Rapid Subtyping of *Yersinia enterocolitica* by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) for Diagnostics and Surveillance

Kristina Rizzardi, Tara Wahab, Cecilia Jernberg

Department of Diagnostics and Vaccinology, Swedish Institute for Communicable Disease Control (SMI), Solna, Sweden

In this study, an alternative to the current traditional bioserotyping techniques was developed for subtyping *Y. enterocolitica* using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The most common pathogenic bioserotypes could easily be distinguished using only a few bioserotype-specific biomarkers. However, biochemical methods should still be used to distinguish biotype 1A from 1B.

The genus *Yersinia* comprises a biochemically heterogeneous group, including 17 different species (1). Three of these are regarded as pathogenic for humans; *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. The last two can cause yersiniosis, which is a zoonotic disease where the identified source of infection most often has been contaminated foods. *Y. enterocolitica* is the most common cause of yersiniosis and can be classified into six different biotypes on the basis of their phenotypic characteristics. Five of these, 1B, 2, 3, 4, and 5, include strains that are pathogenic (2). Biotype 1A, which is the most heterogeneous one, is generally considered to be nonpathogenic; however, a few studies indicate that some strains might cause gastroenteritis (3–6). The six biotypes are further divided by serotyping into more than 50 different O groups, defined on the basis of their O-surface antigen in the lipopolysaccharide membrane (7). Human yersiniosis is mainly associated with bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, and 4/O:3. The predominant pathogenic bioserotypes are geographically distinct (8–11). In Sweden yersiniosis is a reportable disease, with between 300 and 700 cases reported annually. The majority of the

TABLE 2 Clinical isolates used in this study

Biotype	Serotype	No. of isolates
1A	ND ^a	139
1B	O:8	4
2	O:9	20
2	O:5,27	11
4	O:3	20

^a ND, not determined.

cases have domestically acquired infections (75%), and young children dominate (28% between 0 and 4 years old).

Determining the pathogenicity of clinical isolates is crucial for diagnostics, since the majority of *Y. enterocolitica* bioserotypes are nonpathogenic and since nonpathogenic isolates can also be found in asymptomatic carriers in addition to symptomatic patients with gastroenteritis symptoms caused by other agents. Since May 2012 Sweden has a new case definition regarding a laboratory-confirmed case, where biotype 1A has to be excluded when reporting yersiniosis. This would, according to the traditional methods, include the esculin hydrolysis test and/or pyrazinamide hydrolysis test to confirm a pathogenic *Y. enterocolitica* isolate.

Since matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for bacterial identification is both reliable for *Y. enterocolitica* identification (12, 13) and nowadays adopted in many clinical laboratories and hospitals, we have developed and validated a protocol for differentiation between the predominant pathogenic and nonpathogenic *Y. enterocolitica* bioserotypes using the commercial Microflex system with MALDI Biotyper 3.1 software (Bruker Daltonics, Billerica, MA), which can easily be applied and performed by users with limited knowledge of mass spectrometry.

TABLE 1 List of reference strains used in this study

CCUG no.	Biotype	Serotype
8235	1A	O:5
8236	1A	O:6
8237	1A	O:7
8244	1A	O:14
8249	1A	O:19
8251	1A	O:21
8252	1A	O:22
29125	1A	O:7,13
8240	1A	O:10
8238	1B	O:8
8243	1B	O:13
8248	1B	O:18
8250	1B	O:20
12369	1B	O:8
4588	2	O:9
8245	3	O:15
33553	3	O:1(2a,3)
8231	3	O:1
8233	4	O:3
8232	5	O:2,3

Received 13 June 2013 Returned for modification 12 July 2013

Accepted 7 September 2013

Published ahead of print 18 September 2013

Address correspondence to Cecilia Jernberg, cecilia.jernberg@smi.se.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.01416-13>.

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doi:10.1128/JCM.01416-13

TABLE 3 Average intensities and occurrences of the biomarkers needed to identify the most common pathogenic bioserotypes of *Y. enterocolitica*, analyzed with EtOH/FA extraction and ICMS

