OCCURRENCE OF VIRULENCE GENES AND BIOFILM FORMATION IN YERSINIA ENTEROCOLITICA ISOLATED FROM PIGS

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Received: September 24, 2010  Accepted: February 3, 2011

Abstract

Sixty isolates of Yersinia enterocolitica from pigs belonging to 1A and 4 biotypes, and one non-typeable isolate were investigated in order to determine the occurrence of genes directly connected with pathogenicity of Y. enterocolitica by using a multiplex PCR. The multiplex PCR assay was based on the amplification of the ail, ystA, ystB, and yadA genes in one reaction. The bacterial adhesion to hydrocarbon test was used to evaluate cells surface hydrophobicity of Y. enterocolitica. Assay of biofilm formation was performed with the use of polystyrene tissue culture plate. In all isolates (n=33) of biotype 1A and in biochemically not typeable isolate, the ystB-specific amplification product of 68 bp was obtained. In all isolates (n=26) of biotype 4, the specific PCR products for ystA gene and ail gene were obtained. In the majority of this biotype isolates (18/26) the specific PCR product for the plasmid yadA gene was detected. The percentage of isolates with hydrophobic surface of cells was 27.8% and 30.8% within biotype 1A and 4, respectively. All hydrophilic and hydrophobic isolates of Y. enterocolitica adhered to polystyrene, although in different degree. These results suggest that cell-surface hydrophobicity is not important in Y. enterocolitica adhesion and subsequent biofilm formation. The multiplex PCR assay for simultaneous detection the ail, ystA, ystB and yadA genes, enabled fast evaluation of the potential virulence of isolates and differentiation of the pYV plasmid-bearing Y. enterocolitica isolates, the plasmidless isolates, and biotype 1A isolates.

Key words: swine, Yersinia enterocolitica, genes, hydrophobicity, biofilm, multiplex PCR.
various size that could carry novel virulence determinants (15).

Bacteria in natural environments live in aggregates or form biofilms. Bacterial cells within biofilm are physiologically distinct from planktonic counterparts. Reduced metabolic rate of biofilm cells allows them to survive longer and results in an increase in resistance to antibiotics. Biofilms have been involved in the development of several chronic infections and infection of medical implants and tissues (16).

Cell-surface hydrophobicity is an important factor in the adherence and subsequent proliferation of microorganisms on solid surfaces and at interfaces. Microbial adhesion to hydrocarbons (MATH) is considered as a measure of the hydrophobicity of microbial cell surfaces. Strains adhering well to the hydrocarbon are considered to be hydrophobic and strains adhering weakly are considered hydrophilic (3). From a physico-chemical point of view, it can be argued that MATH is a measure of complicated interplay of all factors involved in microbial adhesion to surfaces, most notably long-range van der Waals and electrostatic forces and various short-range interactions (26). In biological systems, hydrophobic interactions can be defined as the attraction between apolar or slightly polar molecules or cells, when immersed in water (27).

The aim of this study was to evaluate of pathogenicity Y. enterocolitica isolates from pigs by using multiplex PCR to identify genetic determinants of virulence and by estimation of hydrophobicity properties and biofilm formation.

Material and Methods

Bacterial strains. A total of 60 Y. enterocolitica isolates were recovered from the tonsils of clinically healthy fattening pigs from various herds in the Northeast Poland. The tonsils were collected from 550 pigs immediately after evisceration. The methods of isolation, identification, and biotyping of Y. enterocolitica isolates were described previously (12). Thirty-three biotype 1A isolates, 26 biotype 4 isolates, and one not typeable isolate were examined.

DNA isolation. Genomic DNA was isolated from Y. enterocolitica using the Genomic Mini commercial kit (A&A Biotechnology, Poland) according to the manufacturer’s protocol. 2.5 µl of the total extracted material from each test sample was used as a template DNA for PCR application.

Primers and PCR conditions. The primers specific for the ail, ystA, ystB, and yadA genes of Y. enterocolitica, synthesised at DNA-Gdansk (Poland), are listed in Table 1. The multiplex PCR was performed in a 25-µl volume containing 2.5 µl of DNA template, 1×PCR buffer, 0.2 mM each dATP, dCTP, dGTP, and dTTP (Fermentas, Lithuania), the ystB-specific primers at 400 nM, the yadA-specific primers at 100 nM, the ail-specific primers, and ystA-specific primers at 50 nM, with 1 U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany). Amplification was carried out in the Multi Gene II thermal cycler (Labnet International, Inc., USA) with following conditions: initial denaturation (94°C, 3 min), followed by 30 subsequent cycles consisting of denaturation (94°C, 1 min), primer annealing (52°C, 1.5 min), extension (72°C, 1.5 min), and final extension (72°C, 10 min). A negative control with each of the reaction components except template DNA, positive control with the genomic DNA from Y. enterocolitica biotype 4 for the ail, ystA and yadA genes, and positive control with genomic DNA from Y. enterocolitica biotype 1A for the ystB gene were included with each test run. The PCR products were analysed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. Molecular size markers (Sigma-Aldrich) were also run for product size verification. The gel was electrophorised in 2 × Tris-borate buffer at 70 V for 1.5 h.

