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Testing cathelicidin susceptibility of bacterial mastitis isolates: Technical challenges and data output for clinical isolates

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ABSTRACT

Bovine mastitis caused by bacterial pathogens, such as *Staphylococcus* (*S.*) *aureus* and *Escherichia* (*E.*) *coli*, is a major economic problem in dairy industry. In order to limit the presence of multi-resistant bacteria in bovine mastitis, alternatives for the treatment with antibiotics are urgently needed. Antimicrobial peptides (AMPs) have recently been discussed as a potential new strategy against bacterial infections. They are key players in the innate immune system, as they can directly act against microorganisms or modulate the immune system. The aim of our study was to test *S. aureus* and *E. coli* mastitis isolates for their susceptibility to the bovine cathelicidins, BMAP-27 and BMAP-28.

Susceptibility testing was performed in analogy to the broth microdilution criteria described by the Clinical and Laboratory Standard Institute (CLSI) to determine MICs of 50 clinical *S. aureus* and 50 clinical *E. coli* isolates for BMAP-27 and BMAP-28. Based on the repetitive testing of four well-selected reference strains, the homogeneity of MIC variances for each peptide as well as the effect of temperature, oxygen level and plastic polymers on MIC testing was determined.

Statistical analysis revealed not only strong peptide-specific variances, but also strain-specific variances in the technical procedure. Finally, using this technique, susceptibility testing of the field isolates revealed statistically significant peptide-specific differences in the MICs. While BMAP-27 showed lower MICs for *E. coli*, BMAP-28 showed lower MICs for *S. aureus*. However, these results clearly illustrate the need of susceptibility testing of AMPs on several unrelated strains and not only on one selected test organism.

1. Introduction

Bovine mastitis is worldwide a leading problem in dairy industry which is often associated with severe suffering of diseased animals and high economic loss. The disease is caused by bacterial infections of the mammary glands – mainly caused by *S. aureus, E. coli* and/or various streptococcal species − and can have either a clinical or a subclinical appearance (Jadhav et al., 2013).

The problem of increasing bacterial resistance to antimicrobial agents with multi-resistant strains, e.g. livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) (Feßler et al., 2010) or ESBL-producing *E. coli* (Freitag et al., 2015), requires alternative treatment strategies (Michael et al., 2015) to prevent any further increase in levels of resistance in bovine mastitis pathogens. Antimicrobial peptides (AMPs) are recently discussed as alternatives to antibiotics (Da Costa et al., 2015). Those peptides are key players of the innate immune system and are expressed in innate immune cells like neutrophils, mast cells, but also T-cells, natural killer cells (NK) cells and epithelial cells. AMPs can directly kill bacteria after they are phagocytosed or come in contact with secreted AMPs during the degranulation process (De Smet and Contreras, 2005). They are small, cationic molecules that can bind to bacterial membranes according to the low cholesterol content (Sood et al., 2008), but also according to the negative charge of the bacterial membrane (Oren et al., 1999). The cell specificity is given by the fact

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that mammalian cells contain higher amounts of cholesterol compared to bacteria and render the eukaryotic host cell more resistant to membrane disruption by the cationic AMPs (Brender et al., 2012). In addition to their ability to exert direct antimicrobial activity over a broad spectrum of pathogens, several AMPs have the capacity to modulate the immune response to control infection and inflammation and are therefore also often named host defense peptides (reviewed by Pachón-Ibáñez et al., 2017).

There are two classes of mammalian AMPs: the defensins and the cathelicidins. Cathelicidins are 12–80 amino acids long and contain a conserved N-terminal sequence – the cathelin region – and a C-terminal domain that is necessary for the antimicrobial activity and can vary in its length (Zanetti et al., 1995). In humans, only one cathelicidin (LL-37) was found, whereas in other species several AMPs are expressed. In cattle, two α-helical cathelicidins are BMAP-27 and BAMP-28 (Kościuczuk et al., 2012), which were shown to exhibit antimicrobial activity against Gram-positive as well as Gram-negative bacteria and even multi-drug resistant bacteria like LA-MRSA (Blodkamp et al., 2015; Zanetti et al., 2002) in the same mode of action as mCRAMP and LL-37 (Skerlavaj et al., 1996). Both peptides show a conserved N-terminal part, whereas the C-terminal part shows a tendency to non-conserved regions (Fig. 1). BMAP-27 and BMAP-28 exhibit the same overall hydrophobicity, especially in the C-terminus (Fig. 1). In our previous work (Blodkamp et al., 2015), both peptides were described as most potent comparing a pool of different mammalian cathelicidins against LA-MRSA isolates.

For testing the susceptibility of bacteria towards antimicrobial agents, minimal inhibitory concentration (MIC) assays are usually performed. This method is standardized for antimicrobial agents according to the recommendations of the CLSI. At present, harmonized protocols for testing the susceptibility of bacteria to AMPs are not available. MIC assays are often performed in analogy to the CLSI standards (CLSI VET01-A4, 2013), but the procedures applied were usually not characterized for the homogeneity/heterogeneity of the results obtained when comparing different peptides or different bacterial isolates.

