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ARTICLE

Characteristics of *Yersinia enterocolitica* biotype 1A strains isolated from patients and asymptomatic carriers

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Abstract *Yersinia enterocolitica* biotype 1A strains are frequently isolated from the environment, foods, and animals, and also from humans with yersiniosis. There are controversial reports on the pathogenicity of biotype 1A strains. In this study, 811 fecal samples from asymptomatic humans from Switzerland were studied for the presence of *Y. enterocolitica*. Nine (1.1 %) of the 811 samples were positive for *Y. enterocolitica* 1A. These strains were compared with 12 *Y. enterocolitica* 1A strains from Swiss patients with diarrhea isolated in the same year. Almost all (20/21) *Y. enterocolitica* 1A strains carried the *yst*B gene, seven strains carried the *hre*P gene, and none carried the *ail*, *yst*A, *myf*A, *yad*A, or *vir*F genes. Most (17/21) *Y. enterocolitica* 1A strains belonged to two major clusters, A and B, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Strains of cluster B were only isolated from humans with diarrhea; however, *yst*B and *hre*P genes were detected in strains from both clinical and non-clinical samples and from strains of clusters A and B. Using ribotyping, six restriction patterns among biotype 1A strains were obtained with *Hind*III enzyme. The most common ribotype (RT I) was found in strains isolated from humans with and without diarrhea. All biotype 1A strains had a unique *Not*I profile by pulsed-field gel electrophoresis (PFGE), showing a very high genetic diversity. In this study, *Y. enterocolitica* 1A strains from clinical and non-clinical samples could not be clearly differentiated from each other. More research is needed in order to prove that biotype 1A strains are a primary cause for human yersiniosis and not only a secondary finding.

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Introduction

Yersinia enterocolitica is an important enteric bacterium causing gastrointestinal problems, long-term sequelae like reactive arthritis, and, sometimes, septicemia due to blood transfusion [4, 9]. *Y. enterocolitica* represents six biotypes (1A, 1B, 2–5) [2]. Strains belonging to biotypes 1B and 2–5 carry the virulence plasmid (pYV) and the chromosomal genes *ail*, *yst*A, *myf*A, and *hre*P, and are, thus, considered pathogenic to humans and animals. Strains belonging to biotype 1A are considered non-pathogenic because they do not carry the pYV and the important chromosomal virulence genes are missing [3, 5].

Biotype 1A strains are widely distributed in the environment and have frequently been isolated from samples of food and animal origin [2]. However, strains of biotype 1A have also been isolated from symptomatic humans [6, 18, 20]. In Finland and Switzerland, biotype 1A strains are common findings in the feces of diarrheic humans [6, 20]. Still, septicemia and reactive arthritis have only rarely been reported [2].

The accurate identification of *Y. enterocolitica* can be difficult if only biochemical tests are used [20]. Especially, *Y. massiliensis*, *Y. mollaretii*, *Y. bercovieri*, and *Y. rohdei* are very easily misdiagnosed as *Y. enterocolitica* with

phenotypic methods. Information of the biotype and virulence genes is needed for a proper assessment of the potential pathogenicity of the *Yersinia* strain [20]. *Y. enterocolitica* 1A strains are serologically very heterogeneous [2] and they show clearly wider genetic diversity than the human pathogenic strains belonging to biotypes 2 and 4 [6, 15]. *Y. enterocolitica* 1A strains have been reported to sometimes carry chromosomal virulence genes like *myf*A, *yst*B, and *hre*P [3].

There are controversial reports on the pathogenicity of clinical and non-clinical *Y. enterocolitica* biotype 1A strains and, thus, *Yersinia* strains from humans with and without diarrhea were collected in Switzerland during 2011 for further characterization using phenotypic and genotypic methods.

