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Simplified Phenotypic Scheme Evaluated by 16S rRNA Sequencing for Differentiation between *Yersinia enterocolitica* and *Y. enterocolitica*-Like species

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Many clinical laboratories are familiar with a sizeable group of "unserotypeable *Yersinia enterocolitica*" strains. Due to identification problems, this group may hide *Y. bercovieri*, *Y. mollaretii*, and *Y. rohdei* strains. We present a simple scheme to distinguish between pathogenic *Y. enterocolitica* and potentially nonpathogenic *Y. enterocolitica*-like strains.

Yersinia enterocolitica, *Y. pseudotuberculosis*, and *Y. pestis* have clearly been shown to cause human disease, while characterization of the remaining eight *Yersinia* species often referred as "*Yersinia enterocolitica*-like" strains has been more limited. Recently, however, these species thought to be nonpathogenic to humans have been found to possess novel virulence mechanisms, and some of them have been associated with human disease (3, 4, 14, 16, 17). Among these species, identification of *Y. bercovieri*, *Y. mollaretii*, and *Y. rohdei* is a problem for clinical microbiology laboratories because the widely used commercial identification systems (for example, API 20 E, API Rapid 32 IDE, Micronaut E, and the Vitek GNI Card) do not list these species in their databases and usually misidentify them as *Y. enterocolitica* (10, 11). The identification of *Yersinia* in clinical microbiology laboratories is generally based on the combination of results from these biochemical identification systems and commercially available antisera against pathogenic serotypes of *Y. enterocolitica.* In addition, a 16S rRNA gene-based PCR assay for identifying and separating *Y. enterocolitica* isolates of European and American origin has recently been developed (12). However, this method involves sequencing, which is not easily applicable in most routine clinical microbiology laboratories. In the present study, we provide readers with simple means of recognizing and identifying *Y. enterocolitica*-like strains, some of which may have pathogenic potential.

In this study, unserotypeable *Y. enterocolitica* strains (n = 67) isolated and identified by Finnish hospital laboratories were retested at the Enteric Bacteria Laboratory (EBL), National Public Health Institute, with API 20 E (bioMérieux, France) at 30°C and by slide agglutination with antisera against *Y. enterocolitica* O:3, O:5, O:8, and O:9 (Denka Seiken, Japan). The strains were also biotyped according to Wauters et al. (18), and 55 strains belonged to biotype (BT) 1A (data not shown). Of the remaining 12 strains, 11 were not biotypeable (at least two reactions diverged from the established biotypes), and one strain (IH 111767) was BT 3 but was unserotypeable (Table 1). Thus, their identification as *Y. enterocolitica* strains was considered doubtful. These strains were further tested for fermentation of sorbose and fucose (at 25°C for 24 and 48 h) and on Congo red-magnesium oxalate agar (CR-MOX test) (13). For comparison, five strains of BTs 1A, 3, and 4 were included (Table 1). The colony morphology of all 17 strains through a stereomicroscope (Olympus [Japan] SZH10 zoom stereomicroscope with an SZH-ILLK illumination base) was examined on cefsulodin-irgasan-novobiocin (CIN) agar (Oxoid) incubated at 30°C for 22 to 24 h and compared to those of *Y. enterocolitica* (ATCC 9610, NCTC 11176, and RH 4823 [BT 1A control strain of EBL, National Public Health Institute]), *Y. mollaretii* (ATCC 43969), *Y. bercovieri* (ATCC 43970), and *Y. rohdei* (ATCC 43380 and 43872) cultured and incubated in parallel.

Genotyping was performed using hybridization with a YeO: 3RS probe as described previously (6). For sequencing, DNA was isolated from bacterial cells by being boiled. Primers (forward, FD1 MOD; and reverse, 533r) were used to amplify the beginning (450 bp) of the 16S rRNA gene sequence (8, 9). Sequences with the 533r primer were determined with an ABIPrism 310 Genetic Analyzer using BigDye fluorescent terminator chemistry (Applied Biosystems, Warrington, United Kingdom).

