017), including colony characteristics on MacConkey agar, Gram staining, motility, indole, ornithine reactions, oxidase, and growth on

triple-sugar iron slants. Presumptive *K. pneumoniae* isolates were further confirmed using partial 16S rDNA sequencing (Barry et al., 1990). Each *K. pneumoniae* isolate was isolated from 1 milk sample collected from a single CM case, with 124 isolates from 124 CM quarter milk samples. Capsule serotypes (K1, K2, K5, K54, and K57) and existence of several virulence genes in *K. pneumoniae* isolates were confirmed using PCR (Gao et al., 2019). Isolates were classified as HMV or non-HMV phenotype via string test: a bacterial loop was used to stretch a “string” of mucus from the bacterial colony, with formation of a viscous string >5 mm defined as a positive test (Fang et al., 2004). Finally, 2 *K. pneumoniae* isolates (HB-AF5 and HLJ-D2) were used in the present study. Both HB-AF5 and HLJ-D2 belonged to K57 capsule serotype and had *entB*, *ybtS*, *iutA*, and *mrkD* genes, although the HLJ-D2 isolate had an extra *kfu* gene (Gao et al., 2019). HLJ-D2 was an HMV isolate, whereas HB-AF5 was a non-HMV isolate.

Bovine Mammary Epithelial Cells

We grew bMEC MAC-T (Shanghai Jingma Biological Technology Co. Ltd., Shanghai, China) to confluence in culture dishes in Dulbecco's modified Eagle's medium (DMEM) with high glucose (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 100 IU/mL penicillin (HyClone), and 100 $\mu\text{g/mL}$ streptomycin (HyClone) at 37°C incubator with 5% CO_2 on cell culture plates (Corning Inc., Corning, NY). At 70 to 80% confluence, cells were detached and plated in 6-well or 96-well plates. Subsequently, cells were challenged with HMV or non-HMV *K. pneumoniae* when they reached 80 to 90% confluence, whereas control cells were cultured only with culture medium. For detection of adhesion and invasion, ultrastructural morphology, apoptosis, and pro-inflammatory gene expression of bMEC, cells were cultured in 6-well plates. Three wells were used to challenge with HMV or non-HMV *K. pneumoniae* in 1 specific time point, whereas the other 3 wells were used as control. For lactate dehydrogenase (LDH) detection, cells were cultured in 96-well plates. Five wells of cells were used to challenge with HMV or non-HMV *K. pneumoniae* in 1 specific time point, whereas 5 wells of cells were used as control. All challenges were repeated 3 times.

Adhesion and Invasion

First, quantities of bMEC per well were calculated using Trypan Blue staining, whereas counts (cfu) were performed using a WGS-2XJ bacterial turbidimeter

(Shanghai Xinrui Instrument Co. Ltd., Shanghai, China). Subsequently, *K. pneumoniae* were diluted using DMEM (Hyclone) for adhesion and invasion detection according to multiplicity of infection (MOI) of 50:1 ratio of *K. pneumoniae* to bMEC. In 6-well plates bMEC were incubated with *K. pneumoniae* (2 mL/well) at 0.5, 1, 1.5, 2, or 3 h at 37°C with 5% CO₂. Additionally, in our preliminary experiments, we tested kanamycin (Sigma-Aldrich; St. Louis, MO) at 25, 50, 100, and 150 µg/mL for 1, 2, and 3 h; we found that 50 µg/mL kanamycin killed *K. pneumoniae* (1×10^7 cfu/mL) within 2 h. Therefore, 50 µg/mL kanamycin for 2 h was used for the invasion assay of *K. pneumoniae*.

Adhesion of *K. pneumoniae* to bMEC was assessed as previously described (Pereyra et al., 2016), with slight modifications; bMEC were cultured in 6-well plates (Corning Inc.). After incubation, samples were washed 3 times using sterile PBS and collected with 0.5 mL of PBS (Solarbio, Beijing, China; pH 7.2; Chen et al., 2017). Cell suspension was 10-fold serially diluted and cultured on trypticase soy agar (TSA) plate (37°C, 20 h) for counting colony-forming units.

