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Molecular phylogenetic and matrix-assisted laser desorption ionization time-of-flight mass spectrometry identification of isolates from horses identified as *Enterobacter cloacae* by biochemical identification

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Enterobacter cloacae is an opportunistic pathogen of horses. Thirty isolates obtained from horses and their environments and identified as Enterobacter cloacae by biochemical methods were reidentified by taxonomic identification based on multilocus sequence analysis (MLSA) and by a commercial identification system based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). MLSA identified the 30 equine isolates as E. ludwigii (9/30), E. asburiae (1/30), or E. cloacae (1/30); 19 isolates were not identified. The MALDI-TOF MS system could not clearly distinguish isolates to the species level, and the limited numbers of reference spectra for Enterobacter species might have contributed to the poor identification.

Key words: *Enterobacter species, horse, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), multilocus sequence analysis (MLSA)*

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Bacteria of the genus *Enterobacter* are Gram-negative facultative anaerobes found in the intestines of humans and animals and in environments such as sewage and soil [4]. They are often isolated as pathogens in horses [19], in which they can cause synovial sepsis or urinary tract infections [5, 21]. They are also often isolated as a cause of pneumonia [20].

Accurate identification of *Enterobacter* species has long been regarded as problematic. Biochemical identification methods have traditionally been used. Among them, the API 20E test is the most frequently used [15], but it can give conflicting results because its reference database has less discriminatory power than the 16S rRNA gene sequence database [12]. Although 16S rRNA gene sequencing is one of the gold standards for bacterial identification [3], it often cannot clearly identify *Enterobacter* species because its results do not correlate well with taxonomic classification at the species level despite good identification at the genus level [3, 8]. Taxonomic evaluation based on multilocus sequence analysis (MLSA) is based on the sequencing of partial housekeeping-protein–encoding genes and is able to identify *Enterobacter* isolates to the species level with strong support by MLSA grouping [1].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used for identifying bacterial species through analysis of the molecular spectra obtained from bacterial cells tested and then collating the spectra in a commercially provided database that includes several thousand spectra. MALDI-TOF MS is recognized as a useful tool for the identification of bacterial isolates from animals [16], and further research would help reinforce it as a valid tool in veterinary medicine.

Enterobacter species are opportunistic pathogens in horses. *Enterobacter cloacae* is known among *Enterobacter* species for its heterogeneity in biochemical and molecular studies [13], and it is important in veterinary medicine to identify *Enterobacter* species accurately. Previous studies have reported some problems in using MALDI-TOF MS

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to identify *Enterobacter* species isolated from humans and the environment [9, 17]; however, it is not clear whether this also applies to identification of *Enterobacter* species from horses.

In this study, we used MLSA to re-identify *E. cloacae* isolates from horses and their environments that had originally been identified by commercial biochemical methods. Based on the MLSA identification results, we then evaluated the accuracy and applicability of a commercial MALDI-TOF MS system for the identification of *Enterobacter* organisms isolated from horses and their environments.

We tested 30 isolates from equine patients and unknown sources probably associated with equine patients or their environments obtained between 1983 and 2016 and stored at −80°C (Table 1). All were preliminarily tested with a biochemical test kit that is known to have varying levels of reliability (API 20E Test System, bioMérieux, Durham, NC, U.S.A.). Based on their biochemical characteristics, they were identified as matching *Enterobacter cloacae* in the APIWEB database (bioMérieux), which included 7 *Enterobacter* species (*E. aerogenes*, *E. amnigenus* 1, *E. amnigenus* 2, *E. asburiae*, *E. cancerogenus*, *E. cloacae*, and *E. gergoviae*).

