

***Arcanobacterium pluranimalium* leading to a bovine mastitis: Species identification by a newly developed *pla* gene based PCR**

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Summary

We are describing a clinical case of bovine mastitis due to *Arcanobacterium pluranimalium* in a Holstein-Friesian heifer, delivering bloody milk on the left hindquarter. Moreover, we report on the development and evaluation of PCR primers based on the pluranimaliumlysin (*pla*) gene for the identification of this species. With the primer pair PlaF/PlaR the *A. pluranimalium* type strain as well as the mastitis isolate 704 revealed a correctly sized amplification product (458 bp), whereas no amplification product was obtained for all non-target strains. The established PCR provides a new and convenient tool for the mastitis diagnostic to differentiate between *A. pluranimalium* and *Trueperella pyogenes*.

Keywords: *Arcanobacterium pluranimalium*, mastitis, PCR, identification

Bovine Mastitis verursacht durch *Arcanobacterium pluranimalium* und Etablierung einer Identifizierungs-PCR basierend auf dem *pla* Gen

Dieser Bericht beschreibt den Fall einer Holstein-Friesian Färse mit klinischer Mastitis (blutige Milch, Hinterviertel links), die durch *A. pluranimalium* verursacht wurde. Zudem wurden im Rahmen dieser Arbeit PCR Primer, basierend auf dem Pluranimaliumlysin (*pla*) Gen, zur Identifizierung von *A. pluranimalium* evaluiert. Sowohl der Referenzstamm wie auch das klinische Isolat 704 zeigten korrekte Amplifizierungsprodukte (458 bp). Alle nicht-Zielstämme waren in der PCR negativ. Die etablierte PCR stellt damit für die Mastitis-Diagnostik ein praktisches Hilfsmittel dar, das zur Unterscheidung zwischen *A. pluranimalium* und *Trueperella pyogenes* herangezogen werden kann.

Schlüsselwörter: *A. pluranimalium*, Mastitis, PCR, Identifizierung

Case History

In May 2012 the diagnostic department of the Institute of Food Safety and Hygiene, University of Zurich, received a milk sample from bovine origin. The cow, a Holstein-Friesian heifer, delivered bloody milk on the left hindquarter since beginning of May 2012 and decreased milk yield was observed. First, the owner assumed that another cow probably hit the cow. He also noticed that this quarter was harder than the other three ones. There was no injury, redness or swelling visible. The cow was in a good general condition. After 2 weeks without any change in milk appearance, a milk sample was aseptically taken and provided for bacteriological analysis. At this time the California Mastitis Test (CMT) from this quarter showed a score of 3 (Schalm et al., 1971).

Diagnostics

The milk was cultured on blood agar and incubated overnight at 37 °C. The next morning there was no visible growth on the plate. After additional 24 hours of incubation small (0.5 to 1 mm), round, translucent to grey colonies producing a total hemolysis were visible (Fig. 1). Such colonies showing a total hemolysis on blood agar are typical for *Trueperella pyogenes* (former *A. pyogenes*; Yassin et al., 2010). Nevertheless, Gram staining of these colonies showed no clear picture. MALDI-TOF based identification also yielded no result, since there was no match with the available spectra. It was therefore decided to further identify these colonies by molecular based methods. The isolate (named isolate 704) was investigated by 16S rDNA (primer pair: 616V, 1492R; Loy et al., 2002), 23 rDNA

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Figure 1: Small, round, translucent to grey colonies of *A. pluranimalium* producing total hemolysis on blood agar.

(primer pair: 23S-1, 23S-2; Ülbegi-Mohyla et al., 2010) and 16S-23S rDNA intergenic spacer region (primer pair: c and b; Kostman et al., 1995; Chanter et al., 1997; Hassan et al., 2008) sequencing using the amplification conditions described in the respective publications. Both, the 948 bp 16S rDNA amplicon (GenBank accession number JX144330) as well as the 663 bp 23S rDNA PCR product (JX44331) yielded a 99% identity to the respective sequences of the corresponding *Arcanobacterium pluranimalium* reference strain M430/94/2 (DSM 13483^T). Intergenic spacer region PCR revealed a 534 bp sized amplicon (JX144332) and a 98% identity to the corresponding *A. pluranimalium* reference strain sequence.

