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Reliable differentiation of a non-toxigenic *tox* gene-bearing *Corynebacterium ulcerans* variant frequently isolated from game animals using MALDI-TOF MS

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ABSTRACT

Corynebacterium (*C.*) *ulcerans* is a zoonotic member of the *C. diphtheriae* group and is known to cause abscesses in humans and several animal species. Toxigenic strains, expressing the *tox* gene encoding diphtheria toxin, are also able to cause diphtheria in humans. In recent years, a non-toxigenic but *tox* gene-bearing (NTTB) variant of *C. ulcerans* has been identified that was frequently isolated from clinically healthy as well as from diseased wildlife animals, especially wild boars (*Sus scrofa scrofa*) in Germany and Austria. The described clinical cases showed similar signs of disease and the isolated corynebacteria displayed common genetic features as well as similar spectroscopic characteristics, therefore being assigned to a so called wild boar cluster (WBC). This study describes the establishment and validation of a method using MALDI-TOF mass spectrometry for a reliable differentiation between various members of the *C. diphtheriae* group at species level as well as a reliable sub-level identification of *C. ulcerans* isolates of the WBC variant. For this study 93 *C. ulcerans* isolates from wildlife animals, 41 *C. ulcerans* isolates from other animals and humans, and 53 isolates from further representatives of the *C. diphtheriae* group, as well as 26 non-*diphtheriae* group Corynebacteria collected via the MALDI user platform from seven MALDI users were used. By assigning 86 *C. ulcerans* isolates to the WBC the extensive geographical distribution of this previously less noticed variant in two Central European countries could be shown.

1. Introduction

Corynebacterium (*C.*) *ulcerans* is one of the members of the *C. diphtheriae* group, which further comprises *C. diphtheriae*, the causative agent of diphtheria in humans, and *C. pseudotuberculosis*, which is the etiologic organism of caseous lymphadenitis in goats, sheep, and for multi-abscesses/ulcerative lymphangitis and edematous skin disease in horses, camelids, pigs, and water buffalos (Guaraldi et al., 2014; Araújo et al., 2018; Quinn et al., 2011). *C. ulcerans* may cause classical respiratory or cutaneous diphtheria in humans. Moreover, it may lead to abscesses, but also causes clinically inapparent infections in a wide range of animal species (Bernard, 2012; Konrad et al., 2015; Guaraldi et al., 2014; Hacker et al., 2016). The major virulence factor of *C. diphtheriae* is the diphtheria toxin (DT), encoded by the *tox* gene, which is linked to lysogenic beta-corynephages (Araújo et al., 2018; Sing et al., 2011). All species of the *C. diphtheriae* group are known to feature members that can harbor the *tox*-gene (Guaraldi et al., 2014; Araújo et al., 2018; Dangel et al., 2019). DT-producing strains reveal a specific

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positive reaction in the immunodiffusion Elek test (Konrad et al., 2010). In recent years, *C. ulcerans* strains that are positive in the Elek test have been isolated even more frequently in human diphtheria cases compared to *C. diphtheriae* strains in Germany and other industrialized countries (Sing et al., 2005; Sing, 2008; Hacker et al., 2016). This situation has prompted the European Centre for Disease Prevention and Control (ECDC) to include *C. ulcerans* in the European Diphtheria Surveillance Network (EDSN) (https://ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/edsn).

