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Differentiation of *Photobacterium damselae* subspecies using Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in fish isolates

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ABSTRACT

Photobacterium damselae is a well-recognized fish pathogen comprising two subspecies (*damselae* and *piscicida*) with significant clinical and epidemiological differences. We explored the suitability of MALDI-TOF MS for discriminating *P. damselae* subspecies in a panel of 45 isolates from diseased fish. MALDI Biotyper approach correctly identified all isolates at species level with average scores values of 2.271 although identification at subspecies level was not reliable. Visual inspection of the spectra using FlexAnalysis revealed five specific peaks (*m/z* 4183 and 8367 for subsp. *damselae* and 4197, 8397 and 8856 for subsp. *piscicida*) that may allow their differentiation. A classification model (GA-5A) was constructed based on these peaks using a genetic algorithm (ClinProTools software). Cross-validation and recognition capability values of this model were 100%. Validation of this model was confirmed by blind identification of 21 isolates of both subspecies resulting in sensitivity and specificity values of 100%. This is the first study describing a classification algorithm for MALDI-TOF MS identification of both *P. damselae* subspecies. This approach proved to be an effective and reliable tool in discriminating within the *P. damselae* subspecies that might be easily integrated into the workflow of microbiological diagnosis laboratories.

Statement of relevance

MALDI based tool for differentiation *P. damselae* subspecies

1. Introduction

Photobacterium damselae is a well-recognized fish pathogen that it is also occasionally associated with disease in other poikilotherms and mammals, including humans. *P. damselae* is classified into two subspecies: *damselae* (formerly *Vibrio damsela*) and *piscicida* (previously known as *Pasteurella piscicida*). These subspecies show important differences regarding their epidemiology and pathogenicity for animals and humans. Although both subspecies are considered marine host pathogens, only subsp. *piscicida* is responsible of photobacteriosis (also called pseudotuberculosis or fish pasteurellosis) causing outbreaks characterized by appearance of multifocal necrosis in different organs (spleen, liver and kidney) leading to high mortality rates (particularly in larvae and juveniles) and important economic losses (Magariños et al., 1996; Andreoni and Magnani, 2014). Isolates belonging to subspecies *damselae* could be recovered not only from diseased fish (causing hemorrhagic/ulcerative lesions in skin and other localizations) but also from uninfected animals as part of the aquatic environment (Rivas et al., 2013). In humans, subspecies *damselae* has been recognized as zoonotic pathogen mainly transmitted through wounds exposed to contaminated water or infected fish (Hundenborn et al., 2013). It can cause a highly severe necrotizing fasciitis and it has also been isolated from patients with bacteremia or meningitis and may lead to a fatal outcome (Rivas et al., 2013). Considering the clinical relevance of this pathogen, a rapid and reliable identification of *P. damselae* clinical isolates is a key step to improve the knowledge of the epidemiology and ecology of both subspecies clarifying their relevance in Public and Animal Health.

Routine diagnosis of *P. damselae* has been traditionally based on culture and subsequent identification according to biochemical characteristics (Andreoni and Magnani, 2014). For subspecies identification, although *P. damselae* subspecies *damselae* and *piscicida* exhibit discriminating biochemical/phenotypical features (Toranzo et al., 1991; Osorio

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et al., 2000), some strains can exhibit aberrant reactions that can lead to their misidentification (Thyssen et al., 1998; Andreoni and Magnani, 2014). To overcome this problem and due to the fact that biochemical diagnostic methods are time-consuming and laborious for routine laboratory workflow, different immunological (Romalde, 2002) and molecular methods have been developed in order to discriminate and identify the two high genetically related subspecies of *P. damselae*. Molecular methods usually include multiplex-PCR, AFLP or PCR-RFLP assays (Osorio et al., 1999; Osorio et al., 2000; Kvitt et al., 2002; Zappulli et al., 2005; Amagliani et al., 2009). However, occasionally problems of specificity have also been reported (Amagliani et al., 2009).

In recent years, Matrix-Assisted Laser-Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has turned into a reliable and rapid bacteriological identification technique allowing accurate classification of uncommon microorganisms (Hundenborn et al., 2013). The application of MALDI-TOF technique for the routine identification of *P. damselae* in microbiology laboratories would offer a rapid and accurate diagnostic tool for this pathogen allowing the prescription of prompt and suitable treatments in patients and, in the case of fish, the implementation of effective control measurements in cases of photobacteriosis outbreaks. To our knowledge, no previous exhaustive studies for the evaluation of the MALDI-TOF performance for the identification of *P. damselae* have been performed. In this context, the present study aimed to evaluate the suitability of MALDI-TOF MS approach as alternative method for the rapid identification of *P. damselae* subspecies *damselae* and *piscicida* isolates.

