Molecular characterization of human clinical isolates of *Yersinia enterocolitica* bioserotype 1B/O8 in Poland: emergence and dissemination of three highly related clones

Running title: *Yersinia enterocolitica* 1B/O8 in Poland

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Abstract

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 *Y. enterocolitica* 1B/O8 highly related clones 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; O9) are prevalent among human isolates (2, 23). This species encompasses three grades of pathogenicity: mostly non-pathogenic strains (biotype 1A), weakly pathogenic strains of biotypes 2 to 6 and highly pathogenic strains (biotype 1B). The high-pathogenicity is attributed to yersiniabactin (Ybt) siderophore-mediated iron uptake system. Ybt is maintained by genes located in the Yersinia enterocolitica high pathogenicity island (Yen HPI) (3). The geographical distribution of Y. enterocolitica 1B/O8 is generally restricted to North America (2, 4), however, this pathogen was sporadically isolated in Japan (8, 9, 11, 15). Recently, a single isolate has been reported in Germany (17). Other reports of Y. enterocolitica O8 occurrence in Europe were published elsewhere (for review see reference 17), however only the 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 form a variety of sporadic cases of human yersiniosis in Poland (Table 1).

The clinical Y. enterocolitica 1B/O8 isolates reported in this study were obtained from the sanitary-epidemiological units and hospitals in Poland. (Geographic distribution of the isolates is shown in supplementary material – Fig.S1). Biotype and serotype 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 bio-serotyping 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 review see reference 2)
were examined by polymerase chain reaction (PCR) to confirm virulence of the tested
isolates. Hence, genes irp1 and irp2 of the yersiniabactin biosynthesis cluster and the
siderophore receptor fyuA, which are located on the Yen HPI, 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 (see supplementary material). All the tested isolates yielded PCR amplicon 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 the highly pathogenic Y.
enterocolitica.

Congo red magnesium oxalate agar medium (14) and the PCR assay with primers for
the yadA gene, which encodes Yersinia adhesine A (2, 6), were used to detect 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 be easily lost when bacteria are cultured in 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 genes ytsLM and ysrS which encode
components of the Yersinia chromosomal type II and III secretion systems (TTSS) termed
Yts1 and Ysa, respectively (12, 21). In addition, putative virulence genes YE2407, YE2447 of
Y. enterocolitica 1B/O8 strain 8081 were investigated (10, 20). All these genes have been
reported to occur exclusively in the highly 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 Yts1 and Ysa gene clusters and share the high pathogenicity *Y. enterocolitica* specific virulence traits reported elsewhere (2, 3, 17, 20).

To determine genetic relatedness of Polish *Y. enterocolitica* 1B/O8 isolates reported 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 CHEF-DR II system (Bio-Rad, USA) and endonuclease *NotI* (Fermentas, Lithuania) with a switching time of 5 to 24 s for 26 h at 14°C and voltage gradient of 6V/cm. Since all the tested isolates were collected within four-years-long period from distinct patients with no epidemiological link, we initially expected diverse genotypes. Surprisingly, 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 genotype A and A1, respectively (Table 1). In contrast, the reference 1B/O8 strain WA-314 revealed distinct *NotI*-pattern (genotype B). Isolates of the A1 genotype could be reproducibly distinguished from the predominating genotype A, even under various PFGE conditions (data not shown).

Interestingly, in the study by Saken and co-workers (16) we found *NotI*-PFGE patterns that strongly resemble patterns of the reported here genotypes A and A1. The resembling patterns were observed for *Y. enterocolitica* 1B/O8 isolates 893/87 and 900/90 collected in Italy and Japan, respectively. Notably, Saken and co-workers used reference strain WA-314 and a comparable PFGE conditions (pulse ramp 8-23; 26h). These findings might suggest that the Polish *Y. enterocolitica* 1B/O8 clinical isolates are related to the isolates from Italy and Japan. Further studies are however required to confirm this thesis.
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 co-workers emphasize that identification of clones must be based on monitoring of several markers of sufficient discriminatory power (18). For this reason, we performed additional PFGE analyses using enzyme *Xba*I (Fermentas, Lithuania). Even though, application of an additional endonuclease has been reported to enhance the genotyping resolution of PFGE (4), all but one Polish 1B/O8 clinical isolates were indistinguishable (genotype II). The only exception was the isolate 180/08 (genotype III) which exhibited single additional band in *Xba*I-pattern (Fig. 1B). The reference strain WA-314 revealed a distinct *Xba*I-pattern (genotype I).

To determine the similarity of the genotypes, PFGE patterns were analyzed using the GelCompar II version 5.10 software (Applied Maths, Saint-Matin-Latem, Belgium). Similarity clustering analyses were performed using the single linkage algorithm and the Dice correlation coefficient with the tolerance of 1.0%. The similarity of the PFGE genotypes distinguished in this study was high and ranged from 94.7% to 96.0% (see Fig.1) for *Not*I and *Xba*I, respectively. When *Not*I and *Xba*I 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, 5 and a single isolate, respectively (Table 1). In conclusion, our results demonstrate that all the tested *Y. enterocolitica* 1B/O8 isolates from Poland are closely related.

In accordance with the criteria proposed by Tenover and co-workers (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 was collected within the last two
years, including all the isolates classified as clone 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 of a number of seropositive patients for both the Y. enterocolitica O8 lipopolysaccharide (LPS) and the Yersinia outer proteins (Yop's) 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 1B/O8 have been reported in Europe (16, 17). The genetic similarity of these isolates has not been yet 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. The 1B/O8 isolates indistinguishable by PFGE were reported in Japan (11, 15) where epidemiological link between patients and contaminated food or direct contact with infected animals has been documented. Consistently, 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 reservoir of the pathogen in Poland and its route of transmission. Our findings indicate also the need to develop novel, high resolution genotyping approaches devoted to Yersinia enterocolitica 1B/O8 subtyping.

