

Identification of *Yersinia enterocolitica* at the Species and Subspecies Levels by Fourier Transform Infrared Spectroscopy[∇]

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Yersinia enterocolitica and other *Yersinia* species, such as *Y. pseudotuberculosis*, *Y. bercovieri*, and *Y. intermedia*, were differentiated using Fourier transform infrared spectroscopy (FT-IR) combined with artificial neural network analysis. A set of well defined *Yersinia* strains from Switzerland and Germany was used to create a method for FT-IR-based differentiation of *Yersinia* isolates at the species level. The isolates of *Y. enterocolitica* were also differentiated by FT-IR into the main biotypes (biotypes 1A, 2, and 4) and serotypes (serotypes O:3, O:5, O:9, and “non-O:3, O:5, and O:9”). For external validation of the constructed methods, independently obtained isolates of different *Yersinia* species were used. A total of 79.9% of *Y. enterocolitica* sensu stricto isolates were identified correctly at the species level. The FT-IR analysis allowed the separation of all *Y. bercovieri*, *Y. intermedia*, and *Y. rohdei* strains from *Y. enterocolitica*, which could not be differentiated by the API 20E test system. The probability for correct biotype identification of *Y. enterocolitica* isolates was 98.3% (41 externally validated strains). For correct serotype identification, the probability was 92.5% (42 externally validated strains). In addition, the presence or absence of the *ail* gene, one of the main pathogenicity markers, was demonstrated using FT-IR. The probability for correct identification of isolates concerning the *ail* gene was 98.5% (51 externally validated strains). This indicates that it is possible to obtain information about genus, species, and in the case of *Y. enterocolitica* also subspecies type with a single measurement. Furthermore, this is the first example of the identification of specific pathogenicity using FT-IR.

The genus *Yersinia* belongs to the bacterial family *Enterobacteriaceae* and encompasses three well-known human pathogens: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. Pathogenic strains of *Y. enterocolitica* cause yersiniosis, an acute enteric disease. In Germany and Switzerland, strains of *Y. enterocolitica* belong to the most frequently isolated pathogens connected with bacterial gastroenteritis (27, 31). *Y. enterocolitica* also causes other clinical syndromes, such as enterocolitis, acute mesenteric lymphadenitis, mimicking appendicitis, postinfectious arthritis, and systemic infections (7, 21). It is assumed that the main contamination source is food of animal origin, especially pork meat or raw milk (8, 21, 27). Therefore, the focus of diagnosis for these bacteria as food-borne pathogens includes the examination of food samples in food inspection and veterinary controls of livestock.

The species *Y. enterocolitica* sensu lato as described by Frederiksen (9) was recently subdivided into several species: *Y. enterocolitica* sensu stricto, *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. mollaretii*, *Y. rohdei*, and *Y. bercovieri* (20). The identification of *Y. enterocolitica* sensu stricto by traditional agar plate techniques (ISO standard 10273:2003) is complicated by the fact that on the commonly used selective agar plates, especially the cefsulodin-irgasan-novobiocin (CIN) agar, several unrelated bacteria also grow (1, 20). In addition, some *Yersinia* strains are inhibited by CIN agar (10). The differentiation of putative *Yersinia* strains isolated from the

CIN agar is additionally impeded because the commonly used commercial identification systems (for example, API 20E or API Rapid 32IDE) do not include all *Yersinia* strains in their databases and usually misidentify them as *Y. enterocolitica* (12). Nevertheless, the biochemical test system API 20E is still used as an affordable tool for the identification of *Y. enterocolitica*. This probably results in a constant misidentification of certain *Yersinia* species, particularly *Y. bercovieri*, *Y. rohdei*, and *Y. intermedia*, as *Y. enterocolitica* (1, 12, 15).

Y. enterocolitica sensu stricto comprises pathogenic and non-pathogenic members. The species can be grouped into various biotypes by biochemical tests and independently into different serotypes by immunological tests. Both types are connected with different pathogenic potential. The most common biotype-serotype combinations associated with human diseases were biotype 1B/serotype O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3 (7). Biotype 1A is deemed to be non- or less pathogenic for humans. Biotype 1B is widespread in the United States and only rarely detected in Europe and Japan (11, 14, 26, 28). Based on different DNA-DNA hybridization values and 16S rRNA gene sequences, it was proposed to name the “American” strains *Y. enterocolitica* subsp. *enterocolitica* (19). Biotypes 2 and 4 are often isolated from yersiniosis patients, and biotype 3 seems to be pathogenic but rare (6, 21).

