Porcine and Human intestinal cells for profiling the capacity of colonization and infection of the foodborne pathogen Yersinia enterocolitica

Abstract

*Y. enterocolitica* is the third bacterial cause of human enteritis in Europe. The species is divided into six biotypes (BT), BT1A regarded as nonpathogenic and pathogenic biotypes 1B, 2, 3, 4 and 5. Pigs, the principal reservoir for human pathogenic strains, do not develop clinical signs. The BT4 is the most frequently biotype isolated from pig and encountered in human yersiniosis. This study investigated the use of *in vitro* cultured cells to assess the ability of *Y. enterocolitica* to adhere and invade pig and human cells. We tested *in vitro* the adhesion and invasion abilities of a collection of 23 *Y. enterocolitica* on intestinal pork cells IPEC-J2 and on human intestinal cells Caco-2. The overall profile of adhesion / invasion was different in the both tests. Nevertheless, in the two tests, the BT1A and the BT5 strains, which are rarely isolated from pigs, show a low capacity to adhere and to invade. These strains were clustered in the class 1. The class 2, forming by strains having a greater efficiency of adhesion and/or a greater efficiency of invasion, contained predominantly strains of BT4. The results obtained in this study reflect the ability of BT4 to colonize pigs and the low capacity to BT1A and BT5 to colonize pigs and humans.

Introduction

*Y. enterocolitica* is a gram-negative zoonotic enteropathogenic bacterium responsible to yersiniosis, the third most commonly reported zoonosis in Europe (EFSA, 2015). Human yersiniosis is associated with a wide variety of clinical symptoms ranging from mild gastroenteritis to invasive syndromes like terminal ileitis (Bottone, 1999). Infection by *Y. enterocolitica* is due to consumption of contaminated food or water. Following ingestion, the bacteria colonize the lumen of the intestine and cross the intestinal tissue barrier by invading M cells (Grutzkau *et al*., 1990, Schulte *et al*., 2000). The invasion of M cells allow the colonization of the underlying tissues and may result in dissemination of the bacteria to the mesenteric lymph nodes and extra-intestinal sites such as spleen, liver or lungs. All *Y. enterocolitica* are not pathogenic for human. The species is divided into six biotypes. The biotype 1A generally regarded as nonpathogenic and the pathogenic biotypes BT1B, BT2, BT3, BT4, BT5. In France and most other countries worldwide, BT4 is the most prevalent biotype isolated from humans (69%), followed by BT2 (30%) and BT3 (Savin and Carniel, 2008). Because the principal source of contamination is due to ingestion of raw or undercooked pork products, pigs are considered as the principal reservoir for human pathogenic strain of *Y. enterocolitica* (Bottone, 1999). Pigs do not develop clinical signs, but they do carry pathogenic *Y. enterocolitica* in their oral cavity and excrete this bacterium in their feces (Thibodeau *et al*., 1999). Two study in France revealed that pathogenic biotypes BT2, BT3, BT4 and BT5 have been recovered from slaughtered pigs (Fondrevez *et al*. 2010, Fondrevez *et al*. 2014) and that *Y. enterocolitica* BT1A considered as nonpathogenic have been recovered from meat samples (Esnault *et al*., 2013). Because *Y. enterocolitica* is found in the intestine of healthy pigs and because the invasion of intestinal epithelial cells is a major step in *Y. enterocolitica* infection in human, two models using porcine and human intestinal *in vitro* cell culture were assessed to profile a collection of 23 strains containing the five biotypes found in France.
Material and Methods

Bacterial strains and growth media

The characteristics of the 23 strains used in this study are listed in Table 1.

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Biotype and origin</th>
<th>N° of strains</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>F endA1 glv44 thi-1 recA1 relA1 gyrA96 deoII napG Φ80lacZAM15 ΔlacZYA-argF U169, hsdR17(rK m-, λ-)</td>
<td>1</td>
<td>(S.G.N. Grant et al., 1990)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>BT1A, pork meat</td>
<td>2</td>
<td>(Esnauld et al., 2013)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>BT2, slaughtered pig, tonsil swab</td>
<td>3</td>
<td>(Fondrevez et al., 2010, and 2014)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>BT3, slaughtered pig, tonsil swab</td>
<td>5</td>
<td>(Fondrevez et al., 2014)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>BT4, slaughtered pig, tonsil swab</td>
<td>12</td>
<td>(Fondrevez et al., 2010, and 2014)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>BT5, slaughtered pig, tonsil swab</td>
<td>1</td>
<td>(Fondrevez et al., 2014)</td>
</tr>
</tbody>
</table>

Overnight cultures of E. coli and Y. enterocolitica were grown at 30°C in BHI (Brain Heart Infusion medium). Human Caco-2 cells and porcine IPEC-J2 cells were cultured at 37°C in the presence of 5% CO₂.

Caco-2 cells were grown in DMEM supplemented with 10% FBS, 1% NEAA (Non-Essentiel Amino Acid Cell Cultur Supplement) and 20mM HEPES (hydroxyethyl piperazineethanesulfonic acid). IPEC-J2 were grown in DMEM supplemented with 10% FBS.