Adherence to xylene. The bacterial adherence to hydrocarbon (BATH) test was performed as proposed by Rosenberg et al. (22). Cultures of Y. enterocolitica were grown on trypticase soy agar (TSA) at 25°C for 24 h. The bacteria were washed from the agar, centrifuged, and then washed twice with 0.85% NaCl. The density of each bacterial suspension was adjusted photometrically to an absorbance of 0.5 at 540 nm (Cecil 1021 Spectrophotometer, Cecil Instruments Ltd, England). One millilitre of xylene was added to 3 ml of bacterial suspensions and vortexed for 1 min. After that, the phases were allowed to separate for 30 min. The aqueous phase was carefully removed and its light absorbance was measured at 540 nm. The results were expressed as the percentage decrease in optical density of aqueous phase compared with the optical density of the bacterial suspension without xylene. Each assay was performed in duplicate and the results were averaged. The isolate was considered hydrophobic if it expressed ≥25% adherence to xylene.

Assay for biofilm formation. The biofilm assay was performed as described by Stepanović et al. (24) with some modifications. The isolates were grown overnight on TSA at 25°C. Next a loop fool of bacterial cells was transferred to 3 ml TSB. The bacterial suspension (200 µl) was inoculated in triplicates to wells of 96-well polystyrene tissue culture plate (Becton Dickinson Labware, USA) and incubated overnight at 37°C. Negative control wells contained only TSB. The content of each well was removed, and the wells were washed three times with 250 µl of sterile PBS (pH 7.4) in order to remove all non-adherent bacteria. The plates were dried at room temperature and attached bacteria were fixed with 200 µl of 99% methanol per well for 15 min. Finally, the attached bacteria were stained for 5 min with 200 µl of 2% crystal violet per well. After rinsing with distilled water the plates were air dried. Then, 160 µl of 96% ethanol was added to each well and mixed.
### Table 1

Oligonucleotide primers used in the study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' → 3')</th>
<th>Amplicon length (bp)</th>
<th>References</th>
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<tr>
<td>ail-a (F)</td>
<td>TGGTTATGCGCAAAAGCCATGT</td>
<td>356</td>
<td>(9)</td>
</tr>
<tr>
<td>ail-b (R)</td>
<td>TGGGAAGTGCGGTGAATTTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ystA-a (F)</td>
<td>GTCTTCATTGGAGGATTGCG</td>
<td>134</td>
<td>(9)</td>
</tr>
<tr>
<td>ystA-b (R)</td>
<td>AATCACTACTGACTCGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ystB-a (F)</td>
<td>AAAGCGGTG(CT)GA(CT)ACTCA(AG)AC</td>
<td>68</td>
<td>(19)</td>
</tr>
<tr>
<td>ystB-b (F)</td>
<td>CA(AG)CATAC(CT)TC(AG)CA(AG)CACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yadA-1 (F)</td>
<td>TAAGATCAGTGTCCTGTCCGGCA</td>
<td>747</td>
<td>(10)</td>
</tr>
<tr>
<td>yadA-2 (R)</td>
<td>TAGTTATTTGCATCCCTAGCAC</td>
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</table>

### Table 2

Occurrence of virulence-associated genes among *Y. enterocolitica* isolates from pigs

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Number of isolates</th>
<th>Results of PCR</th>
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<th></th>
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<tbody>
<tr>
<td>4</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
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<td>1A</td>
<td>33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NT</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

NT - isolate not typeable.

**Fig. 1.** Adhesion of pig-derived *Y. enterocolitica* isolates to polystyrene depending on cell-surface hydrophobicity (A) and biotypes (B).

The absorbance was measured at 595 nm in a microplate reader (Model 550, Bio-Rad, USA). The isolates were classified into four categories: non-adherent, weakly, moderately, or strongly adherent as previously described by Stepanović *et al.* (24). Each assay was repeated three times and the results were averaged.