Pathogenic strains of *Y. enterocolitica* harbor certain virulence factors, such as the plasmid-encoded *yadA* gene and the chromosomally encoded *ail* gene (17, 32). In contrast, apathogenic strains of *Y. enterocolitica* do not contain these two genes. However, the plasmid harboring the *yadA* gene can be lost under certain cultivation conditions in the laboratory (4). This may lead to false-negative results in any test system based on the presence of this plasmid. Therefore, the *ail* gene appears to

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TABLE 1. Strains used in the present study

Species ^a	No. of isolates from:				Total
	Pork meat	Pork feces	Human	Other	
<i>Y. enterocolitica</i> ^d	12			12	24
<i>Y. enterocolitica</i>	20	21	47	1	89
<i>Y. bercovieri</i>			11		11
<i>Y. intermedia</i> group I	20				20
<i>Y. rohdei</i>			2		2
<i>Y. kristensenii</i> / <i>frederiksenii</i> / <i>intermedia</i> group II ^d	8			6	14
<i>Y. pseudotuberculosis</i>				11 ^b	11
<i>Y. ruckeri</i>				12 ^c	12

^a *Y. bercovieri*, *Y. intermedia* group I, and *Y. rohdei* were identified as *Y. enterocolitica* by API 20E and identified earlier by amplified fragment length polymorphism analysis and subsequently by phenotypic analysis (15). The isolates of *Y. frederiksenii/intermedia* group II showed an API profile distinguished from that of isolates of *Y. enterocolitica*. These strains were grouped together with *Y. kristensenii*.

^b Isolated from several mammalian species.

^c Isolated from fish.

^d As determined by API 20E.

be the best-suited marker for the detection of pathogenic *Y. enterocolitica* strains. The product of the *ail* gene is an adhesion and invasion factor (17). Therefore, the detection of the *ail* gene by PCR is used as an indication of the presence of pathogenic strains of *Y. enterocolitica* in selective enrichments or isolated pure cultures (33).

Recently, Fourier transform infrared spectroscopy (FT-IR) has been established as a new method for identification of bacteria, yeasts, and other microorganisms (3, 16, 22, 24, 38). This method analyzes the total composition of all components of the cell using infrared spectroscopy (13, 18). The FT-IR

method is rapid and reliable and therefore can be easily adapted to routine analysis. Furthermore, there accrue almost no costs for consumables during sample preparation and measurements. The technique offers a wide range of applications for differentiation at the species and subspecies levels. It has already been used for the differentiation of several food-borne pathogens, like *Listeria monocytogenes* (25), *Escherichia coli* (13), and *Bacillus cereus* (23, 29). Recently, promising results were obtained by combination of FT-IR and multivariate methods for data processing, in particular artificial neural networks (ANN) (25, 35).

In the present work, FT-IR combined with ANN analysis was applied for classification of *Yersinia* strains at the species level and of *Y. enterocolitica* at the subspecies level. Furthermore, differentiation between pathogenic and apathogenic strains of *Y. enterocolitica* by FT-IR was attempted.

MATERIALS AND METHODS

Bacterial strains. A total of 123 *Yersinia* strains were obtained from human patients, raw retail pork, and pig feces in Switzerland. These strains were previously characterized by API 20E, extended biochemical differentiation as described by Bockemühl and Wong (5) and Wauters et al. (37), and bio- and serotyping (2, 15). In addition, 50 strains from food and veterinary samples which had been isolated at the Chemisches und Veterinäruntersuchungsamt Stuttgart (CVUAS, Germany) were analyzed. A total of eight *Yersinia* strains were provided by the Chemisches und Veterinäruntersuchungsamt Freiburg, Germany, and *Y. enterocolitica* SZ5108/01 was allocated by the Institut für Hygiene und Umwelt, Hamburg, Germany. *Y. enterocolitica* DSM 4780^T (DSMZ, Braunschweig, Germany) was used for comparison. Bacterial strains used in this study are listed in Table 1. In addition, 674 well-defined non-*Yersinia* gram-negative strains belonging to 56 species from 26 genera were involved, including strains of the presumed background flora growing under the same temperature conditions as those indicated in Fig. 1. The *Betaproteobacteria* group contained 33 isolates (nine species from five genera). The *Salmonella* group included 359 isolates, and the third group encompassed *Gammaproteobacteria* (*Enterobacteriales* [249 iso-

