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)

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
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*).

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–JX424888, JX424977–JX425003, JX425012–JX425018, 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 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 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. kobel, 1 of *E. ludwigi, 3 of *E. pulveris, 2 of *E. pyrinus, 1 of *E. radicinicans, 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-

| 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. 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 |
| Entero-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 |
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 including the type strain of E. ludwigii (strain LMG 23768T) 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. asburiae (strain DSM 17506T), and E.cloacae-15 was identified as E. asburiae because it fell into the cluster including the type strain of E. kobei (strain LMG 157, Entero-191, E. cloacae-18, E.cloacae-20, Entero-3, E. hormaechei, 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...
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.
 ANALYSIS OF EQUINE ENTEROBACTER

Entero-190 was assigned to the node including the type clades including type strains of gene. E. cloacae-16 and Entero-181 were not assigned to E. hormaechei –related strain 1 and E. cloacae-10 had 2 deletions and 2 insertions in the value and had a comparatively long branch length (0.159). E. ludwigii with a high bootstrap 27126T), and isolates in clade 2 and clade 3 were called E. hormaechei–related strain 1 and 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

| Isolate    | Enterobacter MLSA | MALDI Biotyper CA System |                      |                      | Consistency |
|------------|-------------------|--------------------------|----------------------|----------------------|-------------|
|            |                   |                          | Best match           | Second-best match    |             |
|            |                   |                          | Species | Score    | Species | Score    |             |
| E. cloacae-1 | Entero. 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. cloacae               | 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. cloacae               | 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. cloacae               | 2.385     |          | E. cloacae | 2.320 | Low     |
| Entero-89   | E. ludwigii       | E. cloacae               | 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-19   | Clade 2           | E. cloacae               | 2.320     |          | E. cloacae | 2.295 | High    |
| Entero-190  | Clade 2           | E. cloacae               | 2.310     |          | E. cloacae | 2.270 | High    |
| Entero-191  | Clade 2           | E. cloacae               | 2.380     |          | E. cloacae | 2.255 | High    |
| Entero-194  | Clade 3           | E. cloacae               | 2.335     |          | E. cloacae | 2.300 | High    |
| Entero-194  | Clade 3           | E. cloacae               | 2.335     |          | E. cloacae | 2.200 | High    |
| Entero-194  | Clade 3           | E. cloacae               | 2.385     |          | E. cloacae | 2.290 | High    |
| Entero-15   | Clade 4           | E. asburiae              | 2.340     |          | E. cloacae | 2.310 | Low     |
| Entero-92   | Clade 4           | E. asburiae              | 2.345     |          | E. cloacae | 2.195 | Low     |
| Entero-100  | Clade 4           | E. asburiae              | 2.300     |          | E. cloacae | 2.085 | Low     |
| Entero-100  | Other isolates    | E. ludwigii              | 2.350     |          | E. cloacae | 2.340 | Low     |
| Entero-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.

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