Materials

In total, 811 fecal samples from asymptomatic humans collected in 2011 in Switzerland were studied for the presence of *Yersinia* spp. The samples were from humans between the ages of 20 and 60 years. Most (641/811) of the samples were from males, 166 samples were from females, and for four samples, the gender was not known. Furthermore, 26 *Yersinia* spp. strains isolated in 2011 from patients with diarrhea in Switzerland were characterized and compared with the strains isolated from asymptomatic humans of the same year (Table 1).

Methods

About a 1-g fecal sample was mixed in 9 ml PMB (peptone broth supplemented with 1 % mannitol and 0.15 % bile salts) [16]. Cold enrichment at 4 °C for 3 weeks was used for all samples before plating on *Yersinia*-selective CIN (cefsulodin–irgasan–novobiocin) agar (Oxoid AG, Basel, Switzerland). The CIN plates were incubated at 30 °C for 24 to 48 h. Presumptive positive colonies were subcultured on blood agar and then tested for the urease enzyme. Urease-positive colonies were identified with API 20E and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [6, 22].

Y. enterocolitica isolates were bio- and serotyped [6]. The biotype was determined using pyrazinamidase and Tween activity, esculin hydrolysis, indole production, and salicin, xylose, and trehalose fermentation, and serotyping was carried out with slide agglutination using commercial *Y. enterocolitica* O:1-O:3, O:5, O:9 (Denka Seiken, Tokyo, Japan), and O:27 antisera (Sifin, Berlin, Germany).

Seven genes were studied by polymerase chain reaction (PCR): two virulence genes (*yad*A and *vir*F) located on the pYV of the pathogenic *Yersinia* spp. and five chromosomal virulence genes (*ail*, *yst*A, *yst*B, *myf*A, and *hre*P) [3, 12, 23, 24]. The DNA was released from bacterial colonies by heating at 99 °C for 10 min, and 1 μl of this liquid was added to 19 μl of the master mix, which contained 1× ready-to-use mix (iQ™ SYBR Green Supermix, Bio-Rad, Hercules, CA) and 200 nM of primers. All genes were studied separately in a single PCR. The fluorescence intensity of the SYBR Green Supermix and the melting curve analysis were studied using the CFX96 system (Bio-Rad). A threshold cycle (Ct) under 30 and a specific melting temperature (Tm) indicated a positive result.

Moreover, the *Yersinia* strains (one isolate per sample) were genotyped. The 16S and 23S restriction fragment length polymorphism (ribotyping) of the strains was studied using *Hind*III restriction enzymes [7] and *Not*I enzyme was used for pulsed-field gel electrophoresis (PFGE) [8]. The Dice correlation coefficient and unweighted pair-group method with arithmetic mean (UPGMA) clustering were used for constructing the dendrogram (Fig. 1).

Table 1 Age and gender distribution of humans with and without diarrhea from which *Yersinia enterocolitica* were isolated in 2011 in Switzerland

Diarrhea	Biotype	No. of strains	Gender		Age (years)		
			Female	Male	<20	20–50	>50
Yes	2 or 4	14	5	9	7	5	2
Yes	1A	12	5	7	1	7	4
No	1A	9	1	8	0	8	1

Results

Nine (1.1 %) of the 811 fecal samples from asymptomatic humans were *Y. enterocolitica* biotype 1A-positive. *Y. enterocolitica* was the only *Yersinia* species isolated from the samples and biotype 1A was the only biotype identified.

In total, 26 clinical *Y. enterocolitica* strains were sent to the *Yersinia* reference laboratory in Switzerland during the year 2011. Fourteen (54 %) of the 26 strains belonged to biotypes 2 or 4 and 12 (46 %) to biotype 1A (Table 1). Seven (50 %) of the 14 strains of biotypes 2 or 4 but only one (8 %) of the 12 biotype 1A strains were isolated from humans under 20 years old.

