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Antibacterial Activity of the Honey Bee Venom against Bacterial Mastitis Pathogens Infecting Dairy Cows

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The antibacterial activity of Korean honey bee venom (KBV) was examined against seven major bacterial mastitis pathogens, *Enterococcus faecium*, *Escherichia coli*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Streptococcus intermedius*, *Streptococcus oralis* and *Streptococcus uberis* isolated from infected mammary quarters of cows. Seven bacterial mastitis pathogens were studied for antibacterial activity of the KBV by disc diffusion assay, minimal inhibitory concentrations (MIC) and bacterial count in milk samples. The KBV showed activity against *Ent. faecium*, *E. coli*, MRSA, *Staph. aureus*, *Strep. intermedius*. The order of susceptibility of the bacteria against the KBV was *Staph. aureus* > MRSA > *E. coli* > *Strep. intermedius* > *Ent. faecium* > *Strep. oralis* > *Strep. uberis*. The MIC against *Staph. aureus*, MRSA and *E. coli* were stronger effect as compared with standard drug. The effect of the KBV (100 μg ml^{-1}) on the viability of *Ent. faecium*, *E. coli*, MRSA, *Staph. aureus*, *Strep. intermedius*, *Strep. oralis* and *Strep. uberis* in milk differed significantly with each other within 12 h incubation period. The results indicate that KBV has significant antibacterial effects against major bacterial mastitis bacteria, *Ent. faecium*, *E.coli*, MRSA, *Staph. aureus*, *Strep. intermedius*. Results of the study indicate the potential use of KBV as alternative to antibiotic therapy. Further investigations are needed though to confirm its efficacy and its effects on the animals.

Key words: Bee venom, *Apis melifera*, Bovine mastitis, Antibacterial activities, Minimal inhibitory concentrations

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Introduction

Bovine mastitis is the most serious dairy problem in terms of economic losses to the dairy industry (DeGraves and Fetrow, 1993). It can lead to increased production costs due to culling, medication, discarded milk, delayed genetic progress, and reduced milk yield and milk quality (Natzke, 1981). Bovine mastitis is classified into contagious mastitis and environmental mastitis. *Staphylococcus aureus* and *Streptococcus agalactiae* are the most contagious agents of bovine acute and chronic mastitis (Hebert *et al.*, 2000; Vasudevan *et al.*, 2003). Environmental mastitis is caused by *Escherichia coli*, *Streptococcus dysgalactiae* and *Streptococcus uberis* (Riffon *et al.*, 2001). In the control of mastitis, antibiotic therapy continues to play an important role (McDonald and Anderson, 1981; Craven *et al.*, 1986). Despite a variety of available antibiotics, treatment of contagious mastitis during lactation is still very low and difficult (Matthews *et al.*, 1994). Several therapeutic methods have been reported, such as antibiotics (Owens *et al.*, 2001; Pengov and Ceru 2003), cytokines (Shkreta *et al.*, 2004), vaccines (O'Brien *et al.*, 2001), immunoglobulin (Barrio *et al.*, 2003) and biologically active substrates (Kawai *et al.*, 2003) but there are factors that influenced their effectiveness. Bacterial strains resistant to antimicrobial agents used in approved intramam-

mary preparations are one of the important reasons for therapy failure. Also, the use of antibiotics to treat bovine mastitis has been the most common source of harmful drug residues in milk (Nair *et al.*, 2005). These serious implications from the use of antibacterial drugs triggered the exploration of alternative therapy against intramammary infections.