Most human *C. ulcerans* infections are caused by zoonotic transmission of *C. ulcerans* between humans and their domestic animals based on epidemiological or molecular typing evidence including next generation sequencing data (Meinel et al., 2014; Hacker et al., 2016). Additionally, the emergence of non-toxigenic *C. ulcerans* is being discussed in clinical context as a source of ulcers and abscesses (Konrad et al., 2015; Berger et al., 2018; Fuursted et al., 2015). Therefore, zoonotic *C. ulcerans* are of special interest with regard to direct and indirect contact of humans to livestock, pet animals, and wildlife (Lartigue et al., 2005; Hogg et al., 2009; Schuhegger et al., 2009; Berger et al., 2011; Meinel et al., 2012; Berger et al., 2019; Moore et al., 2015; Katsukawa et al., 2016; Glawischnig et al., 2018). In the last years, a non-toxigenic but *tox*-gene-bearing (NTTB) variant of *C. ulcerans* has been frequently isolated from healthy and diseased wild boars (*Sus scrofa scrofa*) and in one case from a roe deer (*Capreolus capreolus*) in several regions of Germany and Austria (Contzen et al., 2011; Eisenberg et al., 2014; Rau et al., 2012; Glawischnig et al., 2018). Up to now, 13 of these cases have been described in detail: these cases showed similar pathological changes, such as abscesses of the lymph nodes in wild boars of different ages with similarities to caseous lymphadenitis in goats and sheep caused by *C. pseudotuberculosis* infections (Contzen et al., 2011; Eisenberg et al., 2014). A roe deer infected with *C. ulcerans* had been found dead and revealed a prominent abscess (Rau et al., 2012). The NTTB *C. ulcerans* isolates from all of these cases show an almost identical biochemical pattern and analogous spectra in both Fourier-transform infrared spectroscopy (FT-IR) and in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Contzen et al., 2011; Rau et al., 2012; Eisenberg et al., 2014). Partial sequences of the *tox* genes in these 13 isolates were identical (cf. accession no. GU818742), but different to other known *tox* sequences (Contzen et al., 2011; Dangel et al., 2019). The sequence of the rpoB gene of this NTTB variant of *C. ulcerans* shows some discriminatory variances from other *C. ulcerans* isolates (cf. accession no. GU818735). This NTTB-variant will below also be referred to as belonging to the "wild boar cluster" (WBC), as opposed to the common variant, including the type strain of the species DSM 46325^T. The common variant shows all varieties of toxigenicity features (*tox*-negative, DT-positive, or NTTB) and will be further referred to as non-WBC. MALDI-TOF MS is a widely used technique for species identification of microorganisms in clinical, veterinary and food microbiology (Zimmermann, 2015; Schulthess et al., 2014; Randall et al., 2015; Rau et al., 2016a, b; Pavlovic et al., 2013). Commercial identification systems combine mass-spectrometer and software for acquisition and spectra processing, with a reference spectra collection supplied by the manufacturer (Pranada et al., 2016). For clinically relevant corynebacteria including the *C. diphtheriae* group, different authors have shown the reliability of identification at species level using several commercial MALDI systems and database versions, including the Biotyper (Bruker Daltonik GmbH, Bremen, Germany) or the VITEK MS system (bioMérieux, Marcy l'Etoile, France) (Rajamani et al., 2017; Konrad et al., 2010; Alibi et al., 2015; Farfour et al., 2012). However, differentiation below the species level or the detection of specific epidemiological groups employing MALDI-TOF MS have only rarely been reported (Pérez-Sancho et al., 2016; 2018; Luo et al., 2016) and have not yet been described for *C. ulcerans*. The aim of the present study was to develop a validated MALDI-TOF MS method for the unequivocal identification of *C. ulcerans,* including the differentiation of the WBC and the common variant (non-WBC). Subsequently, this method was applied to the identification of further isolates from Germany and Austria to obtain an overview of the geographical distribution of the WBC variant of *C. ulcerans*. This rapid identification method will be helpful in diagnostics, as well as in tracking down possible human cases in the future, as the zoonotic potential of the WBC of *C. ulcerans* is still unknown.

2. Material and methods

C. ulcerans were collected from governmental veterinary health service laboratories from five Federal States of Germany, and the Friedrich-Loeffler-Institut, Jena (Suppl. 1), including the 13 isolates of the WBC already described (Eisenberg et al., 2014). Additionally, 27 *C. ulcerans* isolated in Austria were included (Glawischnig et al., 2018). The bacteria were isolated during gross pathology of shot and perished game animals submitted for *post mortem* examinations. Other *Corynebacterium* isolates of animal and human origin (Suppl. 1) were selected from the isolate collections of the participating institutes, including the German National Consiliar Laboratory for Diphtheria and the Consiliar Laboratory for C. pseudotuberculosis.

Bacteria were cultivated on solid agar media under aerobic conditions for 24 h. The specific agar/time/temperature combinations for cultivations are listed in MALDI-UP (http://www.maldi-up.ua-bw.de). At the German National Consiliar Laboratory on Diphtheria, all *C. ulcerans* isolates were analyzed by standard workflow, including PCR for the presence of specific *tox* genes, and Elek test for active DT, if indicated (Konrad et al., 2010). Several isolates were further confirmed by partial sequencing of the *rpo*B gene following a protocol published earlier (Khamis et al., 2004; Contzen et al., 2011).

