

## Cathelicidin-1 as a potential biomarker for anestrus detection in cows

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### Abstract

Cathelicidin-1, an antimicrobial peptide, has garnered attention for its potential role as a biomarker in detecting anestrus in cows, providing insights into bovine reproductive health. This study aimed to analyse cathelicidin-1 within the urinary proteome and evaluate its effectiveness as a diagnostic tool for anestrus in cows. The study employed tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry techniques to identify and characterize cathelicidin-1 in the context of anestrus in cows. The analysis confirmed the presence and distinct profile of cathelicidin-1, highlighting its significance in reflecting the physiological and pathophysiological states associated with anestrus. Cathelicidin-1 as a promising diagnostic biomarker for anestrus in cows could revolutionize bovine reproductive management by offering more precise and advanced detection methods compared to the traditional, time-consuming, and sometimes inaccurate approaches, like behavioral observation or hormonal assays. If cathelicidin-1 can be detected non-invasively, it would not only enhance early detection and timely intervention but also reduce the need for invasive procedures, thereby improving animal welfare.

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### Introduction

Anestrus, a physiological state characterized by the absence of estrus cycles, poses significant challenges to cattle reproductive management. Understanding the multifaceted aspects of anestrus is crucial for mitigating economic losses and ensuring optimal herd fertility. Anestrus in cows is broadly defined as a period of sexual inactivity, where cyclic ovarian activity ceases. It can be categorized into physiological anestrus, occurring before puberty, during pregnancy, and post-partum, and pathological anestrus, characterized by extended periods of reproductive quiescence post-partum.<sup>1</sup> Various factors contribute to the onset of anestrus, including nutritional deficiencies, stress, environmental factors, and specific reproductive disorders.<sup>2</sup> Understanding the interplay of these factors is crucial for implementing effective management strategies. Cathelicidins are small, cationic, and host-defense antimicrobial peptides being present in humans and various other species, including cattle, horses, pigs, sheep, goats, chickens, rabbits, and certain fish species.<sup>3</sup> Cathelicidins exhibit a

wide-ranging antimicrobial activity, being effective against bacteria, fungi, viruses, and parasites.<sup>4</sup> Besides their direct antimicrobial actions, cathelicidins safeguard the host through immunomodulatory effects.<sup>5</sup>

The role of cathelicidin-1 as a biomarker of anestrus in cows has not been elucidated earlier. No prior studies indicate the presence of cathelicidin-1 in the urine of cows in anestrus. This is the first study to report the presence of the antimicrobial peptide cathelicidin-1 in the urine of cows with anestrus.

The basis of this study was to address the challenges of anestrus in cattle reproductive management by identifying reliable biomarkers for early detection. Given the complex factors contributing to anestrus, including nutritional, environmental, and physiological elements, the study focused on exploring the presence of urinary peptides as a potential non-invasive biomarker for detecting anestrus. The novelty of our study lies in the identification of cathelicidin-1 in cow urine as a potential biomarker for anestrus detection, using advanced techniques like tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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(tricine-SDS-PAGE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), which could offer a non-invasive and precise method for improving reproductive management in bovine.

## Materials and Methods

**Animal selection and sampling.** Estrus detection was carried out by visual observations for heat symptoms and using a teaser bull for 15 - 20 min in the morning and evening daily. Six Sahiwal parous cows aged 5 - 8 years that had not been observed in estrus or detected by teaser bull for the last 6 or more than 6 months and six normal cycling Sahiwal cows were selected for the study. Per-rectal genital palpation of these animals revealed no active structure on either of the ovaries. They were apparently healthy with normal feed and water intake. Mid-stream urine samples were collected from anestrus and healthy normal cycling cows. Urine was centrifuged at 2,500 *g* for 10 min in a refrigerated centrifuge (4.00 °C). The study was approved by the Institutional Animal Ethics Committee with the approval No. IAEC/21/8, dated 18/03/2021.