2. Material and methods

2.1. Bacteria selection, culture conditions and identification

A total of 45 *P. damselae* clinical isolates (24 *P. damselae* subsp. *damselae* and 21 *P. damselae* subsp. *piscicida*), recovered between 2001 and 2015 from a total of 17 fish farms (in some cases, isolates were recovered from the same farm and date, Table 1) as part of the routine microbiological diagnosis performed in the Health Surveillance VISAVET Centre of the Universidad Complutense (Madrid, Spain), was included in this study. Isolates were recovered from different hosts:

Table 1
Panel of *Photobacterium damselae* clinical isolates included in the present study.

ID. strain	Group[a]	*P. damselae* subsp.[b]	Host	Farm	Year	MALDI TOF score value
01/5483	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	2001	2.186
03/8466	B	*P. damselae* subsp. *damselae*	Rainbow trout	17	2003	2.226
BA05/03138	B	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2005	2.254
BA06/02266	B	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.286
BA06/02613	A	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.264
BA06/02770	A	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.250
BA06/03151	A	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.257
BA06/03222	B	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.282
BA06/03233	B	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.258
BA06/03237	B	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2006	2.114
4696A	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	1999	2.257
4696B	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	1999	2.239
ICM07/01670B	A	*P. damselae* subsp. *damselae*	Gilt-head bream	2	2007	2.353
ICM07/02123	A	*P. damselae* subsp. *damselae*	Gilt-head bream	2	2007	2.240
DICM08_01000-1B	A	*P. damselae* subsp. *damselae*	European sea bass	1	2008	2.296
DICM08_01056-2A	A	*P. damselae* subsp. *damselae*	European sea bass	1	2008	2.336
SAP15/00232-1A	A	*P. damselae* subsp. *damselae*	Gilt-head bream	10	2015	2.353
SAP15/00272-2B	A	*P. damselae* subsp. *damselae*	Gilt-head bream	5	2015	2.173
SAP15/00326-2B	A	*P. damselae* subsp. *damselae*	European sea bass	13	2015	2.323
SAP15/00394A	A	*P. damselae* subsp. *damselae*	Croaker	9	2015	2.295
DICM15/00007-2B	A	*P. damselae* subsp. *damselae*	Gilt-head bream	9	2015	2.235
SAP14/00123-1A	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	2014	2.249
SAP14/00124-1B	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	2014	2.237
SAP14/00125-5B	B	*P. damselae* subsp. *damselae*	Gilt-head bream	15	2014	2.186
7482T	A	*P. damselae* subsp. *damselae*	–	–	–	–
P04/8925-05R	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	3	2004	2.293
DICM0800268	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	6	2008	2.280
SAP15/00393C	A	*P. damselae* subsp. *piscicida*	croaker	9	2015	2.296
01/5480-Bz	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	15	2001	2.261
01/5723 col 1	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	12	2001	2.325
01/5753 col 1	B	*P. damselae* subsp. *piscicida*	Gilt-head bream	7	2001	2.331
01/5755 col 2	B	*P. damselae* subsp. *piscicida*	European sea bass	14	2001	2.290
02/8063 (2)	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	8	2002	2.299
02/8119	B	*P. damselae* subsp. *piscicida*	European sea bass	11	2002	2.245
02/8187 Bz	B	*P. damselae* subsp. *piscicida*	Gilt-head bream	1	2002	2.273
02/8193-D8Bz	B	*P. damselae* subsp. *piscicida*	Unknown	4	2002	2.279
02/8193-D9 Cer	B	*P. damselae* subsp. *piscicida*	Unknown	4	2002	2.250
03/8688	B	*P. damselae* subsp. *piscicida*	Unknown	Unknown	2003	2.326
ICM07/02040	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	2	2007	2.253
DICM07/02176	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	2	2007	2.302
BA05/03162	B	*P. damselae* subsp. *piscicida*	Gilt-head bream	9	2005	2.264
BA05/03166	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	9	2005	2.283
BA05/03267	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	9	2005	2.309
BA05/03375-1	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	9	2005	2.275
BA05/03413-2	A	*P. damselae* subsp. *piscicida*	Gilt-head bream	9	2005	2.307
BA06/03595	B	*P. damselae* subsp. *piscicida*	Unknown	9	2006	2.289
22834^T	A	*P. damselae* subsp. *piscicida*	–	–	–	–

[a] Group A (isolates used for construction of the classification model using ClinProTools), group B (isolates used for external validation of the constructed model).
[b] *P. damselae* subspecies determined by PCR (Osorio et al., 2000).