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Research paper

Bactericidal activity of tracheal antimicrobial peptide against respiratory pathogens of cattle

Khaled Taha-Abdelaziz[a,b], José Perez-Casal[c], Courtney Schott[a], Jason Hsiao[a], Samuel Attah-Poku[b], Durđa Slavić[d], Jeff L. Caswell[a,*]

[a] Department of Pathobiology, University of Guelph, Guelph, ON, Canada
[b] Pathology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt
[c] Vaccine and Infectious Disease Organization-International Vaccine Centre, Saskatoon, SK, Canada
[d] Animal Health Laboratory, University of Guelph, Guelph, ON, Canada

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ABSTRACT

Tracheal antimicrobial peptide (TAP) is a β-defensin produced by mucosal epithelial cells of cattle. Although effective against several human pathogens, the activity of this bovine peptide against the bacterial pathogens that cause bovine respiratory disease have not been reported. This study compared the antibacterial effects of synthetic TAP against *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, and *Mycoplasma bovis*. Bactericidal activity against *M. bovis* was not detected. In contrast, the *Pasteurellaceae* bacteria showed similar levels of susceptibility to that of *Escherichia coli*, with 0.125 μg TAP inhibiting growth in a radial diffusion assay and minimum inhibitory concentrations of 1.56–6.25 μg/ml in a bactericidal assay. Significant differences among isolates were not observed. Sequencing of exon 2 of the TAP gene from 23 cattle revealed a prevalent non-synonymous single nucleotide polymorphism (SNP) A137G, encoding either serine or asparagine at residue 20 of the mature peptide. The functional effect of this SNP was tested against *M. haemolytica* using synthetic peptides. The bactericidal effect of the asparagine-containing peptide was consistently higher than the serine-containing peptide. Bactericidal activities were similar for an acapsular mutant of *M. haemolytica* compared to the wild type. These findings indicate that the *Pasteurellaceae* bacteria that cause bovine respiratory disease are susceptible to killing by bovine TAP and appear not to have evolved resistance, whereas *M. bovis* appears to be resistant. A non-synonymous SNP was identified in the coding region of the TAP gene, and the corresponding peptides vary in their bactericidal activity against *M. haemolytica*.

1. Introduction

Tracheal antimicrobial peptide (TAP), the first-discovered member of the β-defensin family, is a 38 amino acid cationic peptide produced by epithelial cells lining the respiratory tract and other mucosal surfaces (Diamond et al., 2000a,b; Bals, 2000). The antimicrobial activity of TAP is thought to result from disruption of bacterial membranes and later pore formation, which results from the electrostatic interaction of the positively charged peptide and negatively charged phospholipid of the bacterial membrane (Kagan et al., 1994; Hiemstra, 2001). Gene expression of TAP is upregulated following exposure to lipopolysaccharide and other inflammatory stimuli (Russell et al., 1996; Diamond et al., 2000a,b; Yang et al., 2011). However, we have previously shown that glucocorticoids and bovine viral diarrhea virus infection impair

* Corresponding author at: Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Tel.: +1 519 824 4120x54555; fax: +1 519 824 5930.
E-mail address: jcaswell@uoguelph.ca (J.L. Caswell).

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this inducible innate immune response (Al-Haddawi et al., 2007; Mitchell et al., 2007). These findings suggest that impaired TAP expression may be one mechanism by which stress or viral infection predisposes to pneumonia, and this may be a target for novel interventions to prevent the disease.

Although TAP has bactericidal activity against several human pathogens (Lawyer et al., 1996a,b), the effect of this bovine protein on bovine respiratory pathogens is unknown. Some pathogens are known to evade killing by antimicrobial peptides, by blocking the signaling pathways that upregulate their expression (Nizet, 2006), secreting proteins that cleave or trap antimicrobial proteins, or lessening their negative surface charge to reduce their affinity for cationic peptides (Kraus and Peschel, 2006). Thus, although TAP has been shown to kill human respiratory pathogens, bacteria that are adapted to cause pneumonia in cattle may have evolved mechanisms to resist TAP-mediated killing. Thus, the objective of this work was to determine if TAP kills the bacterial pathogens that cause respiratory disease in cattle. Further, we report a non-synonymous single nucleotide polymorphism (SNP) in the coding region of the TAP gene, and characterize its effect on microbicidal activity against *Mannheimia haemolytica*.