Table 1. List of 30 isolates identified as *Enterobacter cloacae* by API 20E from horses and their environments

Isolate	Year of isolation	Source
E.cloacae-1	1983	Unknown
E.cloacae-4	1983	Unknown
E.cloacae-5	1983	Unknown
E.cloacae-6	1983	Unknown
E.cloacae-7	1983	Unknown
E.cloacae-8	1983	Unknown
E.cloacae-9	1983	Unknown
E.cloacae-10	1983	Unknown
E.cloacae-12	1983	Unknown
E.cloacae-14	1983	Unknown
E.cloacae-15	1983	Unknown
E.cloacae-16	1985	Unknown
E.cloacae-17	1985	Unknown
E.cloacae-18	1988	Diarrhea
E.cloacae-20	1991	Metritis
E.cloacae-21	1991	Metritis
E.coli-160	1992	Sepsis
E.cloacae-22	1995	Guttural pouch infection
Entero-3	1999	Pneumonia
Entero-15	2000	Pneumonia
Entero-17	2000	Guttural pouch infection
Entero-19	2000	Pneumonia
Enter-41	2004	Pneumonia
Entero-89	2010	Pneumonia
Entero-92	2011	Pneumonia
Entero-157	2015	Surgical site infection
Entero-181	2016	Abscess
Entero-190	2016	Pleural effusion
Entero-191	2016	Surgical site infection
Entero-194	2016	Cellulitis

Genomic DNA from the 30 isolates was extracted with a commercial DNA extraction kit (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, U.S.A.) in accordance with the manufacturer's instructions. Partial DNA fragments of *gyrB*, *rpoB*, *infB*, and *atpD* were amplified by PCR as described for MLSA analysis for evaluating *Enterobacter* species [1] (which we call "Enterobacter MLSA" in this study). The products were purified with a purification kit (QIAquick PCR Purification Kit, Qiagen, Venlo, the Netherlands). DNA sequencing was outsourced to a commercial service (Greiner Bio-One, Tokyo, Japan). Obtained sequences of the 30 isolates and published sequences of type or reference strains (GenBank accession numbers JX424847–JX424873, JX424882–424888, JX424977–JX425003, JX425012–425018, JX425106–JX425132, JX425141–JX425147, JX425236–JX425262, and JX425271–JX425277) were aligned with ClustalW, and a phylogenetic tree was constructed by the neighbor-joining method with a maximum composite likelihood model using the MEGA 7.0 software [10]. It is reported that reference strains and type strains of *Enterobacter* species form clusters in MLSA trees [1], and we identified the isolates according to the MLSA phylogenetic tree for *Enterobacter* species; isolates included in the same cluster as a type strain were identified as that species. In this study, identification with Enterobacter MLSA was regarded as the gold standard.

For identification using a commercial MALDI-TOF MS system (MALDI Biotyper CA System, Bruker Japan, Yokohama, Japan), isolates were plated on Columbia agar supplemented with 5% horse blood and incubated at 37°C for 24 hr. Each colony was spotted onto a target plate and overlaid with 1 μl of 70% formic acid and 1 μl of α-cyano-4 hydroxycinnamic acid (HCCA) matrix solution. Mass spectra were obtained using the MALDI-TOF MS apparatus and were analyzed with the commercial database v. 8.0.0 of the MALDI Biotyper CA System, which holds the following spectra of *Enterobacter* species: 6 of *E. amnigenus*, 1 of *E. cancerogenus*, 3 of *E. asburiae*, 14 of *E. cloacae,* 1 of *E. hormaechei*, 1 of *E. cowanii,* 11 of *E. gergoviae*, 1 of *E. helveticus*, 1 of *E. kobei*, 1 of *E. ludwigii*, 3 of *E. pulveris*, 2 of *E. pyrinus*, 1 of *E. radicincitans*, and 1 of *E. turicensis*. Spectral data were obtained from two independent assays. The identification system proposes two candidate species for the tested organism—a best-match species and a second-best-match species—with scores indicating the identification probability. In accordance with the manufacturer's instructions, these probability scores can be interpreted as follows: ≥2.00, high-confidence identification (reliable identification at the species level); ≥1.70 to <2.00, low-confidence identi-

fication (identification at the genus level); <1.70, no known organism (unidentifiable). According to the manufacturer's instructions, the consistency of identification is also to be considered. The consistency of identification was evaluated as "high", "low," or "not applicable" based on the scores of the best-match and second-best-match species, as shown in Table 2.