Biotype 1A strains have frequently been isolated in the fecal samples of humans with diarrhea in Switzerland (Table 2). In 2011, biotype 1A was the most common type (46 %) found in the fecal samples of humans with diarrhea, followed by biotypes 4 (35 %) and 2 (19 %).

Most (86 %) of the 14 *Y. enterocolitica* strains belonging to biotypes 2 or 4 were identified with a high ID% using

API 20E (Table 3). Only two of these strains (14 %) had a low or no ID% (https://apiweb.biomerieux.com). Five (24 %) of the 21 *Y. enterocolitica* biotype 1A strains could not be identified with a high ID% using API 20E. One of these strains was even identified as *Serratia marcescens*. Using MALDI-TOF MS, all *Y. enterocolitica* biotype 1A strains were identified as *Y. enterocolitica* and were distinguished from *Y. enterocolitica* biotypes 2 and 4 strains.

All 14 *Y. enterocolitica* strains of biotypes 2 or 4 carried the *ail*, *yst*A, *myf*A, and *hre*P genes in the chromosome, but were *yst*B-negative (Table 4). Most (79 %) of the biotypes 2 and 4 strains also carried *yad*A and *vir*P genes on the pYV. Almost all (95 %) *Y. enterocolitica* 1A strains carried *yst*B in the chromosome, but only one strain was negative. The ribotype pattern (RT VI) of the *yst*B-negative strain differed clearly from the ribotype patterns (RT I–V) of the *yst*B-positive strains (Fig. 1). The *hre*P was detected in 7 (33 %) *Y. enterocolitica* 1A strains (Table 5). None of the *Y. enterocolitica* 1A strains carried the *yad*A or *vir*P genes.

Most (81 %) *Y. enterocolitica* 1A strains belonged to two major clusters, A and B, by MALDI-TOF MS. All but one strain from humans without diarrhea belonged to cluster A and strains of cluster B were only isolated from clinical stool samples (Table 5). Strains carrying *yst*B and *hre*P were found in clinical and non-clinical strains and in strains from both A and B clusters. Using ribotyping, six different restriction patterns (ribotypes), RT I–VI, were obtained with *Hind*III enzyme (Fig. 1). Ten (48 %) of the *Y. enterocolitica* 1A strains expressed ribotype I and they were isolated from humans with and without diarrhea (Table 5). Ribotype II was found in three strains, which were all from clinical samples. Different *Not*I profiles were obtained in all biotype 1A strains and no clear clustering between the strains according source or any other identifiable determinant could be seen.