Invasion of bMEC was determined as described previously (Chen et al., 2017), with minor modifications. We inoculated bMEC with bacteria at an MOI ratio of 50:1 for up to 3 h, washed them with PBS, and treated them with kanamycin (50 µg/mL; 2 h) to kill extracellular *K. pneumoniae*. Cell lysates were then obtained with 1 mL of 5% TritonX-100 (Solarbio). In the control group, cells were also lysed with 1 mL of TritonX-100, and cell lysates were diluted 10-fold, cultured on TSA plate, and incubated at 37°C for 24 h to count colony-forming units (CFU).

Cytotoxic LDH Release Assay

Cytotoxic effects of *K. pneumoniae* on bMEC were assessed using an LDH assay kit (Beyotime Institute of Biotechnology, Beijing, China). Cells cultured at 37°C with 5% CO₂ in 96-well plates (Corning Inc.) were challenged with *K. pneumoniae* at MOI ratio of *K. pneumoniae* to cells of 5:1 for 3, 6, 9, and 12 h. Non-infected cells were similarly incubated as control. After incubation, 200 µL of supernatants from each well was collected and transferred to centrifuge tubes and spun ($8,000 \times g$, 5 min). Then, 120 µL of supernatant was sequentially transferred to a new 96-well polystyrene plate, with 60 µL of reaction mixture added to each well, followed by incubation on a rotary shaker (150 rpm) at room temperature in the dark for 30 min. Absorbance was read at 490 nm (680 Multipurpose Microplate Reader, Biorad, Hercules, CA).

Ultrastructural Morphology

Ultrastructural analysis of bMEC inoculated with *K. pneumoniae* was determined using scanning electron and transmission electron microscopy. After incubation with *K. pneumoniae*, bMEC were washed with PBS and fixed with 2.5% glutaraldehyde (Solarbio; 4°C, 90 min), then washed with PBS, and fixed cells were harvested with a rubber scraper (Thermo Fisher Scientific, Nepean, ON, Canada). Cells were then dehydrated in graded ethanol (from 30 to 100%), dried using the critical-point drying method, and gold-coated with E-1010 Ion Sputter Coater (Hitachi, Tokyo, Japan) as described by Shahid et al. (2017). Finally, the changes in bMEC were observed via scanning electron microscopy (TESCAN 5136, Brno, Czech Republic).

For transmission electron microscopy, bMEC were cultured and challenged as described above. After washing with PBS, cells were fixed with 2% glutaraldehyde-1% paraformaldehyde solution (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China; pH 7.2) for 45 min at room temperature. After washing with PBS, the fixed cells were harvested with a rubber scraper (Thermo Fisher Scientific). Then cells were dehydrated using graded ethanol and acetone (3 changes, 10 min each) and sequentially embedded in epoxy resin acetone mixtures (2:1) for 2 h and in pure resin overnight at 37°C. After resin had polymerized, ultra-thin sections were cut (Leica EM, Wetzlar, Germany), stained with 1% uranyl acetate followed by lead citrate, and viewed with a transmission electron microscope (Hitachi H-7650) at 80 kV. Imaging was performed using a 4k CCD camera (Gatan Inc., Pleasanton, CA) and iTEM software. Damaged mitochondria, desquamation, microvilli breakage, and disruption of cellular cytomembrane were checked to evaluate the damage of bMEC.

Apoptosis

The bMEC inoculated with *K. pneumoniae* were stained with FITC annexin V/propidium iodide (PI), and cell apoptosis was determined using an annexin V-FITC/PI apoptosis detection kit (Beyotime) according to the manufacturer's instructions. Briefly, bMEC were inoculated with *K. pneumoniae* (MOI 5:1) for up to 12 h at 37°C with 5% CO₂. Cells not inoculated with *K. pneumoniae* were used as a control group. After incubation, cells were washed 3 times, collected with cold (4°C) PBS, and centrifuged ($1,000 \times g$, 5 min at 4°C). Subsequently, cells were resuspended with 100 µL of binding buffer and stained with 5 µL of fluorescein isothiocyanate (FITC) annexin V conjugate and 5 µL

of PI (room temperature, 15 min). Binding buffer (400 μ L) was added to samples, and apoptosis was analyzed within 1 h using a BD FACS Calibur flow cytometer (Becton Dickinson, Bergen City, NJ).

