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Short communication

Non-haemolytic *Mannheimia haemolytica* as a cause of pleuropneumonia and septicemia in a calf

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ABSTRACT

Pure cultures of non-haemolytic *Mannheimia haemolytica*, were cultivated from pleural effusion fluid and blood from a 1-month old Belgian Blue bull calf that was presented with apathy and anorexia. The isolates were identified as *M. haemolytica* by 16S rRNA gene sequencing and MALDI-TOF-MS. Since haemolysis on blood agar plates is considered a hallmark of *M.* haemolytica we wanted to elucidate the unusual phenotype of the isolated strain. Therefore the leukotoxin operon (*lktCABD*), responsible for the haemolytic phenotype of *M.* haemolytica and regarded as the most important virulence factor, was completely sequenced. The leukotoxin operon of the isolated strain showed a deletion in the *lktA* gene, resulting in a truncated LktA protein. The absence of a complete LktA protein is responsible for the non-haemolytic phenotype of the strain. To the best of our knowledge, this is the first report of a well-characterized non-haemolytic *M. haemolytica* isolate causing disease in cattle.

1. Introduction

Mannheimia haemolytica (*M. haemolytica*) is a commensal of the bovine nasopharynx and an opportunistic pathogen associated with upper respiratory tract infection, pneumonia, pleuritis and septicemia (Singh et al., 2011). It is one of the best characterized pathogens in the bovine respiratory disease complex (BRDC) (Fulton et al., 2002). It is generally accepted that predisposing factors, such as stress (transport) (Ribble et al., 1995), other (viral, *Mycoplasma*) respiratory tract infections (Burciaga-Robles et al., 2010; Griffin et al., 2010; Leite et al., 2004), overcrowding and air draft (Lundborg et al., 2005) enable *M. haemolytica* to colonize the lower respiratory tract. This results in a variable degree of fibrinous pneumonia and often mortality (Singh et al., 2011).

Several virulence factors contributing to the pathogenesis of *M. haemolytica* infections have been described, including a leukotoxin (LktA), outer membrane proteins, proteases, adhesins, lipopolysaccharide, and capsule (Singh et al., 2011). LktA is generally accepted as responsible for most of the pulmonary damage. The LktA of *M. haemolytica* specifically targets ruminant leukocytes via binding to the CD18 subunit of beta-integrins (Li et al., 1999). In high concentrations LktA is cytolytic, but at sublytic concentrations, it activates neutrophils and induces inflammatory cytokines, degranulation of neutrophils and apoptosis (Tatum et al., 1998; Highlander et al., 2000).

In addition, LktA also displays a hemolytic activity (Murphy et al., 1995). In fact, production of LktA results in the characteristic haemolysis of *M. haemolytica* on blood agar. This haemolytic phenotype is one of the key characteristics used in laboratory routine diagnostics to differentiate *Pasteurellaceae*.

Here we describe pleuropneumonia and septicemia in a Belgian Blue calf associated with a non-haemolytic strain of *M. haemolytica*. The *lktCABD* operon was sequenced and compared to that of previously published sequences of *M. haemolytica* strains.

2. Materials and methods

2.1. Clinical presentation and sampling

A 1 month old Belgian Blue bull calf was presented at the veterinary teaching hospital with the complaint of apathy and anorexia. The calf had not yet been treated before arrival. On farm, there were recurrent episodes of respiratory problems in the calves and on-site analysis showed poor ventilation in the calf stables. Upon presentation, the calf was dehydrated (pale mucosa, cold

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extremities, deep-set eyes), comatose, and showed nervous symptoms (tremor and nystagmus). Lung auscultation revealed bilaterally amplified breath sounds. Blood examination showed dehydration, hypercapnia and hypoglycemia. Transcutaneous ultrasonography of the thorax demonstrated pleural effusion and bilateral extensive translobar consolidation, indicative for pleuropneumonia (Liechtenstein, 2014). After antiseptic preparation, a thoracic aspiration of the pleural fluid was taken under ultrasonographic guidance. Three ml of blood was taken sterile from the jugular vein and directly injected into a vial of soybean-casein digest broth (BD Bactec Plus Aerobic/F) and incubated in the BACTEC 9050 automated blood culture system (Becton Dickinson, New Jersey, USA) (Dumoulin et al., 2010).

2.2. Microbiological identification

The automated blood culture system detected microbial growth after 10 h of incubation and the culture was subsequently subcultured on Columbia agar with 5% sheep blood and Columbia CNA agar with 5% sheep blood (Oxoid, Aalst, Belgium). The sample of pleural effusion fluid was directly plated on Columbia agar with 5% sheep blood and Columbia CNA agar with 5% sheep blood (Oxoid, Aalst, Belgium). Culture plates were aerobically incubated with supplementation of 5% CO_2 at 35 °C for two days. Additionally, the pleural effusion fluid sample was plated on a modified pleuropneumonia-like organism agar (PPLO agar) and incubated in a 5% CO_2 enriched atmosphere for 5 days at 35 °C for detection of *Mycoplasma bovis*. Presumptive identification was obtained by standard biochemical testing (Markey et al., 2013). Antimicrobial susceptibility testing was done with a disk-diffusion test on Iso-Sensitest Agar (Oxoid, Aalst, Belgium) and was performed and evaluated with clinical breakpoints according to the suppliers guidelines (Rosco Neo-Sensitabs, Taastrup, Denmark).