Cell adhesion and invasion assay

For cell adhesion and uptake assays, 1.10⁵ cells Caco-2 or IPEC-J2 cells were seeded and grown 48 hours in individual wells of 24-well cell culture plates before the addition of bacteria.

The overnight cultures of E. coli and Y. enterocolitica bacteria were washed and diluted in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), to obtain a cellular concentration of 2.10⁵ cfu/ml. For the infection, approximately 2.10⁷ bacteria were added to the 2.10⁵ cells and the 24-well cell culture plates were incubated at 37°C in the presence of 5% CO₂. Three hours post-infection, the cells were washed extensively with PBS (Phosphate Buffered Saline). The total number of adherent bacteria was determined by cell lysis using 0.1% Triton X-100 and plating on bacterial media. Bacterial uptake was assessed by adding 100µg of gentamicin per well. After two hours of incubation. The percentage of bacteria that survived killing was determined after plating on bacterial media. For each strain, the relative level of bacterial adhesion and uptake was determined by calculating the number of CFU relative to the total number of bacteria introduced into cells. The experiments were routinely performed in triplicate.

Statistical analysis

Statistical analyses were performed by using logiciel R (R Development Core Team, 2015). Hierarchical cluster and Newman-Keuls test were used for clustering the strains and the influence of biotype was evaluated by performing Fisher’s Exact Test for Count Data.

Results

The hierarchical cluster and Newman-Keuls analysis clustered the strain into two classes. The ten strains which were in the class 1 presented an adhesion capacity inferior to 15% and an invasion capacity inferior to 3%. The 13 strains of the class 2 were more adhesive and/or more invasive.

Among the 23 strains, 43% of the strains (10/23) are in class 1. This class grouped strains having a low capacity of adhesion and a low capacity of invasion. The class 1 contained the two BT1A strains, the strain of BT5 and 67% (2/3) of strains of BT2, 40% (2/5) BT3 strains and 25% (3/12) BT4 strains.

The 23 strains were also tested in vitro on human intestinal Caco-2 model (Figure 2). The clustering of the strains in two classes was based on the adhesion parameter. The first class, characterized by strains possessing an adhesion capacity inferior to 7%, contained 17 strains belonging to the five biotypes.
Material and Methods

Bacterial strains and growth media

The characteristics of the 23 strains used in this study are listed in table 1.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Biotype and origin</th>
<th>N° of strains</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>F’ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR mfd R388 lacZAM15 Δ(lacZYA-argF) U169, hsdR17(rK-, m’K-, λ-), F’</td>
<td>1</td>
<td>(S.G.N. Grant et al., 1990)</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>BT1A, pork meat</td>
<td>2</td>
<td>(Esnault et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>BT2, slaughtered pig, tonsil swab</td>
<td>3</td>
<td>(Fondrevez et al., 2010, and 2014)</td>
</tr>
<tr>
<td></td>
<td>BT3, slaughtered pig, tonsil swab</td>
<td>5</td>
<td>(Fondrevez et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>BT4, slaughtered pig, tonsil swab</td>
<td>12</td>
<td>(Fondrevez et al., 2010, and 2014)</td>
</tr>
<tr>
<td></td>
<td>BT5, slaughtered pig, tonsil swab</td>
<td>1</td>
<td>(Fondrevez et al., 2014)</td>
</tr>
</tbody>
</table>

Overnight cultures of *E. coli* and *Y. enterocolitica* were grown at 30°C in BHI (Brain Heart Infusion medium). Human Caco-2 cells and porcine IPEC-J2 cells were cultured at 37°C in the presence of 5% CO₂.

Caco-2 cells were grown in DMEM supplemented with 10% FBS, 1% NEAA (Non-Essential Amino Acid Cell Culture Supplement) and 20mM HEPES (hydroxyethyl piperazineethanesulfonic acid). IPEC-J2 were grown in DMEM supplemented with 10% FBS.

Cell adhesion and invasion assay

For cell adhesion and uptake assays, 1.10⁵ cells Caco-2 or IPEC-J2 cells were seeded and grown 48 hours in individual wells of 24-well cell culture plates before the addition of bacteria.

The overnight cultures of *E. coli* and *Y. enterocolitica* bacteria were washed and diluted in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), to obtain a cellular concentration of 2.10⁵ CFU/ml. For the infection, approximately 2.10⁷ bacteria were added to the 2.10⁵ cells and the 24-well cell culture plates were incubated at 37°C in the presence of 5% CO₂. Three hours post-infection, the cells were washed extensively with PBS (Phosphate Buffered Saline). The total number of adherent bacteria was determined by cell lysis using 0.1% Triton X-100 and plating on bacterial media. Bacterial uptake was assessed by adding 100µg of gentamicin per well. After two hours of incubation, the percentage of bacteria that survived killing was determined after plating on bacterial media. For each strain, the relative level of bacterial adhesion and uptake was determined by calculating the number of CFU relative to the total number of bacteria introduced into cells. The experiments were routinely performed in triplicate.