API 20 E identified 9 of the 12 doubtful strains as *Y. enterocolitica* with >90% certainty (Table 1). In contrast, sequencing revealed only one *Y. enterocolitica* strain (identical to ATCC 9610 and NCTC 11176) but five *Y. mollaretii* strains (0- to 1-nucleotide difference from ATCC 43969), five *Y. bercovieri* strains (0- to 3-nucleotide difference from ATCC 43970), and one *Y. rohdei* strain (identical to ATCC 43380). Based on the colony morphology on CIN agar, all of the *Y. mollaretii* strains and three of the five *Y. bercovieri* strains (IH 111501, IH 116025, and IH 116028) were tentatively identified as *Y. mollaretii* or *Y. bercovieri*, respectively, compared to the reference strains prior to any other testing (Table 1; Fig. 1). The micro-

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TABLE 1. Further characterization of *Y. enterocolitica* strains identified by clinical laboratories

Strain	Yr	Original ID[a] (37°C)	API 20E profile (30°C)	ST[b]	Morphology[c]	CR-MOX[d]	Reactions (25°C) in BT[e]									srb[f]	fuc	YeO:3RS profile[g]	ID after sequencing[h]
							esc	sal	pyz	lip	xyl	tre	ind	VP	BT				
IH 40555	1988	*Y. enterocolitica*	1114523 (99.7%)	NA	*Y. mollaretii*	−	−	−	+	−	+	+	−	+	NBT	+	−	W	*Y. mollaretii*
IH 41571	1995	*Y. enterocolitica*?	1014763 (53%)	NA	Mostly like *Y. kristensenii*	−	−	−	+	−	+	+	−	(+)	NBT	−	−	ND	*Y. rohdei*
IH 110292	1997	*Y. enterocolitica*	1114523 (99.7%)	NT	*Y. mollaretii*	−	−	−	+	−	+	+	−	−	NBT	+	−	W	*Y. mollaretii*
IH 111322	1999	*Y. enterocolitica*	0114523 (99.9%)	NA	Mostly like *Y. mollaretii*	Ca+	−	−	+	−	+	+	−	+	NBT	−	+	W	*Y. bercovieri*
IH 111501	1999	*Y. enterocolitica*	1114523 (99.7%)	NA	*Y. bercovieri*	−	−	−	+	−	+	+	+	+	NBT	−	+	W	*Y. bercovieri*
IH 111541	1999	*Y. enterocolitica*	1014522 (93.9%)	NA	Mostly like *Y. mollaretii*	(+)	−	−	(+)	−	+	+	−	+	NBT	+	−	W	*Y. mollaretii*
IH 116003	2000	*Y. enterocolitica*?	1114703 (26.2%)	NA	Unique	−	−	−	+	−	+	+	−	(+)	NBT	+	−	W	*Y. bercovieri*
IH 116025	2000	*Y. enterocolitica*	0114523 (99.9%)	NA	Mostly like *Y. bercovieri*	−	−	−	+	−	+	+	−	−	NBT	−	+	W	*Y. bercovieri*
IH 116028	2000	*Y. enterocolitica*	0014523 (97.7%)	NA	Mostly like *Y. bercovieri*	−	−	−	+	−	+	+	−	−	NBT	−	+	W	*Y. bercovieri*
IH 111767	2000	*Y. enterocolitica*	1014522 (93.9%)	NT	Mostly like *Y. mollaretii*	−	−	−	−	−	+	+	−	−	BT3	+	−	W-I	*Y. mollaretii*
IH 111778	2000	*Y. enterocolitica*	0154723 (99.2%)	NT	Atypical *Y. enterocolitica* BT1A	−	+	+	+	+	+	+	−	−	NBT	+	+	W	*Y. enterocolitica*
IH 111799	2000	*Y. enterocolitica*?	1014122 (13.8%)	NT	*Y. mollaretii*	Ca+	−	−	(+)	−	+	+	−	+	NBT	+	−	W	*Y. mollaretii*
Comparison strains																			
IH 111430	1999	*Y. enterocolitica*	1154763 (97.8%)	NT	Mostly like *Y. enterocolitica*	(+)	+	+	+	+	+	+	+	+	BT1A	ND	ND	W	*Y. enterocolitica*
IH 111517	1999	*Y. enterocolitica*	1014523 (96.4%)	O:3	*Y. enterocolitica* O:3	+	−	−	−	−	−	+	−	−	BT 4	ND	ND	3.2a	*Y. enterocolitica*
IH 116007	2000	*Y. enterocolitica*	0114723 (99.9%)	O:3	*Y. enterocolitica* O:3	+	−	−	−	−	−	+	−	−	BT 4	ND	ND	3.11k	*Y. enterocolitica*
IH 41122	1991	*Y. enterocolitica*	1154523 (92.3%)	O:9	*Y. enterocolitica* O:9	+	−	−	−	−	+	+	−	+	BT 3	ND	ND	9.4b	*Y. enterocolitica*
IH 41334	1993	*Y. enterocolitica*	1015723 (92.8%)	O:5	*Y. enterocolitica* O:5,27	+	−	−	−	−	+	+	−	+	BT 3	ND	ND	5.6b	*Y. enterocolitica*