2. Methods

2.1. Preparation of TAP

TAP was synthesized as a linear polypeptide onto a resin, cleaved from the resin, purified by high performance liquid chromatography on a reverse phase column, and eluted with water/acetonitrile solvents. Three peptides were synthesized: 23G, with serine at position 20 and glycine at position 23; 20S with serine at residue 20 and glutamine at residue 23, and 20N with asparagine at residue 20 and glutamine at residue 23 (Table 1). Using MALDI-TOF, the mass spectral analysis confirmed the three synthesized peptides to be pure, with masses as expected: 23G – 4037.8 Da; 20S – 4091.1; 20N – 4118.1 Da. The protein concentrations of the samples were subsequently confirmed (2-D Quant Kit, GE Healthcare, Piscataway, NJ). The linear molecule was oxidized in solution to give the disulfide-bridged molecule, and a mass spectral analysis of the product indeed indicated a loss of 6 mass units confirming that 3 disulfide bonds have been formed during the oxidation. In preliminary experiments, the bactericidal activity of oxidized and non-oxidized peptides was found to have similar activities, so subsequent studies used the oxidized peptides.

Table 1
Amino acid sequences of the 3 synthesized peptides.

Peptide name	Amino acid sequence
23G	npvs cvrnkgicvp ircpgsmkg**g**i gtcvgravkc crkk
20S	npvs cvrnkgicvp ircpg**s**mkqi gtcvgravkc crkk
20N	npvs cvrnkgicvp ircpg**n**mkqi gtcvgravkc crkk

2.2. Assays of antimicrobial activity

The antimicrobial activity of synthetic TAP was measured against isolates of *M. haemolytica* ($n = 8$), *Histophilus somni* ($n = 3$), and *Pasteurella multocida* ($n = 3$) from the lung of cattle with pneumonia; in addition, 1 isolate of *Escherichia coli* from a calf with diarrhea was tested. The initial studies used synthetic peptide 23G. Subsequent studies compared the effect of the polymorphism described below, using peptides 20S and 20N.

Bacteriostatic activity was measured using an agar-based radial diffusion assay (Lehrer et al., 1991; Lawyer et al., 1996a,b). Bacterial isolates were grown in 50 ml tryptose soy broth for 18 h at 37 °C (in 5% CO_2 for *H. somni*), then 500 μl was re-inoculated in 50 ml of fresh broth and incubated for 3–4 h at 37 °C to reach mid-log phase. The bacterial suspension was centrifuged at 900 × *g* for 10 min at 4 °C, re-suspended in 10 ml of 10 mM sodium phosphate buffer (pH 7.4), and the concentration was estimated based on the optical density measured at 620 nm. 10^7 CFU bacteria were mixed with 30 ml of 1% agarose in 10 mM sodium phosphate buffer, 2 mg Tween20 and 30 mg tryptose soy powder. The agar was cooled in a plastic plate, then 3 mm diameter holes were punched in the agar plate, and 0.125–2.0 μg TAP or medium were added to each well. Plates were incubated at 37 °C for 1 h, then covered with 10 ml of melted 1% agarose with 10 mM sodium phosphate and 6 g tryptose soy powder, and incubated for 24 h at 37 °C. The diameters of the zones of inhibition were measured.

In order to measure bactericidal activity, bacteria were prepared as above, and 2×10^4 CFU were exposed to various concentrations of TAP (2-fold dilutions from 100 to 0.78 mg/ml, in sodium phosphate buffer, 50 μl final volume, in triplicate) for 2 h at 37 °C, then plated onto blood agar and incubated at 37 °C for 24 h. The numbers of surviving colony forming units were counted.

A similar technique was used to measure bactericidal activity against three isolates (A, B and C) of *Mycoplasma bovis*, obtained from feedlot cattle with caseonecrotic bronchopneumonia, with the following modifications. Bacteria were grown for 48 h at 37 °C in modified Hayflick's broth with 20% pig serum, centrifuged at 35,800 × *g* for 30 min at 4 °C, resuspended in ¼ strength Ringer's solution containing 18 g/L HEPES, pH 7.6, or sodium phosphate buffer containing 18 g/L HEPES, pH 7.6. The initial concentrations of bacteria were 10, 55 and 83×10^4 CFU/ml for the three isolates. Bacterial suspensions were incubated with 0, 3, 30 and 300 μg/ml TAP for 2 h, then surviving bacteria were enumerated by plating a 10-fold dilution series on modified Hayflick's agar with 20% pig serum for 48 h at 37 °C in 5.5% CO_2.

2.3. Analysis of a non-synonymous SNP

Tracheal samples were obtained at slaughter on 5 different days from 23 beef cattle of unknown breed, using samples from different lots of cattle to avoid genetic relatedness among donors. DNA was extracted (QIAamp DNA Minikit, Qiagen, Valencia, CA), and a 653 bp fragment of the TAP gene including part of the intron and the entire coding region of exon 2 was amplified using the primers (F)