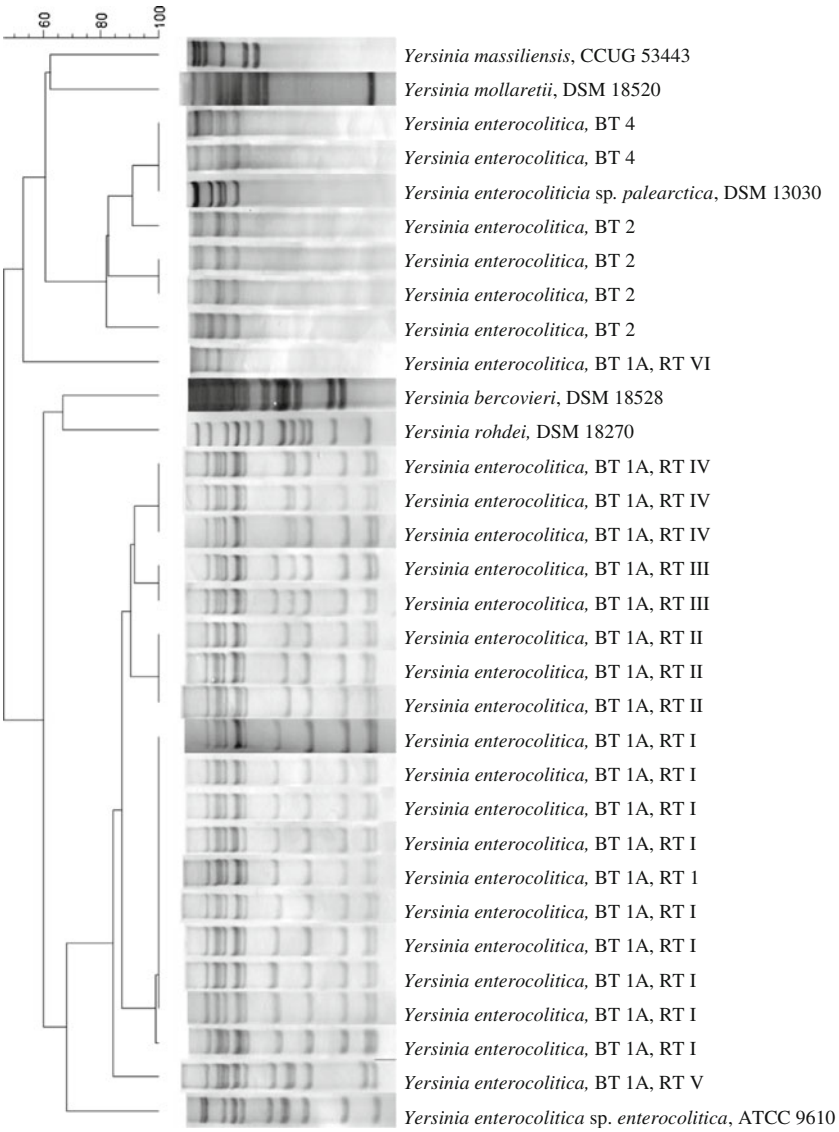

Fig. 1 The ribotypes (RTs) I–VI found among *Yersinia enterocolitica* 1A strains with *Hind*III restriction enzyme

Table 2 Biotypes of 141 *Y. enterocolitica* strains isolated from the fecal samples of patients with diarrhea during the period from 2003 to 2011 in Switzerland

Year	Number of strains (%)				
	1A	2	3	4	NT
2003[a]	5 (38)	4 (31)	0	4 (31)	0
2004[a]	4 (44)	2 (22)	0	3 (33)	0
2005[a]	4 (21)	4 (21)	1 (5)	8 (42)	2 (11)
2006[a]	9 (45)	2 (10)	0	9 (45)	0
2007[a]	10 (71)	1 (7)	0	3 (21)	0
2008[a]	4 (44)	1 (11)	0	4 (44)	0
2009[a]	5 (45)	3 (27)	0	3 (27)	0
2010[a]	8 (40)	5 (25)	1 (5)	6 (30)	0
2011	12 (46)	5 (19)	0	9 (35)	0

NT not typable

[a] Extra-intestinal results have been excluded from the data published by Fredriksson-Ahomaa et al. [6]

Discussion

The prevalence of *Y. enterocolitica* 1A in fecal samples from asymptomatic humans was only 1.1 %, which is surprisingly low, since *Y. enterocolitica* 1A and other non-pathogenic *Yersinia* spp. are frequently isolated from different food samples [2].

During 2011, 26 *Y. enterocolitica* strains from humans with diarrhea were sent to the national reference laboratory for *Yersinia*. Three biotypes (1A, 2, and 4) were identified. The most common biotype was 1A, which has frequently been isolated from the clinical fecal samples of symptomatic patients in Switzerland during the last decade [6]. Direct culturing is mostly used for clinical samples in Switzerland, which indicates that the number of *Y. enterocolitica* 1A is high in fecal samples from humans with diarrhea. Biotype 1A strains have also been reported to be common in Finnish patients. However, in Finland, cold enrichment, which supports the growth of all psychrotrophic *Yersinia* spp., is used also for clinical samples [20].