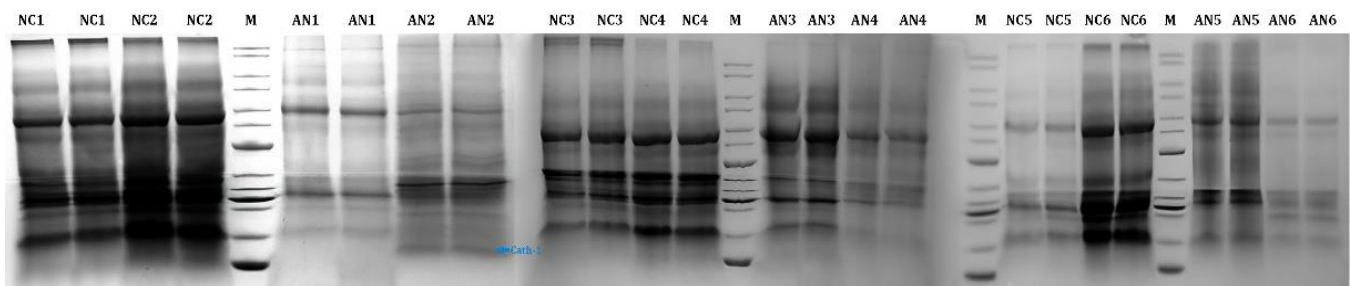
**Extraction of proteins using weak cation exchange beads.** Cationic peptides were extracted from urine using weak cation exchange beads (Macro Prep® CM Resin; BioRad Laboratories, Inc., Hercules, USA). Four-hundred mL of diafiltered urine was mixed with 400 µL of CM-Macro PREP (50.00% slurry) and continuously stirred at room temperature for 2 hr at 4.00 °C. The beads were then sedimented by centrifugation at 200 *g*. The sedimented beads were washed using approximately two-bed volumes of 25.00 mM, pH: 7.50, ammonium acetate (Imperial Life Sciences, Gurugram, India) by centrifugation at 200 *g* for 10 min. This washing step was repeated five times. The cationic peptides were extracted by adding two volumes of 5.00% acetic acid (Imperial Life Sciences) and incubating for a minimum of 20 min at 4.00 °C. The beads were subsequently separated by centrifugation at 200 *g*. To ensure complete recovery of cationic peptides, this extraction step was repeated after 1, 2, 3, and 4 hr, as well as after overnight incubation at 4.00 °C.<sup>6</sup>

**Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Cow urinary proteins were subjected to tricine-SDS-PAGE and compared with the molecular markers ranging from 200 kDa to 10.00 kDa (New England Biolabs, Ipswich, USA). The gels were scanned and analyzed in the Gel Documentation System (Clever Scientific, Rugby, UK) and geneQuant/genePix, respectively, to determine the relative molecular weight of protein bands of the sample appeared on the gel.

**In-gel tryptic digestion, MALDI-TOF-MS, and peptide mass fingerprint (PMF).** In-gel tryptic digestion and MALDI-TOF-MS were performed at Sandor Life Sciences, Hyderabad, India. A MASCOT database PMF search of the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database was first undertaken (NCBI nr release UP9136\_B\_taurus B\_taurus 20220223 (37879 sequences; 22679008 residues; <http://www.ncbi.nlm.nih.gov/>). Protein identification of peptide fragments was performed using the UniProt based on the *Bos taurus* database using the assumption that peptides are mono-isotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Peptide mass tolerance was set to ± 0.80 Da. A MASCOT PMF search of all data was made against the *B. taurus* protein dataset. Significance was established according to the expectancy value transformed into a MASCOT score (with significance at *p*-value < 0.05 at scores over 58.00).

## Results

**Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Tricine-SDS-PAGE analysis of urine fractions collected from ion exchange chromatography of healthy and anestrus animals showed different protein bands (Fig. 1). The separated protein bands ranged from 18.84 to 112.48 kDa for normal cycling animals, whereas they ranged from 15.53 - to 114.54 kDa for the animals in anestrus. The average number of protein bands reported



**Fig. 1.** Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of urine samples in duplicate. NC: Normal cycling cow; AN: Anestrus; NC1, 2, 3, 4, 5, and 6: Normal cycling cows (number one to six); AN1, 2, 3, 4, 5, and 6: Anestrus cows (number one to six). Panel A (Left section): Lanes: NC1, NC2 (two duplicates each), followed by marker (M), then AN1 and AN2 (two duplicates each). Panel B (Middle section): Lanes: NC3 and NC4 (duplicates each), marker (M), then AN3 and AN4 (duplicates each). Panel C (Right section): Lanes: marker (M), NC5 and NC6 (duplicates each), marker (M), then AN5 and AN6 (duplicates each).

were 5.00 and 4.60 in healthy and anestrus animals, respectively. Moreover, the lowest molecular weight of proteins identified in anestrus animals was 15.53 kDa, whereas it was 18.84 kDa for normal cycling animals.