[a] Identified by different hospital laboratories.

[b] Serotype of the strain determined by agglutination with sera O:3, O:5, O:8, and O:9 by Denka Seiken (Tokyo, Japan). NA, not agglutinating with the sera used; NT, not typeable, cross-reacting with the sera used.

[c] Colony morphology on CIN agar (incubation at 30°C for 22 to 24 h) observed through a stereomicroscope and compared to ATCC reference strains.

[d] Congo red magnesium oxalate agar test (13) for the presence of the virulence plasmid at 37°C for 24 and 48 h. −, negative; (+), weak positive; +, positive; Ca+, strain has pinpoint colonies showing calcium dependence (5) but does not bind Congo red.

[e] *Y. enterocolitica* strains divided into BT 1 to BT 5 according to Wauters et al. (18). BT consists of tests for esculin (esc), salicin (sal), pyrazinamidase (pyz), lipase (Tween-esterase) (lip), xylose (xyl), trehalose (tre), indole (ind), and Voges-Proskauer at 25°C for 24 and 48 h. NBT, not biotypeable.

[f] Srb, sorbose; fuc, fucose tests at 25°C for 24 and 48 h. ND, not determined.

[g] Genotyping using *Y. enterocolitica* O:3 repeated sequence probe (6). W, weak hybridization; I, incomplete typing pattern; ND, not determined.

[h] Sequencing of the beginning of the 16S rRNA gene (8, 9).