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## Table 1: Characteristics of tested isolates of *Yersinia enterocolitica* 1B/O8

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year</th>
<th>Region of the origin</th>
<th>Age</th>
<th>Main clinical manifestations</th>
<th>Epidemiological data</th>
<th>pYV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PFGE Clone&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NotI</th>
<th>XbaI</th>
</tr>
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<tbody>
<tr>
<td>WA-314</td>
<td>NA</td>
<td>Reference strain</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>4</td>
<td>B</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>27/04</td>
<td>2004</td>
<td>Konin</td>
<td>38</td>
<td>mesenteritis</td>
<td>+</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>67/04</td>
<td>2004</td>
<td>Poznań</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>152/05</td>
<td>2005</td>
<td>Częstochowa</td>
<td>1</td>
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<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>85/05</td>
<td>2005</td>
<td>Częstochowa</td>
<td>2</td>
<td>diarrhoea</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>82/06</td>
<td>2006</td>
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<td>diarrhoea</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
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<td>86/06</td>
<td>2006</td>
<td>Kielce</td>
<td>14</td>
<td>mesenteritis</td>
<td>+</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>51/07</td>
<td>2007</td>
<td>Kielce</td>
<td>1</td>
<td>ND</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>52/07</td>
<td>2007</td>
<td>Poznań</td>
<td>2</td>
<td>diarrhoea</td>
<td>+</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>71/07</td>
<td>2007</td>
<td>Poznań</td>
<td>1</td>
<td>diarrhoea</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>72/07</td>
<td>2007</td>
<td>Poznań</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>84/07</td>
<td>2007</td>
<td>Poznań</td>
<td>9</td>
<td>diarrhoea</td>
<td>-</td>
<td>2</td>
<td>A1</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>85/07</td>
<td>2007</td>
<td>Poznań</td>
<td>27</td>
<td>caecal abscess</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
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<tr>
<td>89/07</td>
<td>2007</td>
<td>Grodzisk Maz.</td>
<td>36</td>
<td>caecal abscess</td>
<td>+</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
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<td>2007</td>
<td>Zabrze</td>
<td>1</td>
<td>diarrhoea</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>361/07</td>
<td>2007</td>
<td>Kielce</td>
<td>24</td>
<td>pyoperitoneum</td>
<td>-</td>
<td>2</td>
<td>A1</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>379/07</td>
<td>2007</td>
<td>Poznań</td>
<td>28</td>
<td>pyoperitoneum</td>
<td>-</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>404/07</td>
<td>2007</td>
<td>Katowice</td>
<td>25</td>
<td>abdominal ulcer</td>
<td>-</td>
<td>2</td>
<td>A1</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>31/08</td>
<td>2008</td>
<td>Warszawa</td>
<td>3</td>
<td>diarrhoea</td>
<td>+</td>
<td>1</td>
<td>A</td>
<td>II</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> pYV, <sup>b</sup> Clone, <sup>c</sup> Region, <sup>d</sup> ND

*Note:* The table provides characteristics of tested isolates of *Yersinia enterocolitica* 1B/O8, including epidemiological data, PFGE results, and clinical manifestations.
<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>Incidence</th>
<th>Diagnosis</th>
<th>Serotype</th>
<th>Phage Type</th>
</tr>
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<tbody>
<tr>
<td>46/08</td>
<td>Łódź</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>47/08</td>
<td>Zabrze</td>
<td>2</td>
<td>diarrhoea</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>51/08</td>
<td>Kielce</td>
<td>2</td>
<td>diarrhoea</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>61/08</td>
<td>Pułtusk</td>
<td>78</td>
<td>bacteraemia</td>
<td>-</td>
<td>1</td>
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<tr>
<td>93/08</td>
<td>Warszawa</td>
<td>10</td>
<td>diarrhoea</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>128/08</td>
<td>Katowice</td>
<td>1</td>
<td>ND</td>
<td>-</td>
<td>1</td>
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<tr>
<td>146/08</td>
<td>Tarnów</td>
<td>1</td>
<td>diarrhoea</td>
<td>+</td>
<td>1</td>
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<td>Szczecin</td>
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<td>diarrhoea</td>
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<tr>
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<td>Bydgoszcz</td>
<td>6</td>
<td>pseudoappendicitis</td>
<td>-</td>
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</table>

1.  
2.  pYV, the *Yersinia* virulence plasmid
3.  groups of isolates indistinguishable by combined *NotI* and *XbaI* PFGE genotyping
4.  NA, not applicable
5.  ND, not determined
Legend to figure 1

NotI (A) and XbaI (B) PFGE profiles of *Y. enterocolitica* 1B/O8 clinical isolates from Poland (lines 4-9) compared with profiles of bioserotype 1A/O7,8 (lines 1 and 2) and 1B/O8 (line 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. Lines (parts 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 – the reference 1B/O8 strain WA-314, 7 – isolate 51/07. (A) Lines: 4 – 27/04, 5 – 152/05, 6 – 82/06, 8 – 84/07, 9 – 93-1/08. (B) Lines: 4 – 180/08, 5 – 27/04, 6 – 152/05. Size of DNA fragments is given in kilobases (kb).