DNA was extracted from a small loopfull bacteria using lysis buffer (0.25 % SDS, 0.05 M NaOH) and heating at 95 °C for 5 min. DNA was extracted from the pure culture obtained from pleural effusion fluid as well as from the pure culture obtained from the blood derived Bactec® culture. PCR for 16S rRNA gene sequencing was performed as described by Baele et al. (2003) using the conserved primers αβ-NOT (5′-TCAAACTAGGACCGAGTC-3′) and ω_{MB} (5′-TACCTTGTTACTTCACCCCA-3′) and *Taq* Mastermix (Bioline, London, United Kingdom). Amplicons were sequenced using the Sanger dideoxy sequencing technique (GATC Biotech AG, Konstanz, Germany). In addition, identification was confirmed using Matrix Assisted Laser Desorption Ionisation—Time Of Flight Mass Spectrometrie (MALDI-TOF MS) analysis (Bruker Daltonics Biotyper, Database 4613)

2.3. Sequencing of the lktCABD operon

For sequencing of the leukotoxin operon (*lktCABD*), primers as described by Davies et al. (2002) were used, supplemented with primers designed in-house using the whole genome sequence of *M. haemolytica* USMARC 184 (GenBank NZ_CP006957.1) and previously deposited *lktCABD* sequences (GenBank AF314508, AF314511). PCR was performed under standard conditions in a 25 μl reaction volume with *Taq* polymerase (Bioline, London, United Kingdom). The PCR program started with 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 45 s at 62 °C for the *lktA* primers or at 57 °C for the *lktB, C* and *D* primers, and 72 °C for 2 min. The final extension step was 72 °C for 10 min after which samples were cooled to 4 °C. Primers, primer position, product size, and references are given in Table 1. Amplicons were sequenced using the Sanger dideoxy sequencing technique (GATC Biotech AG, Konstanz, Germany).

3. Results

3.1. Clinical evolution

The calf was stabilized with oxygen therapy (6 l/min for 3 days), isotonic fluid therapy with 5% glucose, and flunixine meglumine (1.1 mg/kg/day for 3 days). The pleuropneumonia was initially treated for 5 days with enrofloxacin (5 mg/kg/day for 5 days). After the antibiogram became available, the antimicrobial therapy was switched to a combination of 10.000 IE/kg procaine benzylpenicillin and 5 mg/kg neomycin daily for three weeks. The calf recovered completely without further complications.

3.2. Microbiological identification

The blood derived Bactec® culture and the pleural effusion fluid showed a pure culture of Gram negative, oxidase positive, non-haemolytic (even after removal of the colony) *Pasteurellaceae*. No *Mycoplasma* spp. could be cultivated from the pleural effusion sample. Results of the disk diffusion test showed that the isolated *Pasteurellaceae* strain, named MB1401, was susceptible to beta lactam antibiotics, tetracyclines, quinolones, florfenicol, sulphonamides, trimethoprim and spectinomycin. Inhibition zone diameters obtained with lincosamides, macrolides, apramycin,

Table 1
Target gene, primer name and sequence, product size, position and references for sequencing of the *lktCABD* operon.

	Primer	Sequence (5′ → 3′)	Product size	Position[a]	Reference
lktC	lktCSQFo1	ACACTCCTTTTTCTCTTCTG	723	5218	This study
	lktC/R/2	GCTGTAAGCCACGAATTT		5941	Davies et al. (2002)
lktA	lktA9 (Fo)	TCAAGAAGAGCTGGCAAC	3054	5816	Davies et al. (2001)
	lktASQRe1	GCAGATACACCACCAGCAAT		7009	This study
	lktASQFo2	CGTGTTGCAGCCGGTTTA		6741	This study
	lktAQSRe2	TCCACGGCTATAGTGAACTC		7808	This study
	lktASQFo3	GGTGAAGGTGATGACAAC		7719	This study
	lktA7 (Re)	AGTGAGGGCAACTAAACC		8870	Davies et al. (2001)
lktB	lktB/F/1	CAATTTGCTAGAGCAGCT	2305	8730	Davies et al. (2002)
	lktBSQRe1	GCCATCGCTTTAATCATATTG		9867	This study
	lktBSQFo2	CCGAAATTGACCTTAGTC		9698	This study
	lktB/R/1	TTTTCCATACTTCTRCCC		11035	Davies et al. (2002)
lktD	lktD/F/1	GCAAGCAYCACGAATTACTG	1757	10881	Davies et al. (2002)
	lktDSQRe1	CGATAAACAGCTAGCTCA		11774	This study
	lktDSQFo2	TGAAGGTGCAACACGTAT		11637	This study
	lktDSQRe2	ATTTTTGCGGTAGCCAAGA		12638	This study

[a] Nucleotide position corresponding to the 5'bp of the primer, as in GenBank NZ_CP006957.1.