Observed mass (Da) (<i>m/z</i> ± 500 ppm) ^a	Parameter	Avg value ^b for bioserotype:				
		1A/1B (<i>n</i> = 97)	1A/1B O:21 (<i>n</i> = 46)	2/O:5,27 (<i>n</i> = 11)	2/O:9 (<i>n</i> = 20)	4/O:3 (<i>n</i> = 20)
2,212.5 ± 1.1	EtOH/FA occurrence	—	—	100%	100%	100%
	EtOH/FA intensity ± SD	—	—	3,666 ± 43%	6,683 ± 50%	4,227 ± 37%
	ICMS occurrence	—	—	100%	100%	100%
	ICMS intensity ± SD	—	—	6,014 ± 59%	9,246 ± 37%	10,062 ± 39%
3,865 ± 1.7*†	EtOH/FA occurrence	—	85%	—	—	—
	EtOH/FA intensity ± SD	—	4,039 ± 36%	—	—	—
	ICMS occurrence	—	78%	—	—	—
	ICMS intensity ± SD	—	3,256 ± 85%	—	—	—
3,872 ± 1.7†	EtOH/FA occurrence	100%	—	100%	100%	—
	EtOH/FA intensity ± SD	5,938 ± 41%	—	4,828 ± 40%	3,963 ± 38%	—
	ICMS occurrence	100%	—	53%	100%	—
	ICMS intensity ± SD	2,713 ± 52%	—	2,650 ± 67%	2,025 ± 64%	—
4,921 ± 2.5†	EtOH/FA occurrence	100%	76%	95%	—	—
	EtOH/FA intensity ± SD	2,211 ± 41%	2,224 ± 44%	2,496 ± 33%	—	—
	ICMS occurrence	64%	35%	27%	—	—
	ICMS intensity ± SD	1,075 ± 33%	1,533 ± 61%	1,378 ± 31%	—	—
4,935 ± 2.5†	EtOH/FA occurrence	—	—	—	83%	96%
	EtOH/FA intensity ± SD	—	—	—	1,649 ± 32%	2,317 ± 34%
	ICMS occurrence	—	—	—	39%	96%
	ICMS intensity ± SD	—	—	—	989 ± 22%	1,367 ± 48%
5,830 ± 2.5	EtOH/FA occurrence	—	—	86%	—	81%
	EtOH/FA intensity ± SD	—	—	953 ± 30%	—	833 ± 19%
	ICMS occurrence	—	—	0%	—	26%
	ICMS intensity ± SD	—	—	—	—	752 ± 23%
5,845 ± 2.9	EtOH/FA occurrence	—	—	—	75%	—
	EtOH/FA intensity ± SD	—	—	—	643 ± 6%	—
	ICMS occurrence	—	—	—	0%	—
	ICMS intensity ± SD	—	—	—	—	—
7,730 ± 3.7*	EtOH/FA occurrence	—	100%	—	—	—
	EtOH/FA intensity ± SD	—	16,302 ± 29%	—	—	—
	ICMS occurrence	—	100%	—	—	—
	ICMS intensity ± SD	—	11,556 ± 70%	—	—	—
7,745 ± 3.7	EtOH/FA occurrence	100%	—	100%	100%	—
	EtOH/FA intensity ± SD	19,947 ± 29%	—	19,717 ± 26%	17,618 ± 41%	—
	ICMS occurrence	100%	—	60%	100%	—
	ICMS intensity ± SD	18,059 ± 36%	—	15,072 ± 43%	13,491 ± 32%	—
9,840 ± 4.7	EtOH/FA occurrence	100%	95%	100%	—	—
	EtOH/FA intensity ± SD	3,514 ± 41%	3,416 ± 44%	4,085 ± 34%	—	—
	ICMS occurrence	50%	65%	47%	—	—
	ICMS intensity ± SD	1,875 ± 54%	1,533 ± 61%	2,138 ± 43%	—	—
9,867 ± 4.7	EtOH/FA occurrence	—	—	—	100%	100%
	EtOH/FA intensity ± SD	—	—	—	2,804 ± 41%	4,047 ± 33%
	ICMS occurrence	—	—	—	87%	100%
	ICMS intensity ± SD	—	—	—	1,390 ± 44%	2,621 ± 36%

^a *, biomarker also found in bioserotypes 1B O:13, 1A O:7,13, and 1A O:7,8; †, doubly charged ion.^b Intensity values are in arbitrary units; standard deviations are percentages of the arbitrary units. —, no peak could be detected.

The protocol was generated by analyzing the spectra of 194 clinical isolates and 20 reference strains of *Y. enterocolitica* in triplicate culture plates and extracts (Tables 1 and 2). The spectra were collected with Biotyper RTC, both after ethanol/formic acid (EtOH/FA) extraction and intact-cell mass spectrometry (ICMS) using standard protocols (14). All strains in the study were cultured on Columbia blood agar base plates without blood at 22°C for 24 h; clinical strains were isolated from Swedish patients between 1996 and 2013. Species identification and bioserotype were determined by standard biochemical tests, with an agglutination

assay for serotype determination (antisera O:3 and O:9, Reagensia AB, Sweden; antisera O:5,27 and O:8, Sifin, Germany) (15, 16).