Honeybee (*Apis mellifera*, L) venom has been utilized as pain reliever and treatment of inflammatory diseases during the ancient times (Billingham *et al.*, 1973). It has been extensively studied and used as a meridian therapy among its clinical applications (Kwon *et al.*, 2001; Kim *et al.*, 2003). In Korea, apitherapy which uses live honeybee stings has elucidated therapeutic value for piglets, calves and dairy cows with respiratory diseases such as atrophic rhinitis, pleuropneumonia and Glasser's disease (Choi *et al.*, 2003). The bee venom treatment was reported to be as effective, if not more, as the known antibacterial drugs and imposes no side effects (Eiseman *et al.*, 1982; Akdis *et al.*, 1996; Kwon *et al.*, 2001). The study was carried out to evaluate the antibacterial activity of the Korean honey bee venom (KBV) against the bacterial mastitis pathogens isolated from infected mammary quarters of cows in Korea.

Materials and Methods

Collection and preparation of the bee venom

The European honeybees, *A. melifera* collected in Korea were the source of venom used in the study. Experimental colonies were maintained in the apiary of the National Institute of Agricultural Science and Technology (Suwon, Korea). The bee venom was collected by subjecting the bees to electric shock and the venom is collected using the bee venom collector. KBV was dissolved in distilled water and centrifuged at 12,000 cfg for 10 min to remove insoluble materials. It was then lyophilized using freeze dryer and stored in the refrigerator.

Isolation and preparation of bacterial cultures

Cows with reported systemic and local clinical symptoms of bovine mastitis were identified from dairy farms. Milk samples were collected from these mastitic cows for the study from May 2005 to June 2006 at Yangpyeong province in Korea. One hundred mammary quarters of 67 dairy cow herds were analyzed farmer-diagnosed mastitic quarter in this study. Somatic cell counts farmer-diagnosed mastitic quarter in mammary gland secretion were more than 900,000 cells ml^{-1} (Dehaas *et al.*, 2004). The bacterial strains were isolated from milk samples of 100 farmer-diagnosed mastitic quarters, and seven strains were isolated from 54 quarters. The identification of isolated bacteria was provided with National Veterinary Research & Quarantine Service.Each of *Enterococcus faecium*, *Escherichia coli*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus oralis* and *Strep intermedius* isolated from milk samples were used in the study. Bacteriological media used were purchased from Difco (Sparks, MD). Cultures were maintained on tryptic soy agar (TSA) slants at 4°C, and the purity of each culture was ensured by morphological characteristic on brain heart infusion agar (*Ent. faecium*), mannitol salt agar (MRSA, *Staph. aureus*), blood agar (Streptocicci) or sorbitol MacConkey agar (*E. coli*). For the preparation of inocula, each pathogen isolate was grown separately.

Screening for antibacterial activity

Screening for antimicrobial activity was carried out using the paper disc diffusion method with the following modifications (Lin *et al.*, 2003). The KBV (100 μg) was dissolved to sterilized distilled water and filtered using 0.2 mm syringe filter (Advantec, CA, USA). The bacterial inoculum per plate contained 1.0×10^6 to 3.0×10^8 CFU, which were spread onto the agar surface. Sterile paper discs (7 mm diameter) were placed onto the agar surface and 100 μg of the KBV added per discs in four replicates. The plates were incubated at 37°C for 24 h. The inhibition of bacterial growth was evaluated by measuring the diameter of the transparent zone ground in each disc.

Determination of minimum inhibitory concentrations (MIC)

The MIC were determined by broth dilution method to serve as quantitative data for the antimicrobial KBV (Wu and Hancock, 1999). The bacterial stains were grown in broth media to a mid-logarithmic phase at 1.0×10^6 to 3.0×10^8 CFU/mL. Two hundred microliter of a mid-logarithmic phase culture of bacteria was added to 10 μl of the KBV (range of final concentration; 1-200 μg) in 96 well plate. One well containing 200 μl of bacterial inoculates served as a bacterial control, while another well containing 200 μl of uninoculated broth media and 10 μl of sterilized distilled water were used as a negative control. Culture plates were incubated at 37°C for 24 h. The inhibition of bacterial growth was determined by ELISA reader (UV max, Molecular Devices, USA) measuring the absorbance at 560 nm. Results were expressed as MIC, the lowest concentration of the KBV that reduces growth by more than 90% of the strains.