In this study, we performed MIC assays according to CLSI-protocols for two bovine AMPs – BMAP-27 and BMAP-28 – with two selected clinical *S. aureus* isolates, one clinical *E. coli* isolate and one *S. aureus* laboratory strain. In addition, we tested different temperatures, oxygen level and plastic polymers in our setting. The peptide- and strain-specific homogeneity of the variances in the MIC data was analyzed. Finally, we determined the MIC values for BMAP-27 and BMAP-28 of 50 *S. aureus* and 50 *E. coli* field isolates and statistically compared peptide-specific MIC values of the two bacterial species.

2. Materials and methods

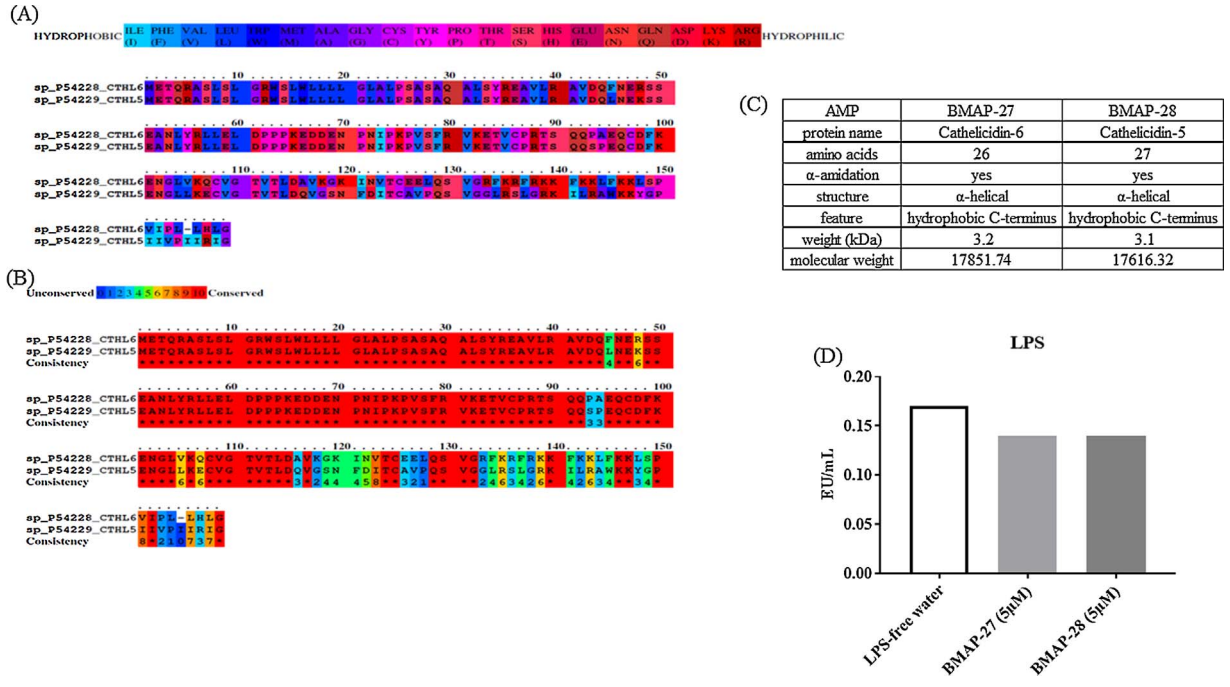
2.1. Bacterial isolates

For the set-up of the technique, four selected reference strains – one clinical field isolate (*S. aureus* RD5, a clinical LA-MRSA isolate from cattle; Feßler et al., 2012), two strains derived from the American Type Culture Collection (ATCC®) (*E. coli* ATCC® 25922, *S. aureus* ATCC® 29213) and one laboratory strain (*S. aureus* Newman Δ*dlt*) – were analyzed. The Δ*dlt* mutant of *S. aureus* Newman is missing D-alanine substituents on its teichoid acids, which is important for bacterial resistance against AMPs. Therefore, this laboratory strains shows lower MICs towards AMPs (Peschel et al., 1999; Simanski et al., 2013).

For validation of the technique, 50 previously characterized clinical *E. coli* as well as 50 methicillin-sensitive *S. aureus* (MSSA) isolates obtained from dairy cattle in Germany suffering from mastitis with clinical or subclinical appearance were tested (Feßler et al., 2012).

2.2. Antimicrobial peptides

Two cathelicidins were used: bovine myeloid antimicrobial peptide 27 (BMAP-27) and BMAP-28. BMAP-27 has 40% and BMAP-28 has 42% hydrophobic residues. Peptides were synthesized as previously described (Andrä et al., 2009). The peptides were purified by HPLC and



(C) AMP	BMAP-27	BMAP-28
protein name	Cathelicidin-6	Cathelicidin-5
amino acids	26	27
α-amidation	yes	yes
structure	α-helical	α-helical
feature	hydrophobic C-terminus	hydrophobic C-terminus
weight (kDa)	3.2	3.1
molecular weight	17851.74	17616.32

Fig. 1. Characteristics of BMAP-27 and BMAP-28.
Hydrophobicity was evaluated according to Eisenberg et al. (1984) (A) and conserved regions were determined using PRofile ALIgNEment (PRALINE) (B). Characteristics are summarized for BMAP-27 and BMAP-28 (C). They both are identical in their structure, amidation status and overall hydrophobicity. The peptides show similarity in molecular weight and length. BMAP-27 (CTHL6) and BMAP-28 (CTHL5) were diluted to a concentration of 5 µM in RPMI and LPS contamination was tested (D). Levels (EU/mL) of both cathelicidins were compared to LPS-free water and show no endotoxin contamination.