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Molecular Characterization of Human Clinical Isolates of *Yersinia enterocolitica* Bioserotype 1B/O8 in Poland: Emergence and Dissemination of Three Highly Related Clones▽†

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Thirty-three clinical isolates of the highly pathogenic *Yersinia enterocolitica* bioserotype 1B/O8 were collected from sporadic cases in Poland from January 2004 to July 2008. The isolates carried major virulence markers and were strongly clonal. This is the first report of the emergence and dissemination of highly related clones of *Y. enterocolitica* 1B/O8 in Europe.

Yersinia enterocolitica is known to be an important human enteric pathogen, causing a large variety of clinical and immunological manifestations (2). Infections in humans are mostly sporadic and related to contaminated food or water. Seven biotypes (1A, 1B, 2, 3, 4, 5, and 6) and more than 60 serotypes have been described for *Y. enterocolitica*. However, certain serotypes (O3; O5,27; O8; and O9) are prevalent among human isolates (2, 23). This species encompasses three grades of pathogenicity: mostly nonpathogenic strains (biotype 1A), weakly pathogenic strains of biotypes 2 to 6, and highly pathogenic strains (biotype 1B). The high pathogenicity is attributed to the yersiniabactin siderophore-mediated iron uptake system. Yersiniabactin is maintained by genes located in the *Yersinia enterocolitica* high-pathogenicity island (3). The geographical distribution of *Y. enterocolitica* 1B/O8 is generally restricted to North America (2, 4); however, this pathogen has been isolated sporadically in Japan (8, 9, 11, 15). Recently a single isolate was reported in Germany (17). Other reports of *Y. enterocolitica* O8 occurrence in Europe were published elsewhere (for a review, see reference 17); however, only strain 893/87 from Italy (16) and the German isolate were confirmed by molecular methods. In Poland, bioserotype 4/O3 is the predominant causative agent of human yersiniosis (5). To the best of our knowledge, bioserotype 1B/O8 was not isolated in Poland until 2004 (13). In the present study, we characterize 33 clinical isolates of *Y. enterocolitica* 1B/O8 collected from January 2004 to July 2008 from a variety of sporadic cases of human yersiniosis in Poland (Table 1).

The clinical *Y. enterocolitica* 1B/O8 isolates described in this study were obtained from sanitary-epidemiological units and hospitals in Poland. (Geographic distribution of the isolates is shown in Fig. S1 in the supplemental material). Biotypes and serotypes were determined as described previously (5, 23). Since *Y. enterocolitica* biotype 1A serotype O7,8 strains, which are widespread in the environment and have the O8 antigenic component, may be confused with the highly pathogenic strains of bioserotype 1B/O8, molecular investigations are recommended to confirm bioserotyping results. (17). The major chromosomal *Y. enterocolitica* virulence marker genes *ystA*, *ail*, and *myfA*, encoding heat-stable enterotoxin, attachment invasion locus protein, and Myf fimbriae, respectively (for a review, see reference 2), were examined by PCR to confirm the virulence of the tested isolates. Hence, the genes *irp1* and *irp2* of the yersiniabactin biosynthesis cluster and the siderophore receptor *fyuA*, which are located on the *Y. enterocolitica* high-pathogenicity island, were investigated to demonstrate that the Polish 1B/O8 isolates belong to the highly pathogenic *Y. enterocolitica* lineage (2, 3). PCRs were carried out as described previously (7) with primers listed in Table S1 in the supplemental material. All of the tested isolates yielded PCR amplicons for the *Y. enterocolitica* 16S rRNA gene and the aforementioned virulence marker genes. There is strong evidence suggesting that the Polish 1B/O8 isolates belong to highly pathogenic *Y. enterocolitica*.

Congo red magnesium oxalate agar medium (14) and the PCR assay with primers for the *yadA* gene, which encodes *Yersinia* adhesin A (2, 6), were used to detect the *Yersinia* virulence plasmid (pYV). The virulence plasmid is considered the gold standard for *Yersinia* virulence determination. However, in contrast to the chromosomal virulence markers, pYV can easily be lost when bacteria are cultured at 37°C (1, 2, 17). In this study, pYV was detected in nine isolates only (Table 1). Given the high sensitivity of PCR, this result may suggest that the majority of tested isolates lost pYV during manipulations in a routine diagnostic laboratory.

