DIVERSITY OF YERSINIA ENTEROCOLITICA POPULATION IN A SLAUGHTERHOUSE BETWEEN 2009 AND 2010 AND DISCRIMINATION ABILITY OF MLVA COMPARED TO PFGE

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Abstract

Yersiniosis is a human disease mainly due to the ingestion of raw or undercooked pork meat contaminated mostly with Yersinia enterocolitica (Ye). In France, 74.3% of pig batches at slaughterhouses carried pathogenic Ye. Among them, biotype 4 (BT4) and biotype 3 (BT3) were often recovered. PFGE is one of the most used methods to type Ye, with the restriction enzymes, XbaI and NotI. Nevertheless, MLVA method based on the diversity of six loci tends to replace PFGE; this method showed a higher discriminatory power in others studies. We investigated the genetic diversity of Ye strains isolated in 2009 and in 2010 in one pig slaughterhouse in France and compared the ability of MLVA and PFGE to discriminate the strains.

During these two years, 335 isolates were collected from pigs. The BT4 represented 88.4% of the strains (296/335) and the BT3 only 11.6% (39/335). PFGE using XbaI enzyme allowed the identification of 12 XbaI-PFGE types and among them only one was common to the both surveys. Because the Simpson’s Index shows a low genetic diversity 31 BT4 strains and 39 BT3 strains were typed using MLVA. For BT3 strains, MLVA had the same index of diversity than PFGE (DI=0.472). In contrary, the index of diversity was significantly higher with MLVA (DI=0.871) than with PFGE (DI=0.665) for BT4 strains.

Our study revealed that the population of Ye in pig varied over the time. The comparison of the both typing methods indicated that MLVA has a better discriminatory power than XbaI-PFGE method for BT4 strains but not for BT3 strains.

Introduction

Yersinia enterocolitica is one of the most relevant biological hazards in the European Union, with yersiniosis being the third most frequently reported foodborne bacterial zoonosis after campylobacteriosis and salmonellosis. The average incidence of human yersiniosis in the European Union in 2015 was 2.2 cases per 100,000 populations (EFSA and ECDC 2016). Yersiniosis cases reported in 2015 in the European Union were mostly due to Y. enterocolitica, and especially to biotype 4 (BT4) (EFSA and ECDC 2016). In France most of the clinical cases are largely due to BT4 strains (Le Guern et al. 2016).

Pigs are considered to be the most important reservoir of Y. enterocolitica. This species can be divided into six biotypes and two groups according to their pathogenicity, biotype (BT) 1A which one is considered as “nonpathogenic”, and BT1B, BT2, BT3, BT4 and BT5 that are known to be pathogenic. To better appreciate the risk that these strains represent to humans, it is very important to characterize them.
Currently, to study the genetic diversity of *Y. enterocolitica*, the most common genotyping method used to discriminate between *Y. enterocolitica* strains is Restriction Fragment Length Polymorphism-Pulsed-Field Gel Electrophoresis (RFLP-PFGE). In other studies, many different restriction enzymes have been used. The most frequent restriction enzymes are *Not*I and *Xba*I (Fredriksson-Ahomaa, Stolle, and Korkeala 2006). Researchers have observed limited diversity among BT4, even among strains of different geographical origin with RFLP-PFGE typing (Fredriksson-Ahomaa et al. 2003; Fredriksson-Ahomaa et al. 2006). That’s why other typing method more discriminatory are needed.

In this context, the MultiLocus Variable-number tandem repeats (VNTR) Analysis (MLVA) have been developed. This method is increasingly used in subtyping *Y. enterocolitica* strains. This technique consists of the detection of six VNTRs. Several studies showed a high discriminatory power of the MLVA methods (Gierczynski et al. 2007; Sihvonen et al. 2011).

The aim of the study was to evaluate the diversity of *Y. enterocolitica* strains isolated from two different years in one pig slaughterhouse and to evaluate the two typing method *Xba*I-RFLP-PFGE and MLVA.

**Materiel and methods**

**Bacterial strains and biotype**

Strains used in this study were collected in one French pig’s slaughterhouses during two surveys. A total of 304 strains were isolated in 2009 (Fondrevez et al. 2010) and 31 in 2010-2011 (Fondrevez et al. 2014). The biochemical assays used to biotype *Yersinia enterocolitica* strains was carried out as described in the ISO 10273:2003 method.