FT-IR was carried out as described before, using a Bruker Tensor 27 equipped with a HTX module (Bruker). For assignment of the WBC of *C. ulcerans* the cluster analysis in Opus software (version 7.2, Bruker) with Ward's algorithm was used (Eisenberg et al., 2014). If the infrared spectrum of an isolate was found in the same branch of the cluster diagram as isolate CVUAS 4292 (Contzen et al., 2011), it was assigned as member of the WBC and subsequently used in the validation process (Table 2).

MALDI-TOF mass spectra were recorded with LT-microflex mass spectrometers (Bruker) in the mass range from 2000 to 20,000 Da, using the standard mode, according to the manufacturer (Table 1). The commercial MALDI Biotyper version MBT 7854 was used for standard microbial species identification. Mass spectra obtained according to the validation concept described by Rau et al. (2016b) were used for validation. To reduce the workflow, measurements from different laboratories of isolates extensively described in several studies were re-utilized (Eisenberg et al., 2014, 2015; Sting et al., 2015; Berger et al., 2019; Sing et al., 2011; Glawischnig et al., 2018; Suppl. 2). The collection and exchange of further single spectra within this study was organized via MALDI-UP (Rau et al., 2016a). Furthermore, veterinary diagnosticians had been invited to contribute to this project via the web site of the German Veterinary Society (http://avid.dvg.net). To analyze MALDI-TOF mass spectra in detail, ClinProTools software (Bruker, version 3.0) was used. This software provides a separate tool to detect potential mass to charge ratio (m/z) marker signals in the mass profiles of bacteria (Pérez-Sancho et al., 2018).

The creation of reference entries, so called main spectra projections (MSP) followed the instructions and standards of the manufacturer. These procedures and the software used have been described elsewhere in more detail (Pranada et al., 2016; Rau et al., 2016b). For relevant MSP used in this study, a selection of data is given in Table 1. This information has been derived from the MALDI-UP catalogue for every isolate used (Rau et al., 2016a). Single spectra of individual isolates were identified following the actual decision rule of the manufacturer: the first hit must show a score value > 2.0 and include the species and/or subspecies information. The second hit, when showing a score value > 2.0 must not be in conflict to the first hit's nomenclatural

Table 1
Reference mass-spectra (MSP) of isolates belonging to the *C. diphtheriae* group, created within this study. Summarized information for the commercial MALDI Biotyper database (MBT 7854, Bruker) is given.

	MBT 7854: entries (no.)	User made entries used for extension of the MBT 7854 database	MUP entry
C. belfantii	8	*Corynebacterium belfantii* CVUAS 3559,2 CVUAS	1014
C. pseudotuberculosis	7	*Corynebacterium pseudotuberculosis equi* NTTB 992 CVUAS	0935
		Corynebacterium pseudotuberculosis ovis CVUAS 5583.2 CVUAS	0958
C. ulcerans	5	*Corynebacterium ulcerans* NTTB-WBC CVUAS 4292 CVUAS	0083
		Corynebacterium ulcerans NTTB-WBC CVUAS 6455 CVUAS	0922
		Corynebacterium ulcerans TTB CVUAS 10306 CVUAS	0945
		Corynebacterium ulcerans TTB 07UVF148 LLBB	0966
		Corynebacterium ulcerans NT 131011719 CVUAS	1015
		Corynebacterium ulcerans NT 141001548 CVUAS	1021

WBC: WBC variant of nontoxigenic but *tox* gene-bearing *C. ulcerans*. NTTB: nontoxigenic but *tox* gene-bearing; TTB: toxigenic and *tox* gene-bearing; NT: non toxigenic; WBC: WBC variant of *C. ulcerans*; MUP entry: MALDI user platform entry number.

Table 2
Validation results for the assignment of known isolates by MALDI-TOF MS using the MALDI Biotyper MBT 7854 database in comparison with the extended MALDI-user-platform-database.