Pro-Inflammatory Genes of bMEC

Samples were collected for RNA extraction from inoculated and control (non-infected) cells at 3, 6, 9, and 12 h post infection (pi). Briefly, bMEC were washed with PBS and harvested with 1 mL of Trizol to extract total RNA, according to manufacturer's protocol (TransGen Biotech Co. Ltd., Beijing, China). Total RNA yield and purity were determined by absorbance at 260 and 280 nm using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with a transcript first-strand cDNA synthesis kit (TransGen Biotech Co. Ltd.), whereas quantitative real-time PCR analysis was performed with a qPCR detection kit (SYBR Green) on an ABI StepOnePlus Real-Time PCR System with Tower (ABI, Redlands, CA). Primers for the house-keeping gene (*GAPDH*; Zbinden et al., 2015) and genes encoding tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, and IL-8 were obtained from published sequences (Beijing Sunbiotech Co. Ltd., Beijing, China; Zbinden et al., 2014, 2015) and presented in Table 1. The cycle threshold (Ct) values (Rotor-Gene software, Version 1.7, Corbett Life Science, Sydney, Australia) and $2^{-\Delta\Delta Ct}$ values were chosen to reflect relative mRNA expression, using *GAPDH* and *ACTB* as reference genes (Table 1).

Production of Pro-Inflammatory Cytokines

Concentrations of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-8 in cell supernatant were measured using an ELISA kit with a detection range of

0.1 to 20.0 ng/mL (Wuhan Huamei Bioengineering Co. Ltd., Wuhan, China). All procedures were performed following manufacturer's instructions. Data acquisition was performed using a Thermo K3 microplate reader (Thermo Fisher Scientific). Cytokines were quantitatively measured in culture supernatants from bMEC inoculated with HMV and non-HMV *K. pneumoniae* at MOI of 5 during 3, 6, 9, and 12 h.

Statistical Analyses

Analyses were performed using one-way ANOVA, followed by the Duncan and LSD multiple tests, using SPSS 20.0 (SPSS Inc., Chicago, IL). All values were expressed as mean $\pm$ standard error of the mean. All measurements were replicated 3 times. Differences were considered significant if $P < 0.05$.

RESULTS

Adhesion and Invasion

Both HMV and non-HMV *K. pneumoniae* quickly and similarly adhered to bMEC (from 0.5 to 1.5 h pi; Figure 1A). However, at 2 h pi, non-HMV *K. pneumoniae* adhered at higher quantities compared with the HMV isolate ($P < 0.01$). Both *K. pneumoniae* isolates rapidly invaded bMEC (1 h pi), followed by higher invasiveness for non-HMV *K. pneumoniae* (until 3 h pi; Figure 1B).

Ultrastructural Damage of bMEC

As assessed by scanning electron microscopy (Figure 2), both HMV and non-HMV *K. pneumoniae* induced cell shrinkage and damage, producing holes on bMEC surfaces (3 h pi) and vacuole formation with cell membrane breakage (6 h pi). At 9 h pi, both *K. pneumoniae*

Table 1. List of primers for real-time PCR¹

Gene	Primer	Sequence 5'-3'	Size (bp)	Annealing temperature (°C)
<i>GAPDH</i>	Forward	GTCTTCACTACCATGGAGAAGG	197	60
	Reverse	TCATGGATGACCTTGGCCAG		
<i>TNFA</i>	Forward	CCACGTTGTAGCCGACATC	155	60
	Reverse	CCCTGAAGAGGACCTGTGAG		
<i>IL6</i>	Forward	CTTCACAAGCGCCTTCACTC	132	60
	Reverse	GTCAGAAGTAGTCTGCCTGC		
<i>IL8</i>	Forward	AGTGCCTACGCACATGTCTTC	149	60
	Reverse	TGCGTCACACAGAACTCGTC		
<i>IL1B</i>	Forward	ATGACTTCCAAGCTGGCTGTTG	114	60
	Reverse	TTGATAAATTTGGGGTGGAAAG		

¹*TNFA* = tumor necrosis factor- α . Cycle threshold (Ct) values were determined and relative mRNA expression was calculated using the comparative Ct method, as described by Zbinden et al. (2015).