**Matrix-assisted laser desorption ionization-time of flight mass spectrometry.** The MALDI-TOF mass spectra resulting from the four randomly selected tryptic-digested gel bands were processed using MassLynx Software (version 4.1; Waters Corp., Milford, USA). The mass-to-charge ratio (m/z) peak lists were automatically produced, using ProteinLynx Software (Waters Corp.). The lists were then entered into MASCOT PMF searches of the complete NCBI nr protein database using the MASCOT search engine. Gel bands from normal animals were identified, with a statistically significant score (significant scores were respectively 61.00 and 64.00), and matched to B-cell translocation gene one protein (Accession No. A0A3Q1M3G0) and peroxidase (Accession No. A0A3Q1MSA0). Gel bands from anestrus animals were identified with a statistically significant score (significant scores were respectively 60.00 and 66.00) for complement C1q subcomponent subunit B and cathelicidin-1 (Accession No. Q2KIV9 and P22226, respectively). All the identified proteins are detailed in Table 1. The MALDI-TOF mass spectra analysis of cathelicidin-1 is presented in Figure 2, providing a detailed examination of the molecular weight distribution. The presence of cathelicidin-1 in the urine of anestrus animals indicates the secretion of antimicrobial

proteins or peptides in response to any infection or other pathophysiological conditions, including anestrus.

## Discussion

Anestrus is a leading cause of economic loss for dairy farmers, with impacts more significant than many realize. It extends the post-partum interval and significantly decreases financial returns from milk sales by reducing the overall number of pregnancies, withdrawal periods following treatment, and the cost of managing prolonged post-partum anestrus.<sup>7</sup> Anestrus is a period of sexual inactivity during which the animal does not exhibit regular estrus cycles and shows no signs of heat.

This study aimed to identify low molecular weight urinary proteins or peptides (cathelicidins) capable of detecting anestrus in cows. Proteins were extracted from urine obtained from normal cycling, as well as anestrus cows, by ultra-centrifugation, separated by tricine-SDS-PAGE and identified by MALDI-TOF-MS followed by PMF. Various techniques have been utilized to concentrate and purify urinary proteins, such as lyophilization, precipitation, ultra-centrifugation, and centrifugal filtration. Ultra-centrifugation is a suitable method for preparing urine samples for SDS-PAGE.<sup>8</sup> Ultra-centrifugations was employed to separate proteins in our study.

Urine serves as a reliable indicator of an animal's physiological state, as it plays a key role in eliminating metabolic waste. In anestrus animals' urine, low molecular weight peptides, cathelicidin-1, were identified. In contrast, in normal healthy animals' urine, no such peptides were reported. The band pattern of peptides in tricine-gel showed nearly similar results across both groups. This study concentrated on endogenous peptides secreted in urine during both the normal cycling phase and anestrus. The observation aligned with the findings of molecular characteristics that the urinary peptides are of low molecular weight. Tricine-SDS-PAGE separates proteins primarily based on mass. Tricine-SDS-PAGE is frequently utilized to separate proteins in the mass range of 1.00 - 100 kDa.<sup>9</sup> This electrophoretic technique is preferred for the resolution of proteins smaller than 30.00 kDa as the concentrations of acrylamide used in the gels are lower than other electrophoretic systems.<sup>10</sup> Urine proteins separated using SDS-PAGE showed a majority of protein bands with molecular weight between 12.00 and 67.00 kDa with a non-significant difference between pregnant and non-pregnant cows.<sup>11</sup> The electrophoretic pattern of urine samples can help detect the types of