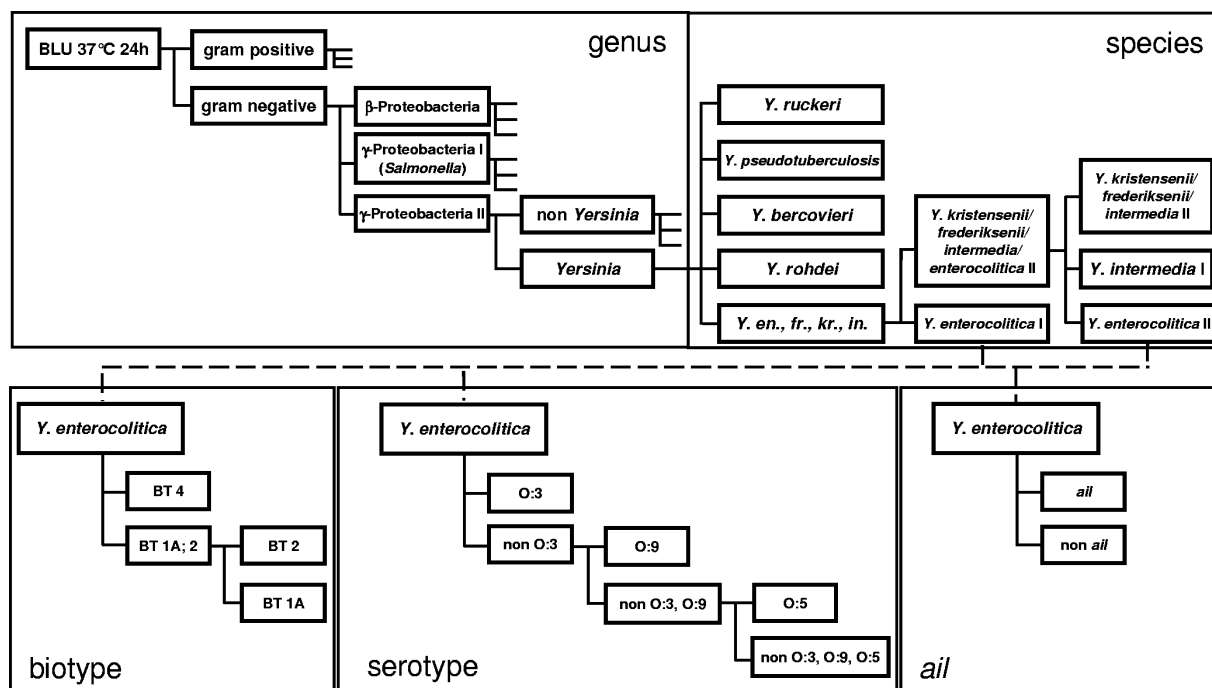


FIG. 1. Hierarchical ANN classification scheme for the differentiation of *Yersinia* strains at the genus and species levels and of the bio-, sero-, and *ail* groups of *Y. enterocolitica*. “*Y. en., fr., kr., in.*” indicates *Y. enterocolitica*/*frederiksenii*/*kristensenii*/*intermedia*.

TABLE 2. Distribution of the *ail* gene among various biotype/serotype combinations of *Y. enterocolitica* and several *Y. enterocolitica*-like species^a

Species	Biotype	Serotype	No. of strains tested	No. of <i>ail</i> -positive strains	% <i>ail</i> -positive strains
<i>Y. enterocolitica</i>	1A	Other ^b	34	0	0
	1A	O:5	7	0	0
	2	O:5	10	10	100
	2	O:9	18	18	100
	3	ND	1	0	0
	4	O:3	19	19	100
	ND	ND	24	10	
<i>Y. bercovieri</i>			11	0	0
<i>Y. intermedia</i> group I			20	0	0
<i>Y. rohdei</i>			2	0	0
<i>Y. kristensenii</i> /frederiksenii/ <i>intermedia</i> group II ^c			6	0	0
Total			160	57	

^a ND, not determined.

^b Serotypes other than O:3, O:5, or O:9.

^c As determined by API 20E.

lates/35 species/16 genera], *Aeromonadales* [3/1/1], *Pasteurellales* [40/3/3], and *Vibrionales* [15/5/1]).

Biotyping and serotyping. Biotypes were characterized previously by discriminatory tests (15) and by reference methods at the Swiss National Center for Enteropathogenic Bacteria (NENT, Lucerne, Switzerland). Serotyping was done by seroagglutination with anti-O:3, -O:5, and -O:9 rabbit antisera (SIFIN, Berlin, Germany) (Table 2).