Y. enterocolitica strains of biotypes 2 or 4 were only isolated from the fecal samples of humans with diarrhea, which shows that asymptomatic humans do not usually shed strains of these biotypes. Strains of biotypes 2 and 4 were frequently isolated from young patients (under 20 years of age). *Y. enterocolitica* 1A strains were isolated from fecal samples from both symptomatic and asymptomatic humans. In Finland, the symptoms and sources of patients with *Y. enterocolitica* 1A strains differed from those patients with strains of biotypes 2 and 4. The patients with biotype 1A strains were adults with more long-lasting, unspecific symptoms, which suggests that the original cause of illness may have been something other than *Y. enterocolitica* 1A [13].

The identification of *Y. enterocolitica* strains by phenotypic methods has been shown to be very laborious and accurate identification is difficult [20]. However, the authors reported that, by combining API 20E and biotyping, *Y. enterocolitica* strains belonging to biotypes 2–5 can be identified reliably. In this study, *Yersinia* strains were identified with API 20E and MALDI-TOF MS. Some discrepancies in the identification of *Y. enterocolitica* occurred if only API 20E was used. All *Y. enterocolitica* 1A strains

Table 3 Identification of *Y. enterocolitica* (YE) isolated from the fecal samples of humans with and without diarrhea in 2011 in Switzerland

Diarrhea	No. of strains	API 20E		Biotype (BT)	MALDI-TOF MS
		Code	*Yersinia enterocolitica* ID%		
Yes	5	1015523	93.8	BT 4	YE BT 2–4
	2	1015522	89.4		
	1	0115523	99.9		
	1	3055723	*Yersinia enterocolitica*[a]		
Yes	3	1155723	98.3	BT 2	YE BT 2–4
	1	1155323	98.9		
	1	1355723	46.8		
Yes	7	1155723	98.3	BT 1A	YE BT 1A
	1	1155763	96.9		
	2	1355723	46.8		
	1	5757723	*Serratia marcescens*[a]		
	1	3355723	*Yersinia enterocolitica*[a]		
No	8	1155723	98.3		
	1	1355723	46.3		

[a]Significant species without ID%

Table 4 Distribution of virulence-associated genes among *Y. enterocolitica* strains isolated from the fecal samples of humans with and without diarrhea in 2011 in Switzerland

Diarrhea	No. of strains	Biotype	Serotype	Virulence-associated genes						
				*yad*A	*vir*F	*ail*	*yst*A	*yst*B	*myf*A	*hre*P
Yes	9	4	O:3 (9)	7	7	9	9	0	9	9
	1	2	O:5,27 (1)	1	1	1	1	0	1	1
	4	2	O:9 (4)	3	3	4	4	0	4	4
	7	1A	O:5 (3), O:8 (2), O:5,8 (1), NT (1)	0	0	0	0	7	0	0
	4	1A	O:8 (1), O:9 (1), O:5,8 (1), NT (1)	0	0	0	0	4	0	4
	1	1A	O:8 (1)	0	0	0	0	0	0	0
No	6	1A	O:5 (3), O:8 (2), NT (1)	0	0	0	0	6	0	0
	3	1A	O:5 (1), O:8 (1), NT (1)	0	0	0	0	3	0	3

were differentiated from *Y. enterocolitica* biotypes 2 and 4 strains by MALDI-TOF MS, which is a convenient method to identify a high number of bacterial strains rapidly [22].