Statistical analysis

Statistical analyses were performed by using logiciel R (R Development Core Team, 2015). Hierarchical cluster and Newman-Keuls test were used for clustering the strains and the influence of biotype was evaluated by performing Fisher’s Exact Test for Count Data.

Results

The adhesion/invasion profile for 23 *Y. enterocolitica* belonging to the five biotypes found in France have been determined for two lines of *in vitro* cultured cells. Because the strains were mainly isolated from pork samples, the adhesion and invasion efficiency were first determined on the intestinal porcine cell line model IPEC-J2 (Figure 1). The strains are clustered in two classes based on their adhesion and invasion capacity.

![Figure 1: Invasion and adhesion profile of 23 Y. enterocolitica into porcine IPEC-J2 cells.](image)

The hierarchical cluster and Newman-Keuls analysis clustered the strain into two classes. The ten strains which were in the class 1 presented an adhesion capacity inferior to 15% and an invasion capacity inferior to 3%. The 13 strains of the class 2 were more adhesive and/or more invasive.

Among the 23 strains, 43% of the strains (10/23) are in class 1. This class grouped strains having a low capacity of adhesion and a low capacity of invasion. The class 1 contained the two BT1A strains, the strain of BT5 and 67% (2/3) of strains of BT2, 40% (2/5) BT3 strains and 25% (3/12) BT4 strains.

Among the 13 other strains are clustered in class 2. Although the class 2 contains the majority of BT3 strains (3/5) and the majority of BT4 strains (9/12), no significant influence of the biotype parameter was observed in the composition of the two classes (Fisher’s Exact Test for Count Data; p=0.16).

The 23 strains were also tested on human intestinal Caco-2 model (Figure 2). The clustering of the strains in two classes was based on the adhesion parameter. The first class, characterized by strains possessing an adhesion capacity inferior to 7%, contained 17 strains belonging to the five biotypes.
Epidemiology and control of hazards in pork production chain – SAFEPORK
One health approach under a concept of farm to fork

Strains of class 1 presented an adhesion capacity inferior to 7% and strains of class 2 an adhesion capacity superior to 7%.

The class 2 contained 33% (1/3) BT2 strains and 42% (5/12) BT4 strains. No significant influence of the biotype parameter was observed in the composition of the two classes (Fisher’s Exact Test for Count Data; p=0.46).

Discussion
Statistical differences in the ability of the isolates to adhere and to invade cultured epithelial cells, between BT1A strains and pathogenic biotypes, have been reported in previous experiment (MC Nally et al., 2006, Schaake et al., 2013). Contrarily to Schaake et al., some strains presented different adhesion and invasion profile for IPEC-J2 test and Caco-2 test. Moreover some strains with a pathogenic biotype are found within the class 1 which is composed of the strains harboring a low capacity of adhesion and a low capacity of invasion. Further analyses have to be done to comfort this observation.

Conclusion
Examination of 23 Y. enterocolitica with two intestinal cell lines, the porcine IPEC-J2 cells and the human Caco-2 cells reflected the ability of BT4 to colonize pigs and the low capacity to BT1A and BT5 to colonize pigs and humans. For characterize more deeply the two classes made in evidence by these tests, more strains have to be analyzed.

Acknowledgements
Financial support for this study was provided by Brittany region in the Program SAD-CYePOD (7890).

References
• Esnault E., Labbé A., Houdayer C. and Denis M., (2013) Yersinia enterocolitica prevalence, on fresh pork, poultry and beef meat at retail level, in France. Proceeding SafePork, Portland, 9-12 september 2013, 72-75

Corresponding author: emile.esnault@anes.fr

Adolescent confection on the control and mitigation of foodborne pathogens in the porcine production chain.
Strains of class 1 presented an adhesion capacity inferior to 7% and strains of class 2 an adhesion capacity superior to 7%.

The class 2 contained 33% (1/3) BT2 strains and 42% (5/12) BT4 strains. No significant influence of the biotype parameter was observed in the composition of the two classes (Fisher’s Exact Test for Count Data; p=0.46).

Discussion

Statistical differences in the ability of the isolates to adhere and to invade cultured epithelial cells, between BT1A strains and pathogenic biotypes, have been reported in previous experiment (MC Nally et al., 2006, Schaake et al., 2013). Contrarily to Schaake et al., some strains presented different adhesion and invasion profile for IPEC-J2 test and Caco-2 test. Moreover some strains with a pathogenic biotype are found within the class 1 which is composed of the strains harboring a low capacity of adhesion and a low capacity of invasion. Further analyses have to be done to comfort this observation.

Conclusion

Examination of 23 Y. enterocolitica with two intestinal cell lines, the porcine IPEC-J2 cells and the human Caco-2 cells reflected the ability of BT4 to colonize pigs and the low capacity to BT1A and BT5 to colonize pigs and humans. For characterize more deeply the two classes made in evidence by these tests, more strains have to be analyzed.