PCR. Molecular detection of the *ail* gene was performed using the modified protocol of Thisted Lambert et al. (33). DNA was extracted from bacterial colonies by thermal lysis. The PCR mixtures (total volume of 25 μ l) consisted of 2 μ l cell lysate containing bacterial DNA; 0.5 U *Taq* DNA polymerase in 1 \times polymerase buffer (Roche, Mannheim, Germany) and 1.5 mM MgCl₂; 1 mg/ml bovine serum albumin (Promega, Madison, WI); 100 μ M each of dATP, dCTP, dGTP, and dTTP (Eppendorf, Hamburg, Germany); and 0.3 μ M each of primers 9A and 10A (Roth, Karlsruhe, Germany) (33). Amplification conditions were as follows: one initial cycle at 92°C for 30 s, 54°C for 1 min, and 72°C for 1 min, followed by three consecutive cycles with a decreasing annealing temperature of 53°C, 52°C, and 51°C, respectively, followed by 30 cycles with a constant annealing temperature of 50°C and a final synthesis step at 72°C for 7 min.

FT-IR. All isolates were cultivated independently in six to nine replicas for 24 h at 37°C on different blood agar plates (Oxoid, Basingstoke, United Kingdom). Cells of each isolate were harvested with a platinum loop and suspended in 80 μ l of deionized water. An aliquot of 35 μ l was placed in the sample zone of a zinc selenide optical plate (BrukerOptics GmbH, Ettlingen, Germany) and dried under reduced pressure for 30 min to a homogeneous solid film, which was used directly for FT-IR spectroscopy. One position on the optical plate was free, to provide a reference reading.

The infrared spectra were recorded for each sample in transmission mode between 4,000 and 500 cm⁻¹ with an FT-IR spectrometer (IFS 28/B, BrukerOptics). The FT-IR spectrometer was equipped with a deuterated triglycine sulfate detector coadding 64 scans. The acquisition and first analysis of data were carried out using OPUS software (version 4.2; BrukerOptics).

The data set for strains used for the construction of the differentiation method (six to nine spectra) was divided into two equal parts. The first one was used to create the method (creation set). The second one was used to verify the created method to gain the recovery rates (internal recovery set). An internal validation was performed when fewer than 20 strains of a respective class were available. If there were more strains available, as is necessary for method construction, the residual strains were used for an external validation (24). Results were given as probability for repeated determination, based on the results of the respective internal and external recovery sets.

The differentiation methods were developed with NeuroDeveloper software (Synthon, Heidelberg, Germany), which is based on an ANN. Generally, the second derivatives of the vector-normalized, five-point smoothed spectra of the creation sets in the wave number range of 1,800 to 500 cm⁻¹ were used for analysis of data in the covar mode by use of a significance of 95%. The 80% randomly selected spectra of the creation set were used as the intrinsic "training set" of the developer module. The internal method optimization of wavelength

TABLE 3. Validation of genus and species classification by FT-IR, given as probability for correct identification of strains (repeated determinations)^a

Organism	<i>n</i> _{ref}	<i>n</i> _{val}	All strains		External strains		
			Identification (%)		Identification (%) ^b		
			Correct	Incorrect	Correct	Incorrect	
<i>Yersinia</i> (genus level)	32	183	92.1	1.3	151	91.5	1.5
<i>Yersinia</i> (species level)	101	183	77.9	2.0			
<i>Y. enterocolitica</i>	48	113	79.9	2.6	65	78.7	3.9
<i>Y. ruckeri</i>	6	12	73.7	1.6			
<i>Y. pseudotuberculosis</i>	5	11	84.3	1.4			
<i>Y. bercovieri</i>	11	11	96.7	0			
<i>Y. rohdei</i>	2	2	100	0			
<i>Y. intermedia</i> I	19	19	45.0	1.3			
<i>Y. kristensenii</i> / frederiksenii/ <i>intermedia</i> II ^c	12	14	89.0	1.3			

^a *n*_{ref}, number of isolates used for reference; *n*_{val}, number of isolates used for validation. For internal validation, all spectra which had not been included in the reference data set were used. The probability of obtaining uncertain results during repeated determinations is given by the residual to 100%.

^b For genus-level determination and for identification of *Y. enterocolitica*, an external validation was applied using all isolates not included in the reference.