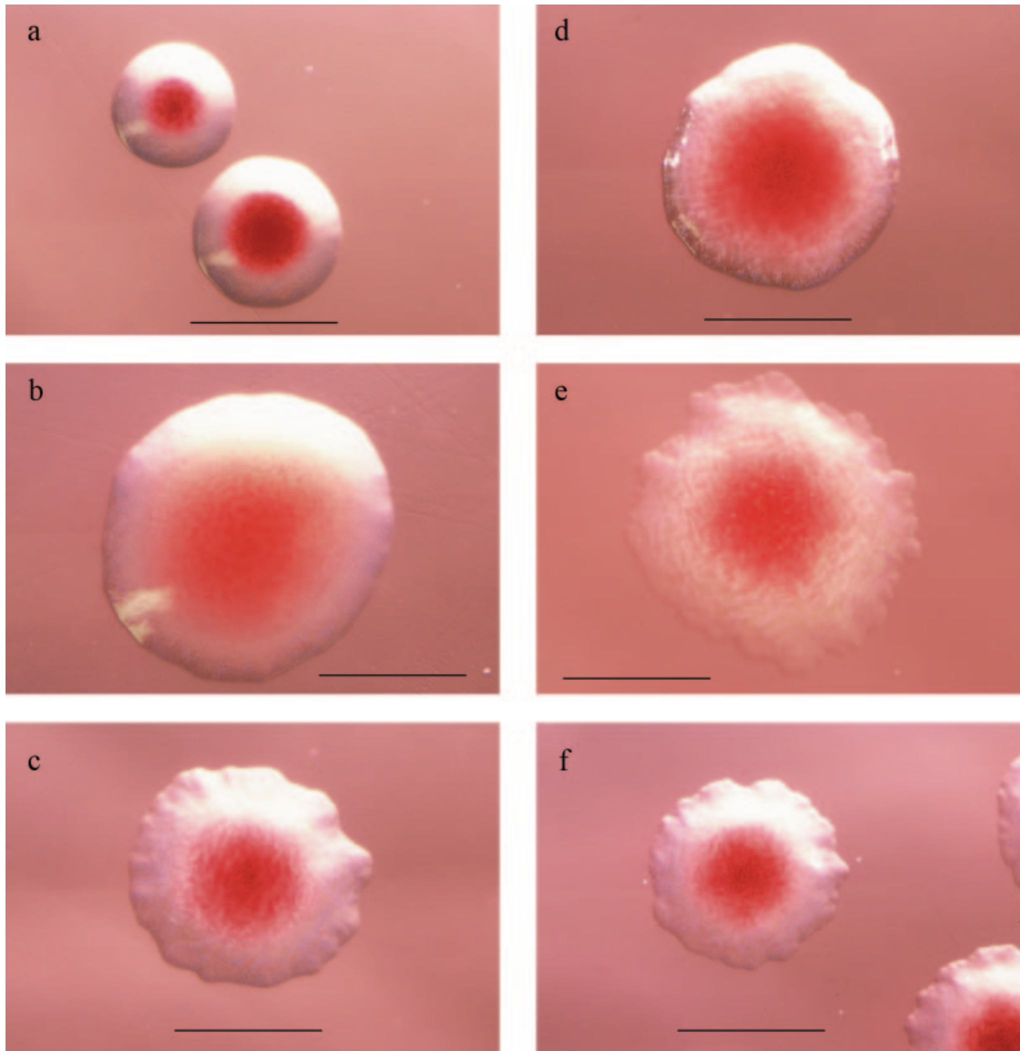

FIG. 1. Differentiating between *Y. enterocolitica*, *Y. bercovieri*, and *Y. mollaretii* on CIN agar (incubation at 30°C, 22 to 24 h) through a stereomicroscope (black bar, 1 mm). (a) *Y. enterocolitica* O:3 (pathogenic serotype, BT 4) appears as characteristically small (approximately <1 mm in diameter), circular colonies with entire edge. The colonies have a small, deep red center (bull's eye) with a sharp border surrounded by a translucent or transparent zone. (b) *Y. enterocolitica* BT 1A (nonpathogenic biotype). Large (approximately 2 mm in diameter), circular colonies with slightly lighter red center surrounded by a translucent to milk-white zone are shown. The center of the colony is large compared to the surrounding zone and has a blurred border. (c) *Y. bercovieri*. Erose-edged, slightly irregular circular colonies (approximately 1.5 mm in diameter) are shown, with a medium-red (sometimes pitting) center with an erose border. The surrounding translucent zone has a characteristic ground-glass appearance (best visible by slightly oblique illumination). (d and e) *Y. mollaretii*. Slightly irregular circular colonies (approximately 1.5 mm in diameter) are shown; a medium-red, diffuse center with no sharp borderline is visible. The surrounding translucent to milk-white zone has a characteristic ground-glass appearance (best visible in slightly oblique illumination). Two types exist: mucoid, with a smoother and more convex appearance (d) and a flat, dry, more irregular- and erose-edged colony (e). It has been noticed in EBL that approximately half of the incoming *Y. mollaretii* strains have a smooth, mucoid colony type and the other half have a dry, flat colony type (unpublished data). (f) *Y. enterocolitica* O:5,27 (pathogenic serotype). Circular, usually erose-edged (almost starlike) colonies (approximately 1 to 1.5 mm in diameter) are shown. A small, deep-red center with a slightly blurred border is visible. The surrounding translucent or transparent zone is large, compared to the center (as with *Y. enterocolitica* O:3), and has a ground-glass appearance.

scopic examination of *Y. mollaretii* and *Y. bercovieri* colonies (approximately 1.5 mm in diameter) revealed characteristic erose edges and ground-glass appearance of the translucent zone surrounding the red center of the colonies (best visible in slightly oblique illumination). These features distinguished them from the colonies of *Y. enterocolitica* BT 1A (approximately 2 mm in diameter, larger center of the colony, and the surrounding zone devoid of ground-glass appearance), serotype O:3 (approximately <1 mm in diameter, smaller, deeper red center of the colony with a sharper border (Fig. 1), and serotype O:9 (data not shown). The rest of the non-*Y. enterocolitica* strains (IH 41571, IH 116003, and IH 111799) had variable colony morphology (Table 1) that clearly differed from the morphology of *Y. enterocolitica*. Of the 11 non-*Y. enterocolitica* strains, it was possible to avoid misidentification as *Y. enterocolitica* for all 11 strains by colony morphology, but only for 3 strains (IH 41571, IH 116003, and IH 111799) with API 20 E.