From the phylogenetic tree constructed on the basis of MLSA, 9 of the 30 isolates (E.cloacae-1, E.cloacae-4, E.cloacae-6, E.cloacae-7, E.cloacae-8, E.cloacae-9, E.cloacae-12, E.cloacae-14, and Entero-89) fell into the cluster with the type strain of *E. ludwigii* (strain LMG 23768^T) and were thus identified as *E. ludwigii* (Fig. 1). Enter-41 was identified as *E. cloacae* because it fell into the cluster including the type strain of *E. cloacae* (strain LMG 2783^T), and E.cloacae-15 was identified as *E. asburiae* because it fell into the cluster including the type strain of *E. asburiae* (strain DSM 17506^T). Among *Enterobacter* species, *E. cloacae*, *E. asburiae*, *E. hormaechei*, *E. kobei*, and *E. ludwigii* are known as the "*Enterobacter cloacae* complex" [13]. They share a major part of their DNA with *E. cloacae* [6], and they are thus difficult to distinguish by 16S rRNA sequencing alone and require other types of genetic investigation, such as MLSA. Enterobacter MLSA, which evaluates 4 protein-encoding genes, can address these taxonomic issues in the genus *Enterobacter* [1] and can delineate species into well-defined clades, helping to identify strains to the species level. MLSA is expected to become an effective method for identifying *Enterobacter* species and may also be useful for identifying related isolates [7, 11]. In fact, E.cloacae-15, which was identified as *E. asburiae* with Enterobacter MLSA, was identified as *E. cloacae* with the biochemical test, even though the biochemical test kit used in this study is capable of identifying *E. asburiae*. The phenotypic characteristics of *E. asburiae* are similar to those of *E. cloacae,* and some *E. asburiae* isolates have been misidentified as *E. cloacae* with biochemical tests [14]. Furthermore, according to the manufacturer's instructions for the biochemical test kit, *E. cloacae* and *E. asburiae* do not have biochemical characteristics by which they can be distinguished from each other; therefore, E.cloacae-15 might have been misidentified as *E. cloacae* in this study.

Nineteen isolates were not able to be identified with Enterobacter MLSA, and 15 of those fell into 4 clades in the phylogenetic tree (Fig. 1). Two isolates (E.cloacae-5 and E.cloacae-17) were included in clade 1, and 3 isolates (E.cloacae-22, Entero-15, and Entero-92) were included in clade 4. Isolates in clade 1 and clade 4 were classified into branches separate from the clusters including type strains of *E. cloacae* and *E. asburiae* with high bootstrap values, respectively, even though the strains and the clusters shared the same root, which suggests that these groups were closely related to each species, and they were considered *E. cloacae*–related strains and *E. asburiae*–related strains, respectiverly, in the Enterobacter MLSA. Ten isolates (E.cloacae-21, E.coli-160, Entero-17, Entero-19, Entero-157, Entero-191, E. cloacae-18, E.cloacae-20, Entero-3, and Entero-194) were included in a branch that could be

Table 2. Conditions and examples for evaluating the consistency of identification

Consistency	Conditions of identification results	Examples	
		Best-match species (Score)	Second-best-match species (Score)
High	The best match was a high-confidence identification, and the second-best-match was a high-confidence identification in which the species was identical to the best match.	Genus (A) species (a) (≥2.00)	Genus (A) species (a) (≥2.00)
	The best match was a high-confidence identification, and the second-best-match was a low-confidence identification in which the species or genus was identical to the best match.	Genus (A) species (a) (≥2.00)	Genus (A) species (a) (≥1.70 to <2.00)
	The best match was a high-confidence identification, and the second-best-match was no known organism.	Genus (A) species (a) (≥2.00)	-** (<1.70)
Low	The best and second-best-match results were high-confidence identifications with unidentical species but the identical genus.	Genus (A) species (a) (≥2.00)	Genus (A) species (b) (≥2.00)
	The best and second-best-match results were low-confidence identifications with the identical genus.	Genus (A) species (a) (≥1.70 to <2.00)	Genus (A) species (a, b...) (≥1.70 to <2.00)
	The best match result was a low-confidence identification, and the second-best-match result was no known organism.	Genus (A) species (a) (≥1.70 to <2.00)	- (<1.70)
NA*	Both the best-match and second-best-match results were high- or low-confidence identifications with different genera.	Genus (A) species (a)	Genus (B) species (b)
	The best-match result was no known organism.	(<1.70)	(<1.70)