In order to gain better insight into the pathogenic potential of Polish *Y. enterocolitica* 1B/O8 isolates, the PCR assay was carried out for the genes *yts1M* and *ysrS*, which encode components of the *Yersinia* chromosomal type II and III secretion systems, termed Yts1 and Ysa, respectively (12, 21). In addition, the putative virulence genes YE2407 and YE2447 of *Y. enterocolitica* 1B/O8 strain 8081 were investigated (10, 20). All of these genes have been reported to occur exclusively in highly

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TABLE 1. Characteristics of tested isolates of *Yersinia enterocolitica* 1B/O8

Strain	Epidemiological data				Presence of pYV[a]	PFGE result		
	Yr of isolation	Region of origin	Age of patient	Main clinical manifestation		Clone[b]	NotI	XbaI
WA-314	NA[c]	NA (reference strain)	NA	NA	−	4	B	I
27/04	2004	Konin	38	Mesenteritis	+	1	A	II
67/04	2004	Poznań	ND[d]	ND	−	1	A	II
152/05	2005	Częstochowa	1	Diarrhea	−	1	A	II
85/05	2005	Częstochowa	2	Diarrhea	−	1	A	II
82/06	2006	Poznań	2	Diarrhea	−	1	A	II
86/06	2006	Kielce	14	Mesenteritis	+	1	A	II
51/07	2007	Kielce	1	ND	−	1	A	II
52/07	2007	Poznań	2	Diarrhea	+	1	A	II
71/07	2007	Poznań	1	Diarrhea	−	1	A	II
72/07	2007	Poznań	3	ND	−	1	A	II
84/07	2007	Poznań	9	Diarrhea	−	2	A1	II
85/07	2007	Poznań	27	Cecal abscess	−	1	A	II
89/07	2007	Grodzisk Maz.[e]	36	Cecal abscess	+	1	A	II
200/07	2007	Zabrze	1	Diarrhea	−	1	A	II
361/07	2007	Kielce	24	Pyoperitoneum	−	2	A1	II
379/07	2007	Poznań	28	Pyoperitoneum	−	1	A	II
404/07	2007	Katowice	25	Abdominal ulcer	−	2	A1	II
31/08	2008	Warsaw	3	Diarrhea	+	1	A	II
46/08	2008	Łódź	ND	ND	+	1	A	II
47/08	2008	Zabrze	2	Diarrhea	−	1	A	II
51/08	2008	Kielce	2	Diarrhea	−	1	A	II
61/08	2008	Pułtusk	78	Bacteremia	−	1	A	II
93/08	2008	Warszawa	10	Diarrhea	−	2	A1	II
128/08	2008	Katowice	1	ND	−	1	A	II
146/08	2008	Tarnów	1	Diarrhea	+	1	A	II
160/08	2008	Szczecin	1	Diarrhea	−	1	A	II
161/08	2008	Szczecin	50	Diarrhea	−	1	A	II
162/08	2008	Kielce	2	ND	−	1	A	II
171/08	2008	Warszawa	ND	Bacteremia	+	2	A1	II
172/08	2008	Warszawa	10	Diarrhea	−	1	A	II
175/08	2008	Zabrze	5	ND	−	1	A	II
180/08	2008	Bydgoszcz	3	Diarrhea	+	3	A	III
181/08	2008	Bydgoszcz	6	Pseudoappendicitis	−	1	A	II

[a] Presence (+) or absence (−) of the *Yersinia* virulence plasmid.
[b] Groups of isolates indistinguishable by combined NotI and XbaI PFGE genotyping.
[c] NA, not applicable.
[d] ND, not determined.
[e] Grodzisk Maz., Grodzisk Mazowiecki.

pathogenic *Y. enterocolitica*. In this study, PCR amplicons of *yts1M*, *ysrS*, YE2407, and YE2447 were detected for all the tested isolates. Our data demonstrate that *Y. enterocolitica* 1B/O8 isolates from sporadic cases in Poland carry the Yts1 and Ysa gene clusters and share the high-pathogenicity-*Y. enterocolitica*-specific virulence traits reported elsewhere (2, 3, 17, 20).