Bacterial count in milk samples

Raw milk sample was autoclaved at 121°C and 103.4 kPa

Table 1. Preliminary screening of antibacterial activities of the KBV on seven bacterial mastitis pathogens isolated from dairy cows in Korea

Bacterial species	Inhibition zone in diameter (mm) *	Antibiotic standard †
Enterococcus faecium	15.1±1.93	14.7±0.89
Escherichia coli	17.4±0.77	14.2±0.44
Methicillin resistant *Staphylococcus aureus*	21.2±1.23	10.8±0.85
Staph. aureus	21.4±0.71	15.6±1.1
Streptococcus intermedius	15.4±0.98	13.5±1.09
Strep. oralis	10.0±0.7	12.8±0.5
Strep. uberis	8.7±0.81	10.5±0.83

*The values are presented as mean±SD (n=4) and represent a KBV inhibition zone in mm, including the 7 mm diameter of the disc. Sterile paper discs were placed onto the agar and 100 μg of the KBV.
†Penicillin was used as standard (20 μg/disc)

pressure for 15 minutes following the procedure done by Nair *et al.*(2005). Milk samples were prepared for each pathogen treatments and then inoculated separately with 100 μl of each pathogen to obtain an inoculation density of about 6.5 log cfu/mL. The KBV was dissolved to a final concentration of 100 μg from 10 ml of whole milk. Control samples were inoculated by bacterial culture in 10 ml of milk only without KBV. All the treated milk samples were incubated at 39°C for 24 h.

Surviving populations of each bacterial pathogens were further cultured by plating 0.1 portions of the milk samples directly or after serial dilutions on duplicate TSA plates containing 0.6% yeast extract (Nair *et al.*, 2005). When growth was observed in the broth, the culture was streaked on mannitol salt agar, blood agar or sorbitol MacConkey agar and observed for growth of typical colonies of each pathogen. Duplicate samples were assayed for each of the treatments, concentrations and specified sampling time. Counting of pathogens was done at 0 min, 12 h, and 24 h.

Statistical analysis

The results were statistically analysed by Duncan's t-tests (SAS enterprise guide 3.0) with repeated measures used to analyse factors influencing the size of the growth inhibition zone and MIC.

Results

The KBV treatment showed wide inhibition zone diameters against the seven bacterial mastitis pathogens screened as summarized in Table 1. The highest inhibitory zones of 21.4 mm was observed from *Staph. aureus*. It was followed by MRSA at 21.2 mm against the standard of 10.8. The KBV exhibited the maximum inhibitory zones with potency almost equal or up to that of standard drugs against *Ent. faecium, E. coli,* MRSA, *Staph. aureus* and *Strep. intermedius*. The most promising KBV was further evaluated for their MIC. The KBV exhibited the inhibitory effect against *Staph. aureus*, MRSA and *E. coli*. The highest inhibition expressed at MIC values of 15.5 μg/mL was recorded against *Staph. aureus* (Table 2). The MIC values against the *Ent. faecium, Strep. intermedius, Strep. oralis* and *Strep. uberis* were 25.4, 36.6, 75.4 and 85.4 μg/mL, respectively. The bacterial count for each of the seven pathogens was determined using the KBV with the most effective antibacterial activity in autoclaved milk. The KBV greatly reduced the bacterial counts of all pathogens compared to the control which has the highest line curve (Fig. 1). The effect of the KBV (100 μg/mL) on the viability of *Ent. faecium, E. coli,* MRSA, *Staph. aureus, Strep. intermedius, Strep. oralis* and *Strep. uberis* in milk differed significantly with each other. The KBV treatment was most effective against *Staph. aureus* with a reduced bacterial count of less than 1.0 log cfu/mL after 12 h of incubation. Similarly, the results indicate that the KBV was effective in inhibiting the growth of *Ent.*

Table 2. Minimum inhibitory concentrations (MIC) of the KBV on seven bacterial mastitis pathogens isolated from dairy cows in Korea