*Consistency was not applied. **No known organism.

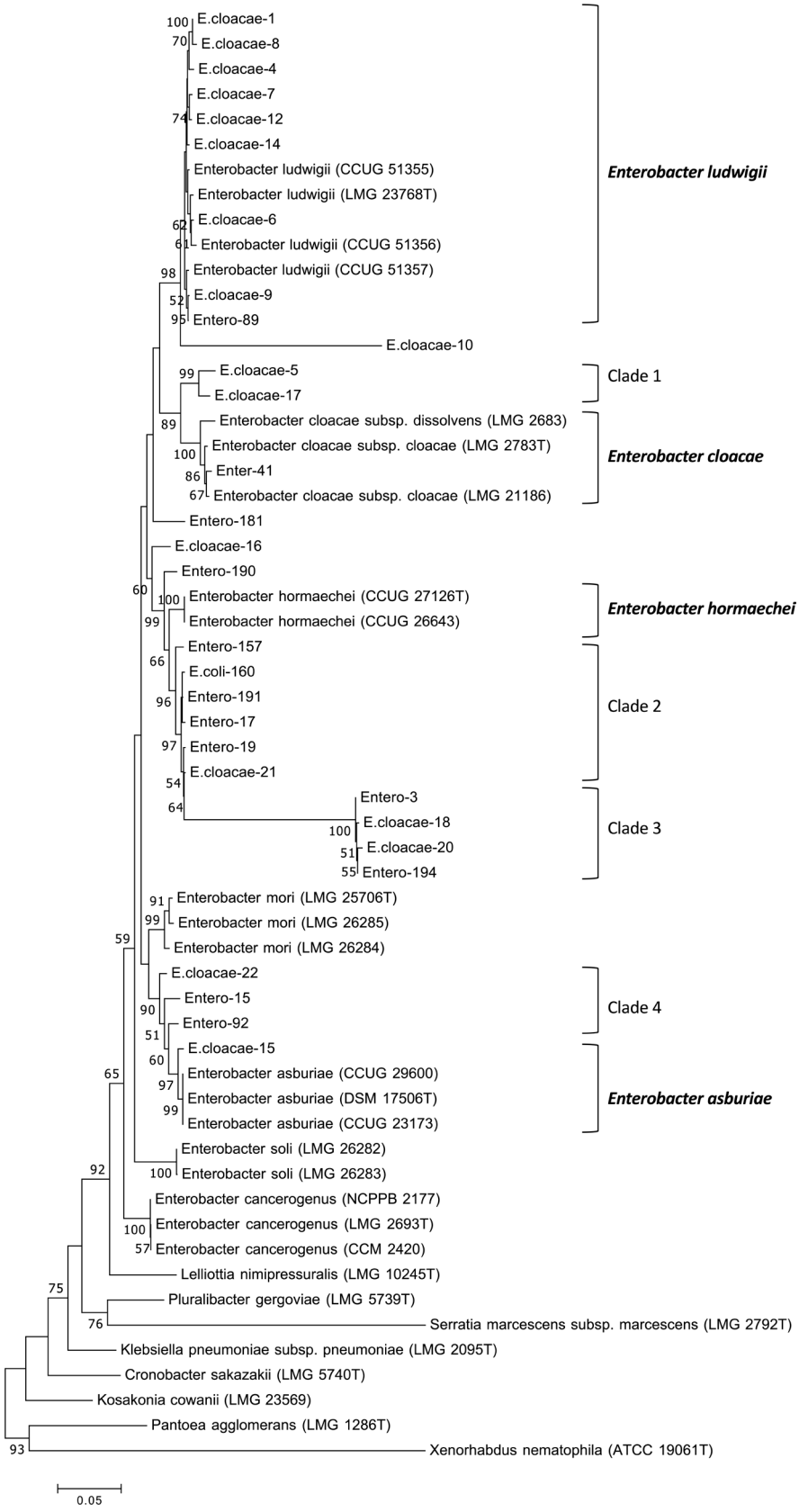

Fig. 1. A maximum likelihood tree of 20 type or reference strains of *Enterobacter* species, 8 strains of related genera, and the 30 isolates shown in Table 1 was constructed on the basis of *gyrB*, *rpoB*, *atpD*, and *infB* sequencing. Bootstrap values of 1,000 replicates are expressed as percentages.

separated into clade 2 and clade 3 with a comparatively long branch length (0.135). Isolates in clade 2 and clade 3 were suggested to be closely related to *E. hormaechei* because they were classified into branches separate from the cluster including the type strain of *E. hormaechei* (strain CCUG 27126^T), and isolates in clade 2 and clade 3 were called *E. hormaechei*–related strain 1 and *E. hormaechei*–related strain 2 in this study. The 4 isolates named as *E. hormaechei*–related strain 2 had a deletion of 5 bps in the middle of the *rpoB* gene, which might make the 4 isolates genetically separate from *E. hormaechei*–related strain 1.

E.cloacae-10 was classified as a branch separate from the clade including *E. ludwigii* with a high bootstrap value and had a comparatively long branch length (0.159). E.cloacae-10 had 2 deletions and 2 insertions in the *infB* gene. E.cloacae-16 and Entero-181 were not assigned to any clades including type strains of *Enterobacter* species. Entero-190 was assigned to the node including the type strain of *E. hormaechei* but was not included in clade 2 or clade 3, which suggests that Entero-190 might be a species related to *E. hormaechei*. The above results indicate that further taxonomic studies are required on the 19 strains not able to be identified by Enterobacter MLSA.

Thirty isolates were evaluated by the MALDI Biotyper CA System (Table 3). Of those, only 12 were identified as *E. cloacae* with high consistency, whereas the remaining 18 could not be identified because of low consistency. Moreover, only one (Enter-41) of those 12 isolates was identified by Enterobacter MLSA as *E. cloacae*, and the remaining 11 isolates were not identified by Enterobacter MLSA. We suggest two possible explanations for this poor identification by the MALDI Biotyper CA System. First, the genetic closeness of *Enterobacter* species would make them difficult to distinguish by MALDI-TOF MS. *Enterobacter* species are known to be close phylogenetically [1]; in particular, clusters including *E. ludwigii* and *E. cloacae* share the same

Table 3. Identification results of 30 isolates by the matrix-assisted laser desorption ionization (MALDI) Biotyper CA System