To determine the genetic relatedness of the Polish *Y. enterocolitica* 1B/O8 isolates described in this study, we carried out pulsed-field gel electrophoresis (PFGE), which is the genotyping standard for *Y. enterocolitica* (4, 16). PFGE was conducted as described previously (22) using the Chef-DR II system (Bio-Rad) and the endonuclease NotI (Fermentas, Lithuania) with a switching time of 5 to 24 s for 26 h at 14°C and a voltage gradient of 6 V/cm. Since all of the tested isolates were collected within a 4-year period from distinct patients with no epidemiological link, we initially expected diverse genotypes. Surprisingly, the resulting PFGE patterns were homogeneous (Fig. 1A). Only two highly similar NotI patterns (genotypes A and A1) could be distinguished in the Polish isolates. Twenty-eight and five isolates were classified as genotypes A and A1, respectively (Table 1). In contrast, the reference 1B/O8 strain WA-314 revealed a distinct NotI pattern (genotype B). Isolates of the A1 genotype could be reproducibly distinguished from those of the predominating genotype A, even under various PFGE conditions (data not shown).

Interestingly, in the study by Saken and coworkers (16), we found NotI PFGE patterns that strongly resemble patterns of the here-described genotypes A and A1. The patterns showing resemblance were observed for the *Y. enterocolitica* 1B/O8 isolates 893/87 and 900/90, collected in Italy and Japan, respectively. Notably, Saken and coworkers used the reference strain WA-314 and comparable PFGE conditions (pulse ramp, 8 to 23; time period, 26 h). These findings might suggest that the Polish *Y. enterocolitica* 1B/O8 clinical isolates are related to the isolates from Italy and Japan. Further studies, however, are required to confirm this thesis.

The remarkable homogeneity of the genetic backbone shown in this study by PFGE may suggest that Polish *Y. enterocolitica* 1B/O8 isolates are clonal. Struelens and coworkers emphasize that identification of clones must be based on monitoring of several markers of sufficient discriminatory power (18). For