Bacterial species	MIC values (μg/mL)
Enterococcus faecium	25.4±0.26
Escherichia coli	19.2±0.42
Methicillin resistant *Staphylococcus aureus*	16.8±0.34
Staph. Aureus	15.5±0.63
Streptococcus intermedius	36.6±0.66
Strep. oralis	75.4±0.2
Strep. uberis	85.4±0.44

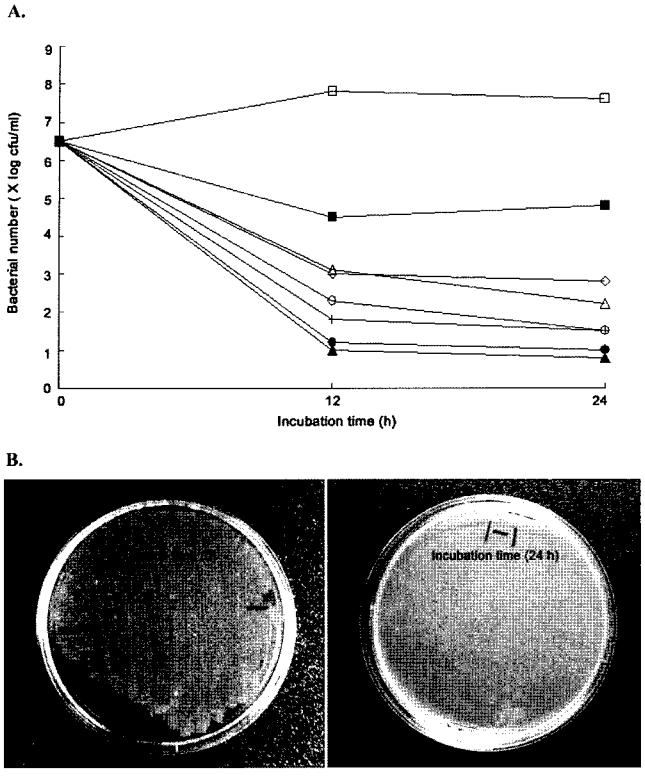

Fig. 1. A) Effect of KBV on seven mastitis pathogens in milk containing KBV (100 μg). Surviving bacterial populations (expressed in log cfu/mL) in milk samples stored at 39°C for 24 h were enumerated at specified time points. Legend: (○) *Enterococcus faecium*, (+) *Escherichia coli*, (●) Methicillin resistant *Staphylococcus aureus*, (▲) *Staphylococcus aureus*, (□) *Streptococcus intermedius*, (◇) *Streptococcus oralis*, (△) *Streptococcus uberis*, and (■) control.
B) Antibacterial activity of KBV against *Staphylococcus aureus* following 24 h incubation in milk containing KBV (100 μg).

faecium, E. coli and MR*SA* together with the other pathogens but at varying degrees. The inhibitory effect on the 3 streptococci species was similar in trend. The average initial population of the pathogens in all samples was approximately 6.5 log cfu μg/mL. The KBV reduced the populations of *Strep. intermedius, Strep. oralis* and *Strep. uberis* by approximately 3.0 log cfu μg/mL within 12 h incubation period. The control samples on the other hand, have an increased population of the tested pathogens at approximately 7.3 - 8.0 log cfu μg/mL after 24 h of incubation.