Biotyper Database MBT 7854										
species	n isolates of species	n of other species (crosscheck)*	Score mean	Score std. deviation	True-positive rate (sensitivity) rate [%]	False-negative	Without decision	True-negative rate* (specificity)	False-positive rate*	Without decision
C. diphtheriae	21	161	2.347	0.118	100	0	0	78.9	0	21.1
C. pseudotuberculosis	32	150	2.325	0.123	100	0	0	77.3	0	22.7
C. ulcerans	103	79	2.166	0.139	88.3	0	11.7	72.2	0	27.8
***other* Corynebacteria**	26	156	1.654	0.375	15.4	0	84.6	92.3	0	7.7
all	182		2.159	0.299	81.3	0	18.7			
WBC isolates ** identified as *C. ulcerans*	48		2.073	0.101	79.2	0	20.8			
Biotyper Database MBT 7854 with addition of personal user-based entries (Table 1 ***)										
C. diphtheriae	21	161	2.367	0.130	100	0	0	98.8	0	1.2
C. pseudotuberculosis	32	150	2.420	0.157	100	0	0	98.7	0	1.3
C. ulcerans	103	79	2.471	0.120	100	0	0	97.5	0	2.5
***other* Corynebacteria**	26	156	2.394	0.218	92.3	0	7.7	100	0	0
all	182		2.439	0.151	98.9	0	1.1			
***C. ulcerans* variants** **										
WBC isolates	48	42	2.510	0.147	100	0	0	100	0	0
non-WBC isolates	42	48	2.435	0.076	100	0	0	100	0	0

* To evaluate the true negative rate and the false positive rate, all divergent spectra from other species/variants were used (crosscheck).
** Only isolates assigned to a cluster by FT-IR (wild boar cluster/WBC, or non-WBC) were used for validation.
*** For the complete list of additional reference entries see MALDI-UP (http://maldi-up.ua-bw.de).

information. Subsequent hits beginning from the third best hit can be neglected in the decision process. A formal validation using mass-spectra from 48 WBC isolates (previously confirmed by FT-IR), 42 non-WBC *C. ulcerans*, toxigenic and non-toxigenic, as well as 53 spectra from other representatives of the *C. diphtheriae* group was performed (Table 2).

3. Results and discussion

Traditionally, *C. diphtheriae* and *C. pseudotuberculosis* have been classified in different biotypes (Araújo et al., 2018). Recently, changes to this taxonomic concept have been suggested based on genomic data. In this respect, Tagini et al. (2018) discussed a division of the species *C. diphtheriae* into two subspecies, while Dazas et al. (2018) proposed to assign *C. diphtheriae* biovar *Belfanti* into a separate species *C. belfantii* (Tagini et al., 2018; Dazas et al., 2018). Similarly, distinct genetic lineages or clusters within the current species *C. ulcerans* have been reported (Subedi et al., 2018; Katsukawa et al., 2016). Thus, a reliable identification of the different species of the *C. diphtheriae* group is particularly important in clinical and veterinary microbiology. This has been confirmed by the ECDC including *C. ulcerans* in the EDSN.

Recently, in Germany a group of NTTB *C. ulcerans* was isolated from game with lymph node abscesses. Isolates of the NTTB type from wild boars were collected by several institutions in various regions of Germany (Suppl. 1). Eisenberg et al. (2014) have already showed that 13 NTTB isolates investigated from 1997 to 2013 form a very homogenous cluster, as observed by FT-IR, by very similar biochemical properties and by their unique and uniform sequences of *rpo*B and *tox* genes (Contzen et al., 2011; Eisenberg et al., 2014).

In the next five years, several institutions in various regions of Germany (Suppl. 1) collected further isolates of the NTTB type from wild boars. These *C. ulcerans* isolates were allocated to the WBC by FT-IR (cf. Eisenberg et al., 2014) and confirmed as *C. ulcerans* of the NTTB-type by the National Consiliar Laboratory on Diphtheria (Konrad et al., 2010; Suppl. 2).

Using MALDI-TOF MS with the commercial MALDI Biotyper (MBT) database 7854, only 79.2% of the 48 confirmed WBC-isolates were directly identified as *C. ulcerans* with score values above 2.0 (Table 2). For the complete dataset of *C. ulcerans* isolates (n = 103), 88.3% were identified at species level. In the MBT version 7854, the identification of *C. ulcerans* and *C. pseudotuberculosis* is accompanied by the information that the "species *pseudotuberculosis / ulcerans* of the genus *Corynebacterium* display very similar patterns and are therefore difficult to be distinguished at species level".