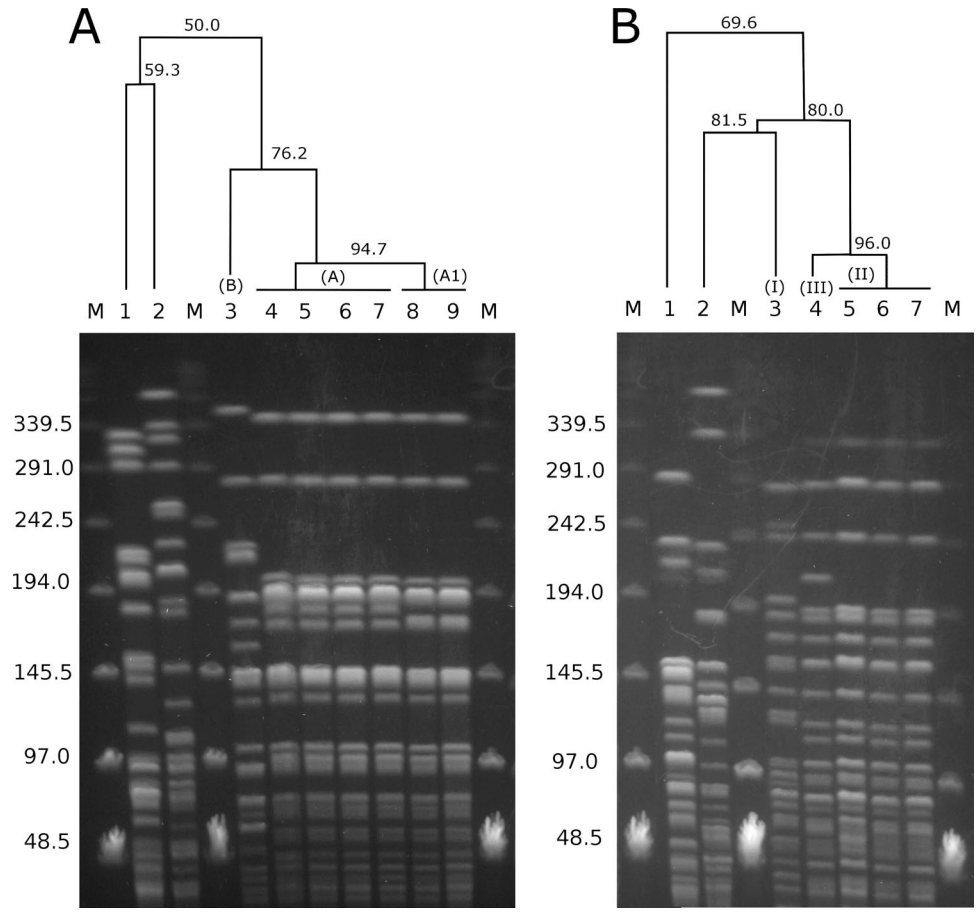

FIG. 1. NotI (A) or XbaI (B) PFGE profiles of *Y. enterocolitica* 1B/O8 clinical isolates from Poland (lanes 4 to 9) compared with profiles of bioserotype 1A/O7,8 (lanes 1 and 2) and 1B/O8 (lane 3) strains. The corresponding dendrograms illustrate genetic similarity of the NotI and XbaI profiles. The similarity values are shown in the dendrograms. PFGE genotypes are indicated in parentheses. Lanes in panels A and B: M, bacteriophage λ DNA ladder; 1, *Y. enterocolitica* 1A/O7,8 strain 323; 2, *Y. enterocolitica* 1A/O7,8 strain UG55; 3, reference 1B/O8 strain WA-314; 7, isolate 51/07; lanes in panel A: 4, 27/04; 5, 152/05; 6, 82/06; 8, 84/07; 9, 93-1/08; lanes in panel B: 4, 180/08; 5, 27/04; 6, 152/05. Sizes of DNA fragments are given in kilobases.

this reason, we performed additional PFGE analyses using the enzyme XbaI (Fermentas, Lithuania). Even though application of an additional endonuclease has been reported to enhance the genotyping resolution of PFGE (4), all but one of the Polish 1B/O8 clinical isolates were indistinguishable (genotype II). The only exception was the isolate 180/08 (genotype III), which exhibited a single additional band in the XbaI pattern (Fig. 1B). The reference strain, WA-314, revealed a distinct XbaI pattern (genotype I).

To determine the similarity of the genotypes, PFGE patterns were analyzed using the GelCompar II software, version 5.10 (Applied Maths, Saint-Matins-Latem, Belgium). Similarity clustering analyses were performed using the single-linkage algorithm and the Dice correlation coefficient with a tolerance of 1.0%. The similarity of the PFGE genotypes distinguished in this study was high and ranged from 94.7% to 96.0% (Fig. 1) for NotI and XbaI, respectively. When NotI and XbaI PFGE profiles were analyzed simultaneously, the Polish 1B/O8 isolates were diversified into three highly similar (97.7%) types (clones), 1, 2, and 3, which comprised 27 and 5 isolates and a single isolate, respectively (Table 1). In conclusion, our results demonstrate that all of the tested *Y. enterocolitica* 1B/O8 isolates from Poland are closely related.

In accordance with the criteria proposed by Tenover and coworkers (19) and recommendations for genotyping data interpretation described by Struelens and colleagues (18), our results may indicate that the tested *Y. enterocolitica* 1B/O8 isolates collected in Poland constitute the same strain comprising three highly related PFGE clones. The genetic homogeneity of the tested isolates may result from the recent emergence of *Y. enterocolitica* 1B/O8 in Poland. Notably, the majority of the tested isolates were collected within the last 2 years, including all of the isolates classified as clones 2 and 3 (Table 1). This might reflect recent speciation events in the predominant clone 1. It is also noteworthy that we have recently observed in Poland a dramatic increase in the number of patients seropositive for both the *Y. enterocolitica* O8 lipopolysaccharide and the *Yersinia* outer protein antigens (13). Taken together, these findings show that the emergence and rapid dissemination of *Y. enterocolitica* 1B/O8 may be a challenge for the public health authorities in Poland.

To date, only a few sporadic isolates of the highly pathogenic

Y. enterocolitica bioserotype 1B/O8 have been found in Europe (16, 17). The genetic similarity of these isolates has not yet been defined. In this study, we characterized a number of clinical isolates of *Y. enterocolitica* 1B/O8 by PFGE. To the best of our knowledge, this is the first report of the dissemination of the closely related clones of highly pathogenic *Y. enterocolitica* 1B/O8 in Europe. 1B/O8 isolates indistinguishable by PFGE were reported in Japan (11, 15), where an epidemiological link between patients and contaminated food or direct contact with infected animals has been documented. In light of these reports, the strong clonality of Polish *Y. enterocolitica* 1B/O8 isolates may suggest a common origin. Further epidemiological investigations supported by local veterinary authorities are required to elucidate the reservoir of the pathogen in Poland and its route of transmission. Our findings also indicate the need to develop novel, high-resolution genotyping approaches devoted to *Yersinia enterocolitica* 1B/O8 subtyping.

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