Distribution of the virulence genes differed between the biotype 1A strains and biotypes 2 and 4 strains. All *Y. enterocolitica* strains of biotypes 2 or 4 carried the chromosomal *ail*, *yst*A, *myf*A, and *hre*P genes. Furthermore, *vir*P and *yad*A located on the pYV were detected in most of the biotypes 2 and 4 strains. All *Y. enterocolitica* 1A strains were *vir*F, *yad*A, *ail*, *yst*A, and *myf*A negative. The pYV has, so far, not been found in *Y. enterocolitica* 1A strains and, thus, *vir*P and *yad*A have also not been detected in biotype 1A strains. In our earlier study, one of the 51 human clinical *Y. enterocolitica* 1A strains carried the *ail* gene in Switzerland [6]. Recently, the *ail* gene was also detected in some biotype 1A strains in Germany and Finland [14, 19]. However, *ail* and *yst*A have very seldom been detected among biotype 1A strains [3]. All but one *Y. enterocolitica* 1A strain carried the *yst*B gene. It has been demonstrated that *Y. enterocolitica* can produce heat-stable enterotoxins. YstB is usually produced by strains belonging to biotype 1A and the enterotoxin YstA by strains belonging to biotypes 1B and 2–5. Singh and Virdi [21] showed that the *yst*B gene is widely distributed among human clinical isolates and that the production of YstB enterotoxin can be induced at the conditions found in ileum (37 °C, pH7.5), indicating that YstB is an important virulence determinant in biotype 1A strains [21]. However, in this study, *yst*B was also detected

Table 5 Distribution of different matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) clusters, ribotypes, and virulence genes among the *Y. enterocolitica* 1A strains isolated from the fecal samples of humans with and without diarrhea in 2011 in Switzerland

Diarrhea	No. of strains	MALDI-TOF MS	Ribotype (*Hind*III)	No. of *yst*B-positive strains	No. of *hre*P-positive strains
No	4	A	I	4	1
	2	A	IV	2	1
	1	A	NT[a]	1	0
	1	A	III	1	0
	1	E	I	1	1
Yes	4	B	I	4	2
	2	B	II	2	2
	1	B	III	1	0
	1	A/B	II	1	0
	1	A/B	IV	1	0
	1	C	V	1	0
	1	D	I	1	0
	1	F	VI	0	0

[a]This strain was restricted with *Eco*RI and it showed an identical ribotype with the *Y. enterocolitica* 1A reference strain CCUG 52868

in all *Y. enterocolitica* 1A strains isolated from asymptomatic humans. Furthermore, the *hre*P gene was detected among clinical and non-clinical strains. The strain carrying the *yst*B and *hre*P genes may have some pathogenic potential, but more research is needed. The *myf*A gene has been shown to be more predominant in Indian biotype 1A strains than in European strains. Batzilla et al. [1] sequenced two *Y. enterocolitica* 1A strains and found, in both strains, *yst*B, *myf*A, and *hre*P, but not *ail* and *yst*A genes. However, *myf*A in both biotype 1A strains showed sequence variability and differed from highly conserved *myf*A in biotypes 1B and 4 strains [1]. This sequence variability may explain the failure to detect the *myf*A gene in biotype 1A strains.

Two major clonal groups of *Y. enterocolitica* 1A have been reported earlier by different genotypic and phenotypic methods [10, 11, 17, 22]. Furthermore, Bhagat and Virdi have shown a correlation between the distribution of virulence-associated genes *myf*A, *yst*B, and *hre*P and the clonal groups [3]. In this study, *Y. enterocolitica* 1A strains were grouped into two major clusters using MALDI-TOF MS; most of the non-clinical strains were grouped into cluster A and most of the clinical strains were grouped into cluster B. However, no correlation between the distribution of the virulence-associated genes and the clonal groups was seen; *yst*B and *hre*P were distributed in strains of both clonal groups. In the earlier study by Bhagat and Virdi [3], the distribution of virulence-associated genes between clinical and non-clinical strains did not significantly differ, which is in accordance with our results. Furthermore, no clear clustering of non-clinical and clinical strains was obtained by ribotyping and PFGE. All 1A strains revealed different *Not*I profiles by PFGE, showing a high genetic diversity among these strains. These results could not show any clear difference between *Y. enterocolitica* 1A strains isolated from humans with diarrhea and without diarrhea. More research is needed in order to prove the significance of biotype 1A strains in human yersiniosis.

Conflict of interest The authors declare that they have no conflict of interest.

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