Figure 1. Adhesion and invasion of hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae* in bovine mammary epithelial cells. (A) Adhesion and (B) invasion of *K. pneumoniae* isolates (up to 3 h post infection). Same isolate but compared with previous time point: * = $P < 0.05$; ** = $P < 0.01$. HMV isolate compared with non-HMV isolate at the same time point: ## = $P < 0.01$. Data are presented as mean $\pm$ SEM of 3 independent experiments.

isolates caused desquamation and microvilli breakage to total disruption of bMEC cytomembranes (Figure 2). These morphological changes were more evident on bMEC infected with the HMV isolate (Figure 2).

Based on transmission electron microscopy, both isolates induced slight swelling, chromatin margination, and cristae degeneration of mitochondria (3 and 6 h pi), followed (9 h pi) by cytomembrane disruption and severe cytoplasm vacuolation with swollen endoplasmic reticulum (Figure 3). The non-HMV isolate induced irregularities of nuclear membranes, whereas the HMV isolate induced severe mitochondria swelling and disruption and cribriform appearance in cytoplasm. The HMV isolate was highly invasive at 12 h, with cell rupture and loss of organelles (Figure 3). Uninfected bMEC lacked morphological changes, with a rich microvilli on the surface and normal structures with intact cytoplasm and organelles (Figure 3).

Cytotoxicity and Apoptosis

At 3 and 6 h pi, we found no difference between HMV and non-HMV isolates for LDH release (Figure 4A). Both isolates induced cytotoxic effects on bMEC at 9 and 12 h pi ($P < 0.01$) as determined by LDH release (Figure 4A).

Similarly, compared with a negative control group, the proportion of apoptotic bMEC in the HMV or non-HMV groups did not notably increase at 3 and 6 h pi, whereas this was different in bMEC incubated with HMV or non-HMV *K. pneumoniae* at 9 and 12 h pi ($P < 0.01$), as determined by annexin V-FITC and PI (Figures 4B and C).

Gene Expression and Production of Pro-Inflammatory Cytokines

For transcriptional mRNA of gene coding, both isolates induced release of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-8 in bMEC at 6 and 9 h pi ($P < 0.01$; Figure 5). In addition, the HMV isolate provoked a more pronounced pro-inflammatory response at 6 and 9 h pi ($P < 0.01$; Figure 5).

Regarding cytokine production, secreted IL-6, IL-8, IL-1 β , and TNF- α were higher in infected bMEC compared with the non-infected group (Figure 6). Compared with controls (non-infected group), the HMV isolate induced higher ($P < 0.01$) production of IL-6 at 12 h pi; IL-8 at 3, 6, and 9 h pi; and TNF- α at 6, 9, and 12 h pi (Figure 6). Furthermore, compared with controls, the non-HMV isolate induced higher ($P < 0.01$) production of IL-8 at 3, 6, and 9 h pi; and IL-1 β at 9 and 12 h (Figure 6). Furthermore, IL-8 and TNF- α production were higher in bMEC exposed to the HMV than to the non-HMV isolate, which was different at 3 ($P < 0.01$) and 6 h pi ($P < 0.05$) for IL-8 (Figure 6B) and at 6 h pi for TNF- α ($P < 0.01$; Figure 6D). In contrast, IL-1 β production was higher in bMEC induced by the non-HMV isolate compared with the HMV isolate at 6 and 9 h pi (Figure 6C; $P < 0.01$).

DISCUSSION

During episodes of bovine CM, bMEC have crucial roles in conferring protection against microbial pathogens by undergoing cell damage and apoptosis and releasing inflammatory cytokines (Chen et al., 2017; Sha-

Figure 2. Use of scanning electron microscopy to assess structural features of bovine mammary epithelial cells (bMEC) after inoculation with hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae*. Yellow arrows indicate broken microvilli. (A) Control; (B) bMEC 3 h after inoculation with HMV; (C) non-HMV at 3 h post infection (pi); (D) HMV at 6 h pi; (E) non-HMV at 6 h pi; (F) HMV at 9 h pi; (G) non-HMV at 9 h pi; (H) HMV at 12 h pi; (I) non-HMV at 12 h pi.