Isolate	Enterobacter MLSA	MALDI Biotyper CA System				
		Best match		Second-best match		Consistency
		Species	Score	Species	Score	
E.cloacae-1	*Enterobacter ludwigii*	*E. ludwigii*	2.340	*E. cloacae*	2.280	Low
E.cloacae-4	*E. ludwigii*	*E. cloacae*	2.345	*E. ludwigii*	2.285	Low
E.cloacae-6	*E. ludwigii*	*E. cloacae*	2.440	*E. ludwigii*	2.295	Low
E.cloacae-7	*E. ludwigii*	*E. ludwigii*	2.375	*E. cloacae*	2.375	Low
E.cloacae-8	*E. ludwigii*	*E. cloacae*	2.380	*E. ludwigii*	2.375	Low
E.cloacae-9	*E. ludwigii*	*E. ludwigii*	2.295	*E. cloacae*	2.220	Low
E.cloacae-12	*E. ludwigii*	*E. cloacae*	2.380	*E. ludwigii*	2.335	Low
E.cloacae-14	*E. ludwigii*	*E. ludwigii*	2.385	*E. cloacae*	2.320	Low
Entero-89	*E. ludwigii*	*E. ludwigii*	2.360	*E. cloacae*	2.325	Low
Enter-41	*E. cloacae*	*E. cloacae*	2.265	*E. cloacae*	2.195	High
E.cloacae-15	*E. asburiae*	*E. asburiae*	2.290	*E. cloacae*	2.125	Low
E.cloacae-5	Clade 1	*E. cloacae*	2.265	*E. asburiae*	2.390	Low
E.cloacae-17	Clade 1	*E. asburiae*	2.225	*E. kobei*	2.115	Low
E.cloacae-21	Clade 2	*E. cloacae*	2.330	*E. cloacae*	2.215	High
E.coli-160	Clade 2	*E. cloacae*	2.315	*E. cloacae*	2.290	High
Entero-17	Clade 2	*E. cloacae*	2.320	*E. cloacae*	2.295	High
Entero-19	Clade 2	*E. cloacae*	2.310	*E. cloacae*	2.270	High
Entero-157	Clade 2	*E. cloacae*	2.380	*E. cloacae*	2.255	High
Entero-191	Clade 2	*E. cloacae*	2.335	*E. cloacae*	2.300	High
E.cloacae-18	Clade 3	*E. cloacae*	2.335	*E. cloacae*	2.200	High
E.cloacae-20	Clade 3	*E. cloacae*	2.385	*E. cloacae*	2.290	High
Entero-3	Clade 3	*E. cloacae*	2.285	*E. cloacae*	2.250	High
Entero-194	Clade 3	*E. cloacae*	2.335	*E. cloacae*	2.315	High
E.cloacae-22	Clade 4	*E. asburiae*	2.340	*E. cloacae*	2.310	Low
Entero-15	Clade 4	*E. asburiae*	2.345	*E. cloacae*	2.195	Low
Entero-92	Clade 4	*E. asburiae*	2.300	*E. cloacae*	2.085	Low
E.cloacae-10	Other isolates	*E. ludwigii*	2.350	*E. cloacae*	2.340	Low
E.cloacae-16	Other isolates	*E. asburiae*	2.220	*E. cloacae*	2.160	Low
Entero-181	Other isolates	*E. kobei*	2.190	*E. asburiae*	2.170	Low
Entero-190	Other isolates	*E. cloacae*	2.420	*E. cloacae*	2.335	High

MLSA, multilocus sequence analysis.

root in the MLSA phylogenetic tree (Fig. 1), which probably confirms their closeness. With *Enterobacter* species that are genetically close, the molecules targeted in MALDI-TOF MS would have protein structures similar to each other. Indeed, the molecular spectra of *E. asburiae, E. cloacae, E. hormaechei, E. kobei*, and *E. ludwigii* in the commercial database were in fact similar to each other (data not shown). Therefore, they might not be correctly distinguished from each other with the MALDI Biotyper CA System. Second, the reference library holds only one spectrum each for *E. cancerogenus*, *E. hormaechei*, *E. cowanii*, *E. helveticus*, *E. kobei*, *E. ludwigii*, *E. radicincitans*, and *E. turicensis.* The overall accuracy of identifying species by MALDI-TOF MS might depend on the number of species in the database used for identification, and a library with multiple patterns of a given species would enable MALDI-TOF MS to better distinguish species that are genetically similar [18]. Poor database composition and depth could lead to unreliable identification by MALDI-TOF MS [9]. An enriched database that contains multiple patterns of each species may recognize minor spectral differences between strains and thus result in more accurate identification. A previous study suggested that enriching the database used for identification enhanced the accuracy and rapidity of identification of yeast by MALDI-TOF MS [2]; therefore, complementing the reference database with the MLSA results of *Enterobacter* species might render the MALDI Biotyper CA System a more reliable identification tool.

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