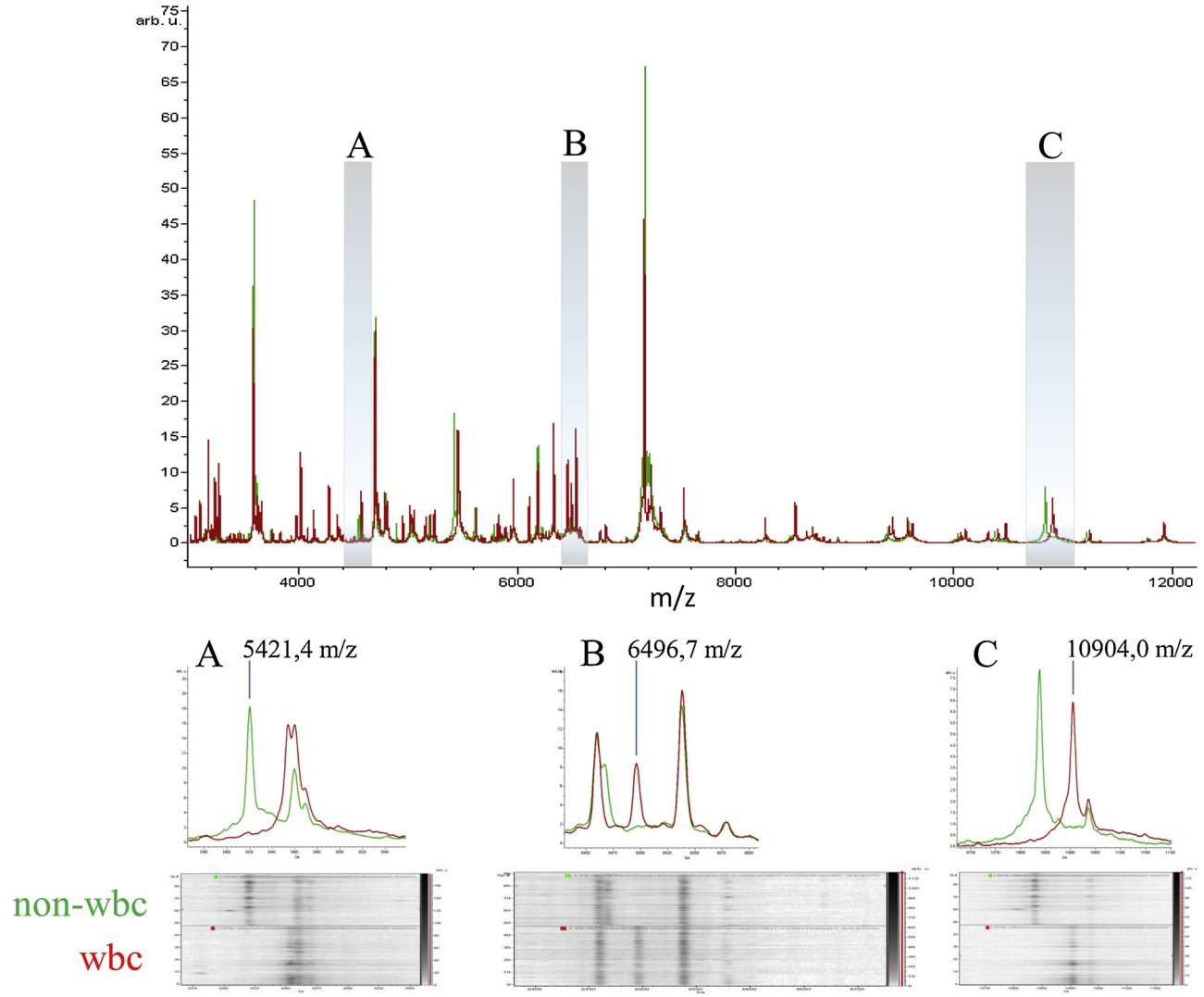

Fig. 1. ClinProTools illustrations: Line 1: Mean mass spectra in the mass to charge ratio (*m/z*) range from 3000 to 12000, obtained from 48 *Corynebacterium ulcerans* isolates of the WBC-variant (red), and from 42 isolates belonging to the non-WBC (green). Exemplary three *m/z* ranges are marked grey (A: 5,400 – 5,700; B: 6,400 – 6,700; C: 10,700 – 11,100). Line 2: Details of the exemplary mass regions A, B and C, showing specific *m/z* signals for both *C. ulcerans* variants. Line 3: Gel view representation obtained from these three ranges of the mass spectra of *C. ulcerans*. WBC: lower part of the diagram; non-WBC: upper part of the diagram.

