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Identification of *Arcanobacterium phocae* isolated from fur animals by phenotypic properties, by MALDI-TOF MS analysis and by detection of phocaelysin encoding gene *phl* as probable novel target

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ABSTRACT

In the present study 12 *Arcanobacterium phocae* strains isolated from fur animals in Finland, including foxes, minks and Finnraccoons, could successfully be identified phenotypically, by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) and genotypically by sequencing 16S rDNA and phocaelysin (PHL) encoding gene *phl*. The PHL of all 12 *A. phocae* strains in the present study and reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 displayed, as typical members of the cholesterol dependent cytolysin-group of toxins, the variant undecapeptide sequence EATGLAWDPWW which appeared to be most closely related to arcanolysin of *Arcanobacterium haemolyticum* and pyolysin of *Trueperella pyogenes*. In addition, gene *phl* could be determined with a newly designed loop-mediated isothermal amplification (LAMP) assay. The detection of mass spectra by MALDI-TOF MS and the LAMP assay based on gene *phl* might help to reliably identify *A. phocae* in future and also elucidate the role this species plays in infections of fur animals.

1. Introduction

According to Yassin et al. (2011) genus *Arcanobacterium* (*A.*) consists of four species, namely *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae*. More recently *A. canis* (Hijazin et al., 2012d), *A. phocisimile* (Hijazin et al., 2013), *A. pinnipediorum* (Sammra et al., 2015), *A. wilhelmae* (Sammra et al., 2017) and *A. urinimassiliense* (Diop et al., 2017) were described as novel species of this genus.

A. phocae was first isolated and characterised in 1997 in mixed culture from various tissues and fluids in common seals (*Phocae vitulina*) and grey seals *(Halichoerus grypus*) of the coastal waters around Scotland, UK (Ramos et al., 1997). However, this bacterium was recovered from pneumonic and septicemic seals but its pathological significance was unclear. Later, Johnson et al. (2003) recovered *A. phocae* from sites of inflammation from live stranded California sea lions (*Zalophus californianus*), Pacific harbour seals (*Phoca vitulina richardii*) and northern elephant seals (*Mirounga angustirostris*) and from sites of inflammation and tissue samples taken during postmortem examinations of the above mentioned marine mammals and from stranded southern sea otters (*Enhydra lutris nereis*) and a common dolphin (*Delphinus delphis*) of the central California coast (USA). In 2010, Giovannini (2010) isolated *A. phocae* from mixed infections of two California sea lions stranded along the southern California coast. *A. phocae* was isolated from pleural fluid of one sea lion and from a wound from the second case.

In addition, *A. phocae* has been involved, together with *Staphylococcus delphini* and *Streptococcus canis,* in the pathogenesis of cases of pododermatitis of the Canadian mink *(Neovison vison)* (Chalmers et al., 2015). Pododermatitis of farmed minks appeared as acute, severe ulceration of footpads with occasional further infection of

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nail beds. This could lead to a chronic hyperkeratotic purulent plantar dermatitis. A link between feeding of seal meat and the emergence of pododermatitis in minks was proposed (Chalmers et al., 2015). Furthermore, *A. phocae* has been suggested to be the aetiological agent of an emerging skin disease of fur animals called fur animal epidemic necrotic pyoderma (FENP). FENP, as multifactorial disease, is an emerging disease among Finnish fur animals in which *A. phocae* as well as other infectious factors, such as a novel *Streptococcus* sp., are involved (Nordgren et al., 2014). This previous study suggested a species shift of *A. phocae* from marine mammals to fur animals. In Finland, Nordgren et al., 2016 also studied experimentally possible routes of transmission by an infection of minks with *A. phocae,* either alone or concurrently with a novel *Streptococcus* sp. Typical signs and gross- and histopathological findings for FENP were detected when naïve minks were infected with the tissue extract of minks with FENP using a subcutaneous or intradermal but not a peroral infection route. However, predisposing factors such as the environment, the general condition of the animals, temperature and skin trauma might also contribute to the development of FENP (Nordgren et al., 2016). Molenaar et al., 2017 described the isolation of *A. phocae* in minks in three cases of severe postvaccination wounds at the injection site.

The identification of hitherto described *A. phocae* was performed with phenotypic methods (Ramos et al., 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Ülbegi, 2010; Hijazin et al., 2013), MALDI-TOF MS analysis (Hijazin et al., 2012b, 2012c, 2012d), Fourier transform infrared (FT-IR) spectroscopy (Nagib et al., 2014) and genotypically by sequencing 16S rDNA (Ramos et al., 1997; Johnson et al., 2003), 16S-23S rDNA intergenic spacer region (Hassan et al., 2008; Chalmers et al., 2015) and the genes *rpo*B (Ülbegi-Mohyla et al., 2010), *cpn*60 (Hijazin et al., 2012a), *tuf* and *gap* (Wickhorst et al., 2016). Also, a real-time PCR was applied for detecting *A. phocae* directly from DNA extraction from mink footpad tissue without the interference of other bacterial species (Chalmers et al., 2015). More recently a complete genome sequence of *A. phocae* DSM 10002^T was described by Varghese and Submissions (2016) (accession number: LT629804).

The present study was designed to investigate the usefulness of phenotypic properties, MALDI-TOF MS analysis and phocaelysin encoding gene *phl* as a novel target gene for identifing of *A. phocae* strains isolated from fur animals.