Figure 3. Use of transmission electron microscopy to assess structural features of bovine mammary epithelial cells (bMEC) after inoculation with hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae*. Yellow arrow indicates damage on cell surface; red arrows indicate bMEC that have invaded bMEC.

Annexin V

Figure 4. Cytopathic effects of hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae* in bovine mammary epithelial cells (bMEC). (A) Effects of *K. pneumoniae* isolates on lactate dehydrogenase activity of bMEC. (B) Percentage of apoptotic cells stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI; up to 12 h post infection). Data are presented as mean $\pm$ SEM of 3 independent experiments. Compared with control group: * $P < 0.05$, ** $P < 0.01$. HMV isolate compared with non-HMV isolate at the same time point: # $P < 0.05$, ## $P < 0.01$.

hid et al., 2017). Although virulence of *K. pneumoniae* isolates from cattle has been studied (Bannerman et al., 2004; Kanevsky-Mullarky et al., 2014; Osman et al., 2014), cellular pathogenicity of *K. pneumoniae* in bovine mastitis is not known. In the present study, *K. pneumoniae* isolated from CM adhered rapidly and invaded bMEC. Adherence of pathogens to bMEC is the initial stage of pathogenicity, whereas subsequent invasion into host cells protects bacteria from antimicrobial treatment (Chen et al., 2017). Rapid adherence of *K. pneumoniae* may facilitate intracellular invasion and contribute to development of chronic infections. In addition to high antimicrobial resistance of bovine *K. pneumoniae* in vitro (Cheng et al., 2019a), we speculated that capability to adhere and invade would increase *K. pneumoniae* resistance within the bovine udder. Compared with HMV *K. pneumoniae*, the non-HMV isolate had more pronounced adhesive and invasive abilities,

suggesting that the latter had greater potential to damage mammary tissues. Regardless, our results did not implicate HMV phenotype as a key factor to increase the invasion ability of *K. pneumoniae* in bovine CM.

In this study, internalization of *K. pneumoniae* quickly caused obvious cellular damage (after 3 h pi), including membrane disruptions, fragmentation of nuclear membranes, chromatin condensation, swollen mitochondria, and even cellular collapse. Combined with subsequent cell apoptosis (6 and 9 h pi), we confirmed that *K. pneumoniae* was highly cytotoxic in mastitis, consistent with infections in humans (Shon et al., 2013; Cubero et al., 2016). Compared with non-HMV *K. pneumoniae*, the HMV isolate induced more severe damage in bMEC, implying greater cytotoxicity. In the first week after *Klebsiella* mastitis, cows have a rapid decline in milk production that lasts for the remainder of the lactation (Gröhn et al., 2004). Based on present results,

Figure 5. Relative mRNA expression of IL-6, IL-8, IL-1 β , and tumor necrosis factor- α (TNF- α) in bovine mammary epithelial cells after inoculation with hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae*. Data are presented as mean $\pm$ SEM of 3 independent experiments. Compared with control group: * = $P < 0.05$, ** = $P < 0.01$. HMV isolate compared with strain non-HMV at the same time point: # = $P < 0.05$, ## = $P < 0.01$.

we hypothesized that cellular damage and apoptosis induced during *Klebsiella* CM causes substantial loss of mammary epithelial cells, substantially decreasing milk yield, even after apparent recovery from CM. Therefore, non-steroidal anti-inflammatory drugs (e.g., meloxicam) are recommended for treating CM caused by *Klebsiella*, to reduce inflammation and cellular damage (van Soest et al., 2018).