^c As determined by API 20E.

combinations was carried out routinely, with the remaining 20% of the spectra put in the "validation set" (30, 35).

The hierarchical classification at the genus and species levels was integrated in a database of 674 non-*Yersinia* gram-negative isolates as indicated in Fig. 1. The methods for the subspecies analysis of *Y. enterocolitica* were performed independently for serotype, biotype, and the *ail* marker.

RESULTS AND DISCUSSION

Construction of a hierarchically structured method for the identification of members of the genus *Yersinia* using an artificial neural net. A collection of 183 *Yersinia* strains, which had been isolated from different food and animal sources in Germany and Switzerland, was analyzed. These strains had been identified previously by various phenotypic tests (Table 1) and also partially further differentiated by bio- and serotyping. The FT-IR spectra obtained were separated into classes, and an ANN (NeuroDeveloper software) was used to construct a hierarchically structured modulated method (35, 36). The hierarchical model obtained consisted of a top level and various subsequent subclassification levels. If an FT-IR spectrum of an unknown isolate was predicted to belong to one of the nets in the higher levels, this spectrum was projected to the next level to distinguish between the respective classes available in the accordant subnet.

The strains were initially divided into three main classes, which were used as a preliminary filter. The strains from the genus *Yersinia* belonged to the third group, which was differentiated in a second step down to the genus level (Fig. 1).

For validation, 4,500 FT-IR spectra were obtained from 674 different gram-negative isolates which did not belong to the genus *Yersinia* and classified by the newly created method. The probability for an incorrect identification of these strains as *Yersinia* was less than 0.2% at the genus level (data not shown).

The probability for correct identification of *Yersinia* strains at the genus level was 91.5% (*n* = 151, external validation), with an error of only 1.5% (Table 3). The difference to 100% was caused by the additional existence of uncertain results.

TABLE 4. External validation of the IR spectral database for subspecies classifications^a

Type	Biotype				Serotype					<i>ail</i>				
	<i>n</i> _{ref}	<i>n</i> _{val}	Identification (%)		Type	<i>n</i> _{ref}	<i>n</i> _{val}	Identification (%)		<i>ail</i> status	<i>n</i> _{ref}	<i>n</i> _{val}	Identification (%)	
			Correct	Incorrect				Correct	Incorrect				Correct	Incorrect
1A	26	15	100	0	O:3	9	10	99.7	0	Positive	31	26	99.9	0
2	12	16	99.2	0	O:5	12	7	78.3	1.8	Negative	31	25	91.8	1.3
4	9	10	94.2	0	O:9	6	12	99.5	0					
					Other ^b	21	13	88.0	8.5					
Total	47	41	98.3	0	Total	48	42	92.5	2.9	Total	62	51	98.5	0.7

^a *n*_{ref}, number of isolates used for reference; *n*_{val}, number of isolates used for validation.

^b Serotypes other than O:3, O:5, or O:9.

Identification of different *Yersinia* species by FT-IR plus ANN analysis. The identification of *Yersinia* strains at a species level was developed in several steps. First, *Y. ruckeri*, *Y. pseudotuberculosis*, *Y. bercovieri*, and *Y. rohdei* isolates were separated clearly by the ANN analysis of their FT-IR spectra from the group containing *Y. enterocolitica*, *Y. frederiksenii*, *Y. kristensenii*, and *Y. intermedia*. For further classification it was helpful to divide *Y. enterocolitica* isolates into two IR groups, because it facilitates the separation of the remaining subgroup in the following step. Strains of *Y. enterocolitica* group I (comprises *Y. enterocolitica* biotype 4) were separated in the second level of the species identification, and finally in the next level, *Y. enterocolitica* group II could be differentiated from strains of *Y. intermedia* group I (Fig. 1). These strains of *Y. intermedia* were identified as *Y. enterocolitica* by API 20E but identified as *Y. intermedia* by biochemical analyses (5, 37). In addition to *Y. intermedia* I, in this step a heterogeneous group of strains was separated from *Y. enterocolitica* group II. This group was not further differentiated and consisted of isolates of *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia* II. These strains could also be differentiated from *Y. enterocolitica* by biochemical profiles. So finally, the FT-IR/ANN analysis resulted in the differentiation of eight classes at the species level (Fig. 1).

In an external validation, 78.7% (*n* = 65) of *Y. enterocolitica* sensu stricto isolates were identified correctly, with an error of only 3.9% (Table 3). All other *Yersinia* species comprised less than 20 isolates. Therefore, an internal validation was performed, and 75% of these strains were classified correctly.