2. Materials and methods

2.1. Bacterial strains

The strains investigated in the present study included 12 *A. phocae* isolated from fur animals in Finland (Nordgren et al., 2014), type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and other reference strains of genus *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012d, 2013; Sammra et al., 2015). The origin and date of isolation of the *12 A. phocae* strains isolated from fur animals are summarised in Supplementary material (Table S1 and Table 1). Two of the *A. phocae* strains (strain 66 and 83) came from the same farm, all the other strains came from different farms.

2.2. Phenotypical characterisation of the bacteria

The bacterial culturing of the *A. phocae* strains was carried out on 5% sheep blood agar for 48 h at 37° C in a candle jar. The strains were investigated phenotypically, by determining haemolysis, synergistic or reverse CAMP-like reactions, with a commercial identification system (API-Coryne test system, bioMérieux, Nürtingen, Germany), tablets containing substrates (Medco Diagnostika GmbH, München, Germany), with 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany) and some other biochemical tests described previously (Ülbegi-Mohyla et al., 2009; Hijazin et al., 2013).

Table 1
Phenotypical properties of the 12 *A. phocae* strains in the present study, type strain *A. phocae* DSM 10002^T and *A. phocae* DSM 10003.

Biochemical properties	*A. phocae* (n = 12)	*A. phocae* DSM 10002^{T*}	*A. phocae* DSM 10003*
Haemolysis on SBA	+(+) (10); + (2)	+(+)	+(+)
CAMP-like haemolytic reactions**			
Staphylococcus aureus β-haemolysin	− (12)	–	–
Streptococcus agalactiae	+ (12)	+	+
Rhodococcus equi	+ (12)	+	+
Reverse CAMP reaction	+ (12)	+	+
Nitrate reduction	$-^1$ (12)	$-^1$	$-^1$
Pyrazinamidase	$-^1$ (8); $+^1$ (4)	$-^1$	$-^1$
Pyrrolidonyl arylamidase	$+^1$ (10); $-^1$ (2)	$-^1$	$-^1$
Alkaline phosphatase	$+^1$ (12)	$+^1$	$+^1$
β-Glucuronidase	$-^{1,3}$ (12)	$-^{1,3}$	$-^{1,3}$
β-Galactosidase	$+^{1,3}$ (12)	$+^{1,3}$	$+^{1,3}$
α-Glucosidase	$+^{1,2,3}$ (12)	$+^{1,2,3}$	$+^{1,2,3}$
N-Acetyl-β-Glucosaminidase	$+^{1,3}$ (12)	$+^{1,3}$	$+^{1,3}$
Esculin	$-^1$ (12)	$-^1$	$-^1$
Urease	$-^1$ (12)	$-^1$	$-^1$
Gelatine	$-^1$ (12)	$-^1$	$-^1$
Fermentation of:			
Glucose	$+^1$ (12)	$+^1$	$+^1$
Ribose	$+^1$ (9); $-^1$ (3)	$+^1$	$+^1$
Xylose	$-^1$ (11); $(+)^1$(1)	$-^1$	$-^1$
Mannitol	$-^1$ (12)	$-^1$	$-^1$
Maltose	$+^1$ (12)	$+^1$	$+^1$
Lactose	$+^1$ (12)	$+^1$	$+^1$
Saccharose	$+^1$ (7); $-^1$ (5)	$+^1$	$+^1$
Glycogen	$+^1$ (9); $-^1$ (3)	$+^1$	$+^1$
Catalase	+ (10); − (2)	+	+
Serolysis on Loeffler agar	− (12)	–	–
Starch hydrolysis	+ (12)	+	+

* Results according to Ülbegi-Mohyla et al. (2009); Ülbegi (2010) and Hijazin et al. (2013); () = number of strains showning positive or negative reactions; SBA = sheep blood agar.

** Synergistic or reverse CAMP-like reaction with indicator strains; the reactions are shown as follows: +(+) = enhanced positive reaction; + = positive reaction; (+) = weak reaction; − = negative reaction; ^{1} = Api-Coryne test system (bioMérieux, Nürtingen, Germany); ^{2} = tablets containing substrates (Medco Diagnostika GmbH, München, Germany); ^{3} = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

2.3. Proteomic analysis by MALDI–TOF MS

The 12 *A. phocae* strains were analysed by MALDI-TOF MS as described previously (Hijazin et al., 2012b, 2012c, 2012e) using the Bruker Daltonik MALDI Biotyper software package version 4.0 (Bruker Daltonik, Bremen, Germany) as follows: A few colonies of freshly cultured bacteria were suspended in 75% ethanol. After centrifugation, the pellet was resuspended in 30 µl 70% formic acid and with the same volume of pure acetonitrile. The suspension was centrifuged and 1 µl of the supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. This was done for 8 spots per sample. The sample was overlaid with 1 µl matrix (10 mg α -cyano-4-hydroxy-cinnamic acid ml^{-1} in 50% acetonitrile/2.5% trifluoroacetic acid). Mass spectra were acquired using a microflex mass spectrometer (Bruker Daltonik) in the linear mode and a mass range of 2–20 kDa using the automated functionality of flexControl 3.4 software (Bruker Daltonik). At least 20 of the 24 acquired raw spectra were used to generate a main spectrum.The software calculates a similarity score [log (score)] by considering the proportion of matching