By using the ClinProTools software (Bruker) several specific *m/z* signals were observed as common in the mass spectra of the 48 WBC isolates, differentiating this variant from the 42 isolates of the non-WBC defined by FT-IR (Fig. 1). Based on the observed mass differences, the creation of appropriate reference spectra was possible. To extend the commercial MBT database 7854 for the special WBC variant of *C. ulcerans*, two additional MSP were created using the well characterized strains CVUAS 4292 and CVUAS 6955, that had been isolated from abscesses of a wild boar and a roe deer, respectively (Contzen et al., 2011; Rau et al., 2012). Furthermore, four non-WBC isolates were used to generate additional reference entries of the non-WBC *C. ulcerans* (Table 1).

Using this extended MALDI-TOF MS database, the success of identification at species level was increased from 88.3% to 100% for all 103 *C. ulcerans* isolates used in this validation. Likewise, the mean score values increased significantly (Table 2).

Using the extended reference database, we did not find any restrictions for the differentiation of *C. ulcerans* and *C. pseudotuberculosis*. This is in contrast to the restrictive note in the Bruker database MBT 7854. The data obtained in this study prove the validity of the method for successful identification of all three species, i.e. *C. diphtheriae* (incl. *C. belfantii)*, *C. ulcerans* and *C. pseudotuberculosis*. This also applies to the differentiation below species level for the WBC and non-WBC *C. ulcerans* variants (Table 2). These further discriminations are particularly relevant for wild boars. However, reports of the isolation of *C. pseudotuberculosis* and *C. ulcerans* from domestic pigs (Oliveira et al., 2014; Schuhegger et al., 2009; this study) and even from a babirusa (*Babyrousa celebensis*) kept in a zoo (this study) indicate that these further discriminations could also be relevant for veterinary diagnostics of pigs (*Suidae*) in general.

The MBT system supports the creation of own entries as well as the exchange with other users through a simple import/export function. This does not only facilitate a timely adaptation of identification of corynebacteria to the expected taxonomic changes in the *C. diphtheriae* group but also offers an option to implement the described differentiation of the two observed variants of *C. ulcerans*. In addition, using spectra of reliably assigned isolates from different laboratories offers the opportunity to significantly extend the database and therefore improve the precision of bacterial identification.

So far, the isolation of corynebacteria of the WBC variant is geographically as patchy as the regional competencies of the institutions participating in this study (Fig. 2). Up to now, the WBC has predominately been isolated from free ranging wild boars, and in one single case from a roe deer. Due to the fact, that available geographical