This demonstrated that the differentiation of *Yersinia* strains at the species level using FT-IR in combination with ANN analysis has great potential. Thus, for some *Yersinia* strains, which were misidentified as *Yersinia enterocolitica* by API 20E, the probability of being correctly classified as *Y. intermedia*, *Y. bercovieri*, or *Y. rohdei* was 65.6%.

Differentiation of *Y. enterocolitica* isolates at the subspecies level. A main problem in the identification of enteropathogenic strains of *Y. enterocolitica* is that only certain groups of *Y. enterocolitica* sensu stricto appear to be associated with human pathogenicity. Therefore, for practical purposes it is not sufficient to identify *Y. enterocolitica* sensu stricto; in addition, it is necessary to identify the enteropathogenic strains within this species. This has traditionally been attempted by the classification of biotypes and serotypes, and it has been found that enteropathogenic isolates are found mainly in certain biotype-serotype combinations, although recently some doubts have been raised about the significance of this classification (34).

It has previously been shown that FT-IR provides sufficient resolution power to distinguish microbes even at a subspecies level (24, 25). Therefore, two independent methods were established by use of ANN analysis for classification of *Y. enterocolitica* isolates at a subspecies level. One method was created to represent biotypes 1A, 2, and 4 of *Y. enterocolitica* (Fig. 1) using FT-IR-spectra from 47 isolates with well-defined biotypes (Table 4). An external validation used spectra of *Y. enterocolitica* strains belonging to the same biotypes, which were obtained independently from those taken for method development. In the experiments, 98.3% of the strains (*n* = 41) were allocated to the correct biotype.

For differentiation of serotypes O:3, O:5, and O:9, a second method was created by use of ANN analysis based on 48 serotyped isolates (Table 4). The classification of the serotypes by FT-IR was correct in 92.5% of the cases (*n* = 42, external validation). Most misidentifications were due to difficulties in IR separation of serotype O:5 from the other class excluding serotypes O:3, O:5, and O:9.

Differentiation of *Y. enterocolitica* strains containing the *ail* gene by FT-IR. Pathogenic strains of *Y. enterocolitica* harbor the virulence factor which is encoded by the *ail* gene. Therefore, the presence of the *ail* gene was analyzed with various *Yersinia* species by PCR. The gene was detected in 57 strains of *Y. enterocolitica* which represented the biotype-serotype combinations 2/O:5, 2/O:9, and 4/O:3. These combinations of biotype and serotypes were already shown to encompass the majority of pathogenic *Y. enterocolitica* strains (7). In contrast, all strains of *Y. enterocolitica* biotype 1A, *Y. bercovieri*, *Y. intermedia*, and *Y. rohdei* were *ail* negative. Therefore it was attempted to differentiate *ail*-positive and *ail*-negative strains using FT-IR spectra. Thus, a database of 62 isolates of *Y. enterocolitica* was established, and another method was created by use of ANN analysis for separation of *ail*-positive and *ail*-negative strains (Fig. 1). The external validation with 51 *Y. enterocolitica* isolates resulted in a 98.5% probability for correct classification, with only 0.7% of identifications incorrect (Table 4). All 33 strains of *Y. bercovieri*, *Y. intermedia*, or *Y. rohdei* which could not be separated from *Y. enterocolitica* by using the API 20E test system were allocated to the non-*ail* class using this method.

The present study demonstrated that it is possible to identify *Yersinia* strains at the species level as well as at different subspecies levels using FT-IR in combination with various ANN subnets. The promising external validation of the three different strategies for subspecies differentiation, especially for the

differentiation of the pathogenicity marker *ail*, reflects the usefulness of FT-IR for the risk evaluation of contaminations in food and veterinary samples. The integration of the various classification systems allows, in the case of *Y. enterocolitica* strains, the simultaneous classification at the species level and at the serotype or biotype level in a single analysis. In addition to the great accuracy, the FT-IR analysis is also significantly faster than the conventional test systems. Thus, it is possible to differentiate *Yersinia* species by FT-IR in combination with ANN-based methods in 1 day with better results than those from the commonly used API 20E system for determination of presumptive pathogenic strains of *Y. enterocolitica*.

FT-IR methods are very flexible concerning separation of various bacterial strains at the genus, species, or subspecies level. It is very likely that it is possible to extend the methods established here for other rarely detected *Yersinia* species or *Y. enterocolitica* biotypes.

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