In EBL, it has been found useful to start the identification of API 20 E-verified *Y. enterocolitica* strains received from clinical microbiology laboratories by examining the microscopic colony morphology on CIN agar and by CR-MOX testing. The strains are then forwarded for serotyping (i.e., the appearance of pathogenic serotypes O:3 and O:9), biotyping (the appearance of other strains), and additional biochemical testing (*Y. enterocolitica*-like appearance and presence of nonbiotypeable strains) if necessary. For example, *Y. bercovieri* and *Y. mollaretii* strains, misidentified as *Y. enterocolitica*, are easily revealed by colony morphology and by negative reactions to esculine, salicine, and lipase and a positive reaction to pyrazinamidase. *Y. enterocolitica* serotype O:5,27, which is generally less frequently isolated, has a colony morphology sometimes similar to that of *Y. bercovieri* and *Y. mollaretii* (Fig. 1), but it can be distinguished by a negative pyrazinamidase reaction with biotyping. In this study, the only exception was the strain IH 111767 with a negative pyrazinamidase reaction, placing it to *Y. enterocolitica* BT 3, but confirmed as *Y. mollaretii* after sequencing. Distinguishing between *Y. bercovieri* and *Y. mollaretii* can then be made by tests for fucose and sorbose (Table 1) (2, 15). Sequencing is necessary only for a few strains remaining unidentified after these steps, including rare cases of *Y. rohdei*, pyrazinamidase-negative *Y. bercovieri* or *Y. mollaretii* with colony morphology similar to that of *Y. enterocolitica* O:5,27, and *Y. enterocolitica* with coinciding atypical morphology and biotype. After introduction of the microscopic morphology in the identification process in EBL, it has been noticed that approximately half of the incoming *Y. mollaretii* strains have a smooth, mucoid colony type; the other half have a dry, flat colony type (unpublished data).

The cross-reactions of the commercial antisera in serotyping are typical of *Y. enterocolitica*-like strains and *Y. enterocolitica* BT 1A (1, 19). In EBL in 2000, the nonserotypeable strains represented about 40% of the incoming *Y. enterocolitica* strains (7). Therefore, building the identification of *Y. enterocolitica* solely on the use of these sera, together with a diagnostic kit like API 20 E, is inadequate. For laboratories that have limited capacity for biotyping, the simplest way to avoid misidentifications is to compare the colony morphology of a API 20 E-identified *Y. enterocolitica* strain with the *Y. enterocolitica* control strains representing serotypes and/or biotypes O:3/4, O:5,27/2 or O:5,27/3, O:8/1B, O:9/2, O:9/3, and BT 1A.

The probe YeO:3RS contains a region upstream of the *Y. enterocolitica* O-antigen cluster, a repeated sequence (RS) that is present in multiple copies in the genome. In our previous study (6), the RS was shown to be present only in the genome of the "European" pathogenic serotypes (namely, O:3, O:5,27, O:9, O:1, and O:2) of *Y. enterocolitica*. The sequence was absent from the genomes of other *Y. enterocolitica* serotypes and *Yersinia* species, resulting in a weak or incomplete typing pattern of those strains in that study. The current results are in accordance with the previous observations; none of the *Y. enterocolitica*-like strains were typeable with the probe compared to the complete typing pattern of the "European" pathogenic bioserotypes in this study. The latter strains were also clearly positive by a CR-MOX test (Table 1).

To summarize, a simple scheme for identification of *Y. enterocolitica*-like strains is presented. Without accurate identification, already in the primary diagnostics, it is impossible to gain insight into the true clinical significance of *Y. enterocolitica*-like species. Although the advanced molecular methods are constantly developed, they still may not be available in many routine clinical microbiology laboratories. Therefore, it was interesting to notice how easily the straightforward comparison of the colony morphology of *Yersinia* isolates can effectively prevent the misidentification of a strain as *Y. enterocolitica*.

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