Initially an in-house *Yersinia* library was created following Bruker's protocol with the reference strains obtained from the University of Göteborg Culture Collection (CCUG) to assess whether an increased amount of bioserotype-specific spectra could discriminate between subtypes. Screening the clinical isolates against the Bruker library and the in-house library showed that the Biotyper software could not correctly discriminate between bioserotypes. In order to produce a protocol that could be

FIG 1 *Y. enterocolitica* bioserotype identification flow chart. A simplified flow chart for detection of the most common pathogenic bioserotypes is shaded in gray. Pyz/Esc test, pyrazinamide and/or esculin hydrolysis test, recommended for biotype determination. †, doubly charged ions.

applied in routine diagnostics, we searched for bioserotype-specific biomarkers using the FlexAnalysis software. Peak selection was performed using a centroid algorithm, a signal-to-noise (S/N) ratio of 5, a minimum intensity threshold of 250, top hat baseline subtraction, and Savitzky/Golay smoothing. A study by Stephan et al. (17) addressed this problem in a similar way; however, that study does not include biotype 1B or differentiate between bioserotypes 2/O:5,27 and 2/O:9. Furthermore, the protocol is complex, and performing it for routine diagnostics requires extensive knowledge of mass spectrometry and database manipulation.

By analyzing the CCUG reference strains and the clinical isolates, we found biomarkers that are specific to one or more bioserotypes of *Y. enterocolitica*. In order to develop the least laborious and most reliable protocol, we compared the peak intensities and occurrences of specific biomarkers needed to identify the most common bioserotypes both with EtOH/FA extraction and ICMS. As shown in Table 3, peak intensities and occurrences were lower after ICMS. Thus, initial species identification can be performed by ICMS, but subsequent subtyping should be done using EtOH/FA extractions with a total spectral intensity above 10,000 arbitrary units (AU).

A correct identification of the common pathogenic bioserotypes 2/O:9, 2/O:5,27, and 4/O:3 was obtained from just a few serotype-specific biomarkers. We developed a flow chart (Fig. 1) for identification of the most prevalent pathogenic bioserotypes of *Y. enterocolitica*. A more detailed view of the spectral differences

for the biomarkers needed to identify the most common bioserotypes of *Y. enterocolitica* is shown in Fig. S1 in the supplemental material.

A set of 63 *Yersinia* sp. isolates was used to independently validate the protocol (see Table S1a in the supplemental material). The results showed a 100% correlation between the MALDI-TOF assay and the traditional methods, within the limitations of the MALDI-TOF protocol.

Interestingly, within the cohort used to generate the protocol we found spectral outliers. Seven isolates contained biomarkers from more than one bioserotype. Following further isolation and reanalysis of individual colonies, we could determine that these were cocultures. Four had erratically been subtyped only to biotype 1A. However, in a coculture containing both biotype 1A and a pathogenic biotype, 1A masks the pathogenic biotypes by giving a positive result in the esculin and/or pyrazinamidase hydrolysis test (see Table S1b in the supplemental material).

The clinical isolates that had been subtyped to bioserotype 1B/O:8 showed a broad heterogeneity in their spectra. However, by comparing these with the spectra from the reference strains we could conclude that three out of four of the isolates of biotype 1B were actually bioserotype 1B/O:21. Of the 132 isolates of biotype 1A, we found 44 that were bioserotype 1A/O:21. The reference strain for 1A/O:21 and the clinical isolates of 1A/O:21 and 1B/O:21 had all been serotyped to O:8 by agglutination. This indicates that the antiserum for bioserotype 1B/O:8 cross-reacts with that

for serotype O:21 within biotypes 1A and 1B, as assessed by MALDI-TOF MS.

This gives a new perspective on the epidemiological picture of *Y. enterocolitica* infections in Sweden and possibly in other countries where biotype 1B is predominant. In order to further assess the possible cross-reactivity, agglutination with O:21 antiserum has to be done. This protocol can, therefore, not only be applied for diagnostic purposes but also be a tool for epidemiological surveillance.

Due to the lack of well-characterized reference strains and the small number of clinical isolates of biotypes 1B, 3, and 5, in Sweden, we cannot determine with certainty the specificity of the serotype-specific biomarkers detected in these biotypes, although the biomarkers of these isolates were completely absent from all the isolates of biotype 1A. We encourage other reference laboratories to adopt, validate further, and improve this protocol.

In this work we have elaborated a protocol for use in routine diagnostics for bioserotype differentiation of *Y. enterocolitica*. The total time required for obtaining a correct identification at species and bioserotype level is less than 30 min using the EtOH/FA extraction protocol.

ACKNOWLEDGMENTS

We acknowledge the Swedish Clinical Laboratories that isolated the strains included in the study. We also thank Anneli Rasmusson, Kristina Schönmeier, and Eva Borg for assistance in bio- and serotyping the isolates.

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