Previous studies have shown that bMEC could exhibit a pro-inflammatory cytokine response (TNF- α , IL-1 β , IL-6, and IL-8) that can regulate the host cells' damage (Fitzgerald et al., 2007; Günther et al., 2010; Fujisawa et al., 2019). In the present study, we found elevated mRNA transcriptions of TNF- α , IL-1 β , IL-6, and IL-8 genes in cells inoculated with both *K. pneumoniae* isolates, as well as production of more TNF- α ,

IL-1 β , and IL-8 in either HMV or non-HMV isolate, which implied that these are main epithelial effectors against *K. pneumoniae*. Bovine MEC have an important role in protecting the mammary gland from severe inflammation caused by invading pathogens (Wellnitz et al., 2006). Pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 are main immunomodulators produced by bMEC during the early stage of infection and are responsible for endotoxic shock in acute mastitis (Breyne et al., 2018), which could initiate the immune response and activate leukocytes in the udder after stimulation by molecules released from microorganisms (Cheng et al., 2019b).

A previous study found increased concentrations of TNF- α , IL-1 β , and IL-8 in mammary glands infected with *K. pneumoniae* (Bannerman et al., 2004), consis-

Figure 6. Protein expression of IL-6, IL-8, IL-1 β , and tumor necrosis factor- α (TNF- α) in bovine mammary epithelial cell supernatant after inoculation with hypermucoviscous (HMV) or non-HMV *Klebsiella pneumoniae* isolates. Data are presented as mean $\pm$ SEM of 3 independent experiments. Compared with control group: * = $P < 0.05$, ** = $P < 0.01$. HMV compared with non-HMV isolate at the same time point: # = $P < 0.05$, ## = $P < 0.01$.

tent with our results in the bMEC infection model. Tumor necrosis factor- α and IL-1 β are potent inducers of fever and acute-phase protein synthesis. In this study, TNF- α and IL-1 β mRNA and protein levels increased earlier (<9 h) than reported in the previous study (>12 h; Bannerman et al., 2004); therefore, we inferred that bMEC may contribute to initial immune defense responses. Interleukin-8 is a chemotactic agent that facilitates neutrophil recruitment to the site of infection to kill invading pathogens (Leitner et al., 2000). An immediate increase in IL-8 (<6 h) released by bMEC after *K. pneumoniae* challenge implied rapid recruitment of neutrophils. Additionally, we found increased production of IL-6, IL-8, and TNF- α in bMEC exposed to the HMV isolate, implying a more profound pro-inflammatory response.

Our intention was to select 2 isolates with similar virulence profiles, as determined in our previous study (Gao et al., 2019), to characterize differences in HMV phenotype and its role in pathogenicity of bovine *K. pneumoniae*. However, the importance of the HMV phenotype was not clearly elucidated. The major limitation was that only 1 isolate for each phenotype (HMV-positive and HMV-negative) was compared. Although we used 2 isolates that were similar for several important virulence factors, other virulence factors that were not detected in the previous study (Gao et al., 2019) may have differed between the 2 isolates and perhaps contributed to differences in pathogenicity of bovine *K. pneumoniae* on bMEC. Therefore, we must emphasize that this was a preliminary exploration of the role of the HMV phenotype on pathogenicity of bovine *K. pneumoniae*. In a further study, whole-genome sequencing of *K. pneumoniae* isolates should be performed, to compare these 2 isolates for other virulence factors. In addition, as a next step, in vivo infection models (mice and cows) should be developed to better characterize virulence.

CONCLUSIONS

Klebsiella pneumoniae isolated from bovine CM adhered to and invaded bMEC, inducing damage and causing apoptosis. In addition, *K. pneumoniae* increased transcriptional expression of *IL6*, *IL8*, *IL1B*, and *TNFA* genes and production of TNF- α , IL-1 β , and IL-8 in inoculated bMEC. The HMV *K. pneumoniae* isolate had lower adhesive and invasive ability compared with a non-HMV isolate, but induced more serious damage to bMEC. Therefore, *K. pneumoniae* was cytopathogenic on bMEC and induced pro-inflammatory responses. However, the HMV phenotype did not have a key role in pathogenicity of *K. pneumoniae* on bMEC. This study preliminarily explored the pathogenic mechanism

of *Klebsiella* mastitis at the cellular level and indicated that more attention should be paid to reduction of milk yield due to damage of bMEC.

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