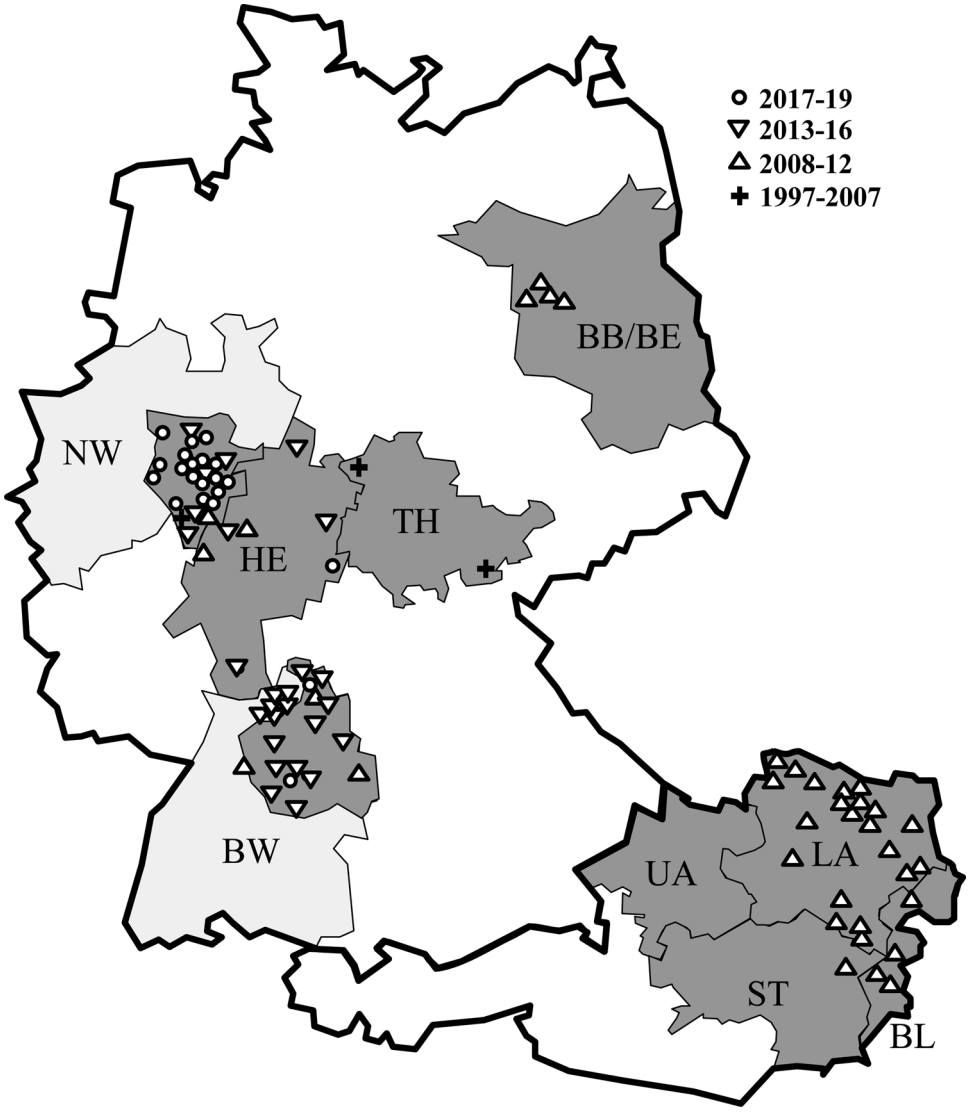

Fig. 2. Map of Germany and Austria with marked localities (accuracy +/- 20 km) where confirmed WBC *C. ulcerans* were isolated. Sample submission regions of the participating institutions are filled. States of Germany: BB: Brandenburg; BE: Berlin; BW: Baden-Wuerttemberg; HE: Hesse; NI: Lower Saxony; NW: North-Rhine-Westphalia; TH: Thuringia; States of Austria: BL: Burgenland; LA: Lower Austria, ST: Styria, UA: Upper Austria.

data are incomplete, we assume a higher percentage of infected or colonized wild animals in, around and between the marked regions. Recently, Glawischnig et al. (2018) reported a high prevalence of 11.4% of *C. ulcerans* in wild boars in parts of Austria. In their study, specifically mandibular lymph nodes from wild boars hunted down routinely in 2011/2012 were tested. The authors observed the same pathological changes of lymph nodes as described by Contzen et al. (2011) in some cases. Using the extended and validated database, the MALDI-TOF mass spectra of 27 *C. ulcerans* isolates from Austria all could be assigned to the WBC variant (Table 1). In contrast, *C. ulcerans* isolates from wild animals from Japan (Katsukawa et al., 2016) do not cluster with the European WBC isolates in *tox*-gene alignment (data not shown).

We expect that overpopulation of wild boars, intensive hunting and the increased awareness of hunters and competent authorities faced by the risk of African swine fever (ASF) in Western Europe, will further increase collection of isolates. Correct identification of these isolates could be accomplished by our reference entries and user-based single spectra supporting validation via MALDI-UP (Rau et al., 2016a).

4. Conclusion

WBC-isolates of *C. ulcerans* can be unequivocally assigned using MALDI-TOF MS, shown by the formal validation concept according to Rau et al., 2016b). Improved diagnostics of isolates of the WBC-variant supported by reliable identification of MALDI-TOF MS analysis will help to gain more insight into the significance of these particular corynebacteria in animals and possibly in humans considering the zoonotic potential of the members of the *Corynebacterium diphtheriae* group. The taxonomic assignment of such isolates has to be clarified in further studies.

Declaration of Competing Interest

None.

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JR conceived the study. RS, MP, PK, TE, HH, AB, AS, HL, MC and JR carried out diagnostics and experiments. JR and MC wrote the manuscript and all the authors read and approved the final text.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108399.

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