Discussion

The biochemical component and pharmacological effect of the bee venom have been reported by Kim *et al.*(2005). Honeybee venom was said to contain several peptides like melittin, apamin, adolapin, mast cell degranulating peptide, enzymes, biologically active amines, and non-peptide components (Lariviere and Melzack, 1996; Kwon *et al.*, 2002). Enzymes are also composed of phospholipase A2, hyaluronidase, acid phosphomonoesterase, a-D-glucosidase, and lysophospholipase (Banks and Shipoline, 1986; Somerfield *et al.*, 1984). The honeybee venom has been studied to determine its antibacterial and anti-inflammatory effects by Fennel *et al.*(1968), Somerfield *et al.*(1984), and Saini *et al.*(1997). Fennel *et al.* (1968) reported that the venom contains mellitin which is active against gram-positive more than gram-negative bacteria. In contrast, a stronger activity on *E. coli* had been reported previously for bee venom (Stocker and Traynor, 1986; Perumal Samy *et al.*, 2007). The study provides evidence that bee venom has antibacterial effect against both gram-positive and gram-negative bacteria isolated from milk of bovine mastitis. The six species of gram-positive bacteria *Ent. faecium*, MRSA, *Staph. aureus*, *Strep. intermedius*, *Strep. oralis*, *Strep. uberis* and one of gram-negative bacteria, *E. coli*, causing bovine mastitis were all inhibited by the KBV equivalent to that shown by standard antibiotic. There were also broader spectrum of antibacterial activity in all the tested bacteria. A strong activity was shown not only against *Ent. faecium*, MRSA, and *Staph. aureus* but also *E. coli*.

Further studies showed that the MIC of the KBV was very much higher against *Staph. aureus*, *Ent. faecium*, MRSA and *E. coli* at the lowest dilution of MIC 16 μg/mL. *Staph. aureus*, *Ent. faecium*, MRSA and *E. coli* were more sensitive to the KBV than *Strep. intermedius*, *Strep. oralis* and *Strep. uberis*. Moreover, the antibacterial effect of the KBV in autoclaved milk did not differ significantly. In general, The KBV resulted to a rapid decline in the populations of all the bacterial species within 12 h of treatment with *Staph. aureus* being the most sensitive to the KBV. Most mastitis-causing strains of *Staph. aureus* are surrounded by a layer of exo-polysaccharide or pseudo-capsule slime that interfere with antibacterial agents so that when lipids come in direct contact with the bacterial cell membranes the antibacterial activity happens (Sutra *et al.*, 1990; Pengov and Ceru, 2003). In comparison with the gram-negative mastitis pathogen, *E. coli*, the differences in the cell wall structure could have contributed to the comparatively high antibacterial activity of the KBV. Investigators have reported that antibacterial effect to gram-negative and gram-positive bacteria differs among antibacterial agents (Monk *et al.*, 1996). Although the exact mechanism of KBV's action on bacteria is not yet known, it was evident from the study that the KBV has indeed an antibacterial effects against both gram-negative and gram-positive bacteria.

The emergence of antibacterial resistant strains of animal pathogens and their potential health risk to humans through food-born transmission have captured the attention of the public and the scientific community (Pitkala *et al.*, 2004; Nair *et al.*, 2005). The extensive use of antibiotics enable many pathogens to acquire multiple drug-resistance genes that restrict treatment options (Owens *et al.*, 2001). The pressure to limit antibiotic use in food-producing animals is one of the biggest challenge for the dairy industry in spite of the fact that antibiotics remain the most effective tool to combat mastitis (Bradley, 2002). Therefore, there is a need for an effective alternative strategy for treating bovine mastitis that would reduce antibiotic us age (Komine *et al.*, 2006). Bee venom being a natural product that has been successfully used as antibacterial agent for a long time, it has the potential to be an alternative to antibiotic therapy.

The results of the study indicate that the KBV has a potential antibacterial effects against mastitis pathogens. In conclusion, tested the KBV at its lowest concentration was bactericidal against the major bovine mastitis pathogens *Ent. faecium*, MRSA, *Staph. aureus*, *Strep. intermedius*, *Strep. oralis*, *Strep. uberis* and *E. coli* found in milk. This study provides justification for the evaluation of the KBV as an alternative to antibiotics for the treatment of bovine mastitis. Future experiments are needed, however, to ascertain the in vivo efficacy of KBV as intramammary treatment of bovine mastitis and determine their potential effects on the mammary gland tissue.

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