The original description of *A. pluranimalium* is based on an isolate from a harbour porpoise in Scotland and another from a fallow deer from Sweden (Lawson et al., 2001). In the years from 2001 to 2009, *A. pluranimalium* was recovered from 22 sheep samples as reported by Foster and Hunt (2011) including material from abortion, semen, abscesses, viscera and one case each of naval ill and peritonitis. Within this study a number of other host species were examined but there was only one further isolate of *A. pluranimalium* which was recovered from a milk sample collected from a cow with mastitis. Overall, this suggests that ovine animals are the major host of *A. pluranimalium*, with other host animals rarely affected.

Treatment and Progress

After collecting a milk sample for the microbiological analysis, the left hindquarter of the cow was treated intramammarily with Neo-M (Dr. E. Graeub, Switzerland), which contain 1 Mio. U.I. Benzylpenicillinum procainum and 350 mg Neomycinum per 10 g. Four injectors were administered with an interval of 12 hours. A fifth injector was administered 24 hours after the fourth one. After the treatment the milk returned to normal character and milk yield increased again. Three weeks after milk sam-

pling the quarter still showed a hardening but the CMT was negative.

Development and evaluation of a pluranimaliumlysin (*pla*) gene based *A. pluranimalium* specific identification PCR

Until recently the genus *Arcanobacterium* comprised nine species: *A. haemolyticum*, *A. pyogenes*, *A. bernardiae*, *A. phocae*, *A. bialowiezense*, *A. bonsai*, *A. hippocoleae*, *A. abortisuis* and *A. pluranimalium*, many of which were associated with animal hosts and/or pyogenic and opportunistic infections (Collins et al., 1982, Lehnen et al., 2006). In 2010, comparative chemotaxonomic and phylogenetic studies performed by Yassin et al. (2010) indicated that the genus *Arcanobacterium* was not monophyletic and it was proposed that the genus *Arcanobacterium* should be restricted to *A. haemolyticum*, *A. hippocoleae*, *A. phocae* and *A. pluranimalium*. The other species *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* and *A. pyogenes* were reclassified in a new genus *Trueperella*, as *Trueperella abortisuis*, *Trueperella bernardiae*, *Trueperella bialowiezensis*, *Trueperella bonasi* and *Trueperella pyogenes*.

So far, *T. pyogenes* and *A. pluranimalium* have been associated to bovine mastitis. Based on colony morphology and the hemolytic activity on blood agar, these two species cannot be differentiated from each other. Since no *A. pluranimalium* specific identification PCR had previously been available, a further aim of this study was to design and evaluate specific PCR primers for the identification of this species. A primer pair (PlaF: 5'-TCG CCA ATC AGA ATC TCG-3' and PlaR: 5'-GTT GTT GAC TCC GCG TGC-3') was designed based on the partial pluranimaliumlysin (*pla*, EMBL_CDS: CBY79009.1) gene sequence of *A. pluranimalium* type strain 13483^T. Specificity of the PCR assay was evaluated using the *A. pluranimalium* type strain as a positive control and eight non-target strains (*A. haemolyticum* DSM 20595^T, *A. hippocoleae* DSM 15539^T, *A. phocae* DSM 10002^T, *Trueperella abortisuis* DSM 19515^T, *Trueperella bernardiae* DSM 9152^T, *Trueperella bialowiezense* DSM 17162^T, *Trueperella bonsai* DSM 17163^T, *Trueperella pyogenes* DSM 20630^T). For amplification, mixtures (total volume 50 µl) containing GoTaq Green Master Mix (Promega, Madison, WI) with a final concentration of 1.5 mM MgCl₂, 200 µM dNTPs each and primers at 10 pmol concentration were prepared. Thermal cycling was carried out using an initial denaturation step of 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec and elongation at 72 °C for 30 sec. The amplification product was resolved on a 1% agarose gel followed by ethidium bromide staining and examination under UV light. The PCR assay was successfully performed using either (10 ng) of extracted DNA (DNeasy® Blood &

Tissue Kit, Qiagen, Switzerland) or boiled colony material. With the primer pair PlaF/PlaR the *A. pluranimalium* type strain as well as isolate 704 target revealed a correctly sized amplification product (458 bp), whereas no amplification product was obtained for all non-target strains (100 % specificity). The sequenced product of isolate 704 (JX144333) displayed 100 % identity to the respective sequence of the type strain. The established PCR provides a convenient tool for the mastitis diagnostics to differentiate *A. pluranimalium* from *T. pyogenes*.

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