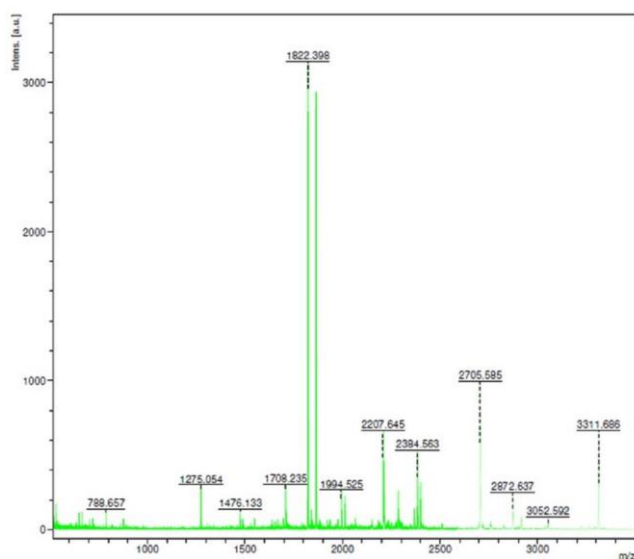


Fig. 2. Protein spectra of cathelicidin-1.

Table 1. The identified proteins from urine.

Proteins	MASCOT score	Accession No.	Species	Groups	Molecular weight (kDa)
Cathelicidin-1	66.00	P22226	<i>Bos taurus</i>	Anestrus	17.93
Complement C1q subcomponent subunit B	60.00	Q2KIV9	<i>Bos taurus</i>		26.61
B-cell translocation gene 1 protein	61.00	A0A3Q1M3G0	<i>Bos taurus</i>	Healthy animals	42.66
Peroxidase	64.00	A0A3Q1MSA0	<i>Bos taurus</i>		158.75

proteins leaking into the urine, providing valuable diagnostic and prognostic information.<sup>12</sup> Estrus urine showed the presence of two volatile compounds, ethylbenzene and 2,2-dimethylbutane, that may serve as a biomarker during pregnancy in cows.<sup>13</sup> The SDS-PAGE analysis of urine proteins from cardiomyopathy-affected calves revealed aberrant electrophoretic patterns compared to the healthy animals.<sup>14</sup> This may also serve as a basis for investigating the urinary protein pattern during anestrus in cows. Various antimicrobial proteins and peptides, namely defensin and S10A8, were identified from normal cycling healthy cow's urine in our previous studies using different electrophoretic and mass spectrometric methods,<sup>15,16</sup> and it was assumed that these proteins may show variation in their expression in disease conditions.

This is the first study elucidating the presence of cathelicidin-1 in the urine of cows with anestrus. The study found that electrophoretic analysis of urinary proteins in conjunction with MALDI-TOF-MS, followed by PMF searching of databases, might be a useful tool for detecting some novel protein markers, such as cathelicidin-1, capable of detecting animals in anestrus. This technique provides an improved method to generate the urinary protein database. The MALDI-TOF-MS/PMF is the significantly cheaper strategy that can be employed where the genome sequence of the particular organism under study is available.<sup>17</sup>

Proteomic analysis of urinary proteins is a promising tool for studying reproductive physiology and pathophysiology, as well as identifying biomarkers for anestrus. Our proteome profile of the urine of anestrus animals revealed the presence of an interesting antimicrobial peptide, cathelicidin-1. The urinary proteome of healthy animals contained anti-proliferative protein (B-cell translocation gene 1 protein) and anti-oxidant agents, like peroxidase. Gel spot from anestrus animals was matched to cathelicidin-1, which is an antimicrobial protein being subjected to up- or down-regulation in the case of any infection or pathophysiological conditions. Another protein identified from anestrus animals is complement C1q subcomponent subunit B, playing an important role in complement activation and innate immune response.<sup>18</sup>

Though this study identified cathelicidin-1, other potential biomarkers for anestrus might have been unnoticed. A broader proteomic analysis could yield additional relevant biomarkers. The findings are based on a limited sample set, which may not represent the full diversity of cow populations, affecting the generalizability. While cathelicidin-1 was identified as a potential biomarker, its direct correlation with anestrus pathology, and its sensitivity and specificity in diagnosing anestrus conditions require further investigation.

Future research should explore a wider range of urinary biomarkers alongside cathelicidin-1 for a more

comprehensive anestrus diagnosis in cows. Large-scale validation across different breeds and conditions is needed to confirm the reliability. Our findings suggest the potential to develop non-invasive diagnostic kits or automated systems for detecting anestrus in cows by incorporating cathelicidin-1 and other identified biomarkers. Comparative studies on cathelicidin-1 levels in various reproductive states (*e.g.*, normal cycling, pregnancy, and anestrus) are also essential to assess the diagnostic accuracy.

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### Conflict of interest

The authors declare no competing interests.

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