### Results and Discussion

The detection of the specific sequences within plasmid and cDNA has been applied to the rapid and specific finding pathogenic *Y. enterocolitica*. The sequences homologous to the *ail* and *virF* genes are the most commonly used (5). These sequences have been used for detection of pathogenic *Y. enterocolitica* belonging to 1B, 2, 3, 4, and 5 biotypes. The detection of sequences homologous to the *yadA* and *virF* genes allowed identification of *Y. enterocolitica* isolates with the virulence plasmid pYV. In our study sequence homologous to *yadA* gene was detected. Additionally, we used a primer pair amplifying the *ystB* gene in order to identify *Y. enterocolitica* biotype 1A. The results of multiplex PCR searching for *ail*, *ystA*, *ystB*, and *yadA* genes within biotypes of *Y. enterocolitica* are listed in Table 2. In all isolates of
biotype 1A, the ystB-specific amplification product of 68 bp was obtained. This product was also obtained in biochemically not typeable isolate. No products specific for ystA, ail, and yadA genes were obtained within biotype 1A. The presence of the ystB gene in nearly all pig-derived isolates of biotype 1A was shown by Platt-Samoraj et al. (18). In our previous research (13), it was shown that the ystB gene was present both in the clinical strains and in those isolated from pigs. The presence of the ail and ystA chromosomal genes of virulence was also evaluated. In all the isolates of biotype 4, the specific PCR products for ystA gene and ail gene were obtained. In the majority of this biotype isolates (18/26), the specific PCR product for the plasmid yadA gene was detected. The ail and ystA genes are present in Y. enterocolitica strains, which belong to biotypes regarded as pathogenic (13, 14, 18). Results confirming the presence of the ail gene in biotypes 1B, 2, 3, 4, and 5 were obtained by Wannet et al. (28), who used the ail gene as a specific indicator enabling a quick detection of invasive strains of Y. enterocolitica.

In our research we also evaluated cell surface hydrophobicity of Y. enterocolitica based on bacterial adherence to xylene. The majority of Y. enterocolitica isolates (71%) showed hydrophobic cells surface. The percentage of isolates with hydrophobic surface of cells was 27.8% and 30.8% within biotype 1A and 4, respectively. The high overall proportion of hydrophophilic isolates found in this study suggests that cell-surface hydrophobicity is not important in Y. enterocolitica adhesion and subsequent biofilm formation, because all hydrophilic and hydrophobic isolates of Y. enterocolitica adhered to polystyrene, although in different degree. The isolates with hydrophilic surface of cells adhered weakly (31.8%), moderately (50%), and strongly (18.2%). The percentage of hydrophobic isolates, which adhered to polystyrene weakly, moderately, or strongly was 30%, 60%, and 10% respectively (Fig. 1A). The highest percentage of isolates showed moderate adhesion both among isolates of biotypes 4 (63.6%) and 1A (47.6%). The same percentage of isolates belonging to biotype 4 adhered weakly (18.2%) and strongly (18.2%). The high percentage (38.1%) of biotype 1A isolates showed weak adhesion (Fig.1B).

According to Rosenberg (23), hydrophobicity test and adhesion assay are not always positively correlated. The significance of hydrophobicity in many instances of microbial adhesion is still not clear, particularly role of hydrophobic interaction in mediating adhesion to host tissues and environmental surfaces. Adherence of pathogenic bacteria to host tissues is necessary for infection. The ability of cells to form biofilms is important because physiological changes in cells forming biofilm can protect the bacteria from various stresses. Kim et al. (11) suggested that flagella play a critical role in biofilm formation in Y. enterocolitica. The presence of rotating flagella is required for the bacteria to gain access to micromiches on abiotic surfaces. The ability of biofilm formation by Y. enterocolitica may constitute an adjustment of the bacteria to persist in environmental niches, rather than a mechanism of virulence, because higher temperature in an infected host inhibits the production of flagella. Results obtained by Kim et al. (11) also suggest that flagella are not the only determining factor for the development of biofilms by Y. enterocolitica, because very little biofilm was observed after 6 h, and by 24 h the cydA and crp mutants exhibited wild-type levels of biofilms, although these mutants were completely defective for flagellar expression.

The multiplex PCR assay in this study was developed for simultaneous detection of the ail, ystA, ystB, and yadA genes, which enabled fast evaluation of the potential virulence of the isolates. The multiplex PCR assay was shown to be a sensitive tool for differentiation between the pYY plasmid-bearing Y. enterocolitica isolates, the plasmidless Y. enterocolitica, and Y. enterocolitica biotype 1A isolates. Biofilm formation by all isolates may be a form of adjustment developed by Y. enterocolitica to persist in environmental niches.

References
10. Kaperud G., Vardund T., Skjerve E., Hornes E., Michaelsen T.E.: Detection of pathogenic Yersinia enterocolitica in foods and water by immunomagnetic