**Restriction fragment length polymorphism-pulsed-field gel electrophoresis (RFLP-PFGE)**

RFLP-PFGE was done using *Xba*I (XbaI-PFGE) restriction enzymes (Roche, Boulogne-Billancourt, France). Strains were sub-cultured on Plate Count Agar (PCA) at 30°C for 24h. Bacterial suspension in TE buffer (0.01 M Tris-EDTA buffer, pH 8.0) were adjusted to an optical density (600 nm) of 1.5 and mixed with 1% agarose for the plug preparation. Plugs were incubated for 48h at 50°C in lysis solution (Na2EDTA 0.5M, pH9, N-lauryl-Sarcosyl 1%, proteinase K 1 mg/ml). A total of five washes with TE buffer were used to remove excess reagents and DNA was then digested with 40U of *Xba*I at 37°C for 4 hours. The electrophoresis conditions had an initial switch time of 1.5s, with final switch time of 18.0s, for 25h. Electrophoretic patterns were compared using BioNumerics software (Applied Math, Sint-Martens-Latem, Belgium). The Simpson’s index (DI) was determined as described by Hunter and Gaston (1988) to assess the diversity of the populations.

**Multi-locus variable number tandem repeat (MLVA)**

MLVA was performed using the technique developed by Gierczynski et al. (2007) and improved by Sihvonen et al. (2011). Six Variable Number Tandem Repeat (VNTR) were considered; they are coded V2A, V4, V5, V6, V7 and V9. We used the same
primers as Gierczynski et al. (2007). The six VNTR were amplified in two distinct multiplex PCRs. The first one amplified the VNTRs V2A, V4 and V6 with the forward primers labelled respectively by 6-FAM, HEX and Cy3 fluorescent Dye. The second amplified the VNTRs V5, V7 and V9 with the forward primers labelled respectively by 6-FAM, HEX and Cy3 fluorescent Dye.

The multiplex PCRs were performed with QIAGEN Multiplex PCR kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions in a total volume of 25 μl. The PCR conditions were the same as those described by Sihvonen et al. (2011). The two PCR products of each strain were diluted to 1/100 in sterile water, and run separately in a capillary electrophoresis with an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA) using D (DS-30) fragment analysis chemistry according to the manufacturer’s instructions. The Geneflo™ 625 ROX labelled (EurX, Gdańsk, Poland) was used as an internal size standard. Electrophoretic patterns were analyzed using BioNumerics 7.6 software (Applied Math, Sint-Martens-Latem, Belgium). The Simpson’s index (DI) was determined to assess the diversity of the populations.

Results

Among the 335 Y. enterocolitica strains isolated from one french slaughterhouse, the majority of the strains were from BT4 (88.4%). BT3 was less abundant (11.6%) and was only recovered in 2009 probably because there were many more strains isolated in 2009 (304 strains) than in 2010 (31 strains) in this slaughterhouse.

The 335 strains were distributed in 12 XbaI-PFGE profiles coded X1 to X12 with a DI of 0.647. We obtained eight XbaI-PFGE profiles (X1 to X8) in 2009 and five in 2010 (X1 and X9 to X12) with only X1 common over the two years (Figure1).

![Figure 1. XbaI-PFGE profile (X1 to X12) distribution between the two years. The Simpson’s Index (DI) and the number of XbaI-PFGE profile (p) and the number of strains (n) are indicated for 2009, 2010 and for the two years.](image)

The XbaI-PFGE shows also a low discriminatory power among years with a DI of 0.661 in 2009 and 0.631 in 2010. Moreover, BT4 and BT3 strains showed three similar profiles (X3, X8 and X10). So another typing method is necessary.

We evaluated the discriminatory power of MLVA compared to PFGE on 70 strains including 31 BT4 strains and 39 BT3 strains. We obtained a total of 16 MLVA
genotypes, and no one was common between BT4 and BT3. The BT4 strains were distributed in 13 MLVA genotypes (M1 to M13) and the BT3 strains were distributed in three MLVA genotypes (coded M14 to M16), (Figure 2).

The MLVA compared to XbaI-PFGE on 70 strains presents a significantly higher DI which is respectively 0.813 and 0.647 (Table 1). For the BT4 strain the DI was equal to 0.871 and significantly higher than the DI obtained from PFGE (DI=0.665). For the BT3 strains the DI was identical for the two methods (DI=0.472).

Table 1. Simpson’s index (DI) of the RFLP-PFGE and MLVA on the 70 strains studied in MLVA, and the confidence interval at 95% (CI95%).

<table>
<thead>
<tr>
<th></th>
<th>Number of strains</th>
<th>RFLP-PFGE</th>
<th>MLVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DI</td>
<td>CI95%</td>
</tr>
<tr>
<td>Total strains</td>
<td>70</td>
<td>0.647</td>
<td>[0.596 ; 0.699]</td>
</tr>
<tr>
<td>BT4</td>
<td>31</td>
<td>0.665</td>
<td>[0.559 ; 0.770]</td>
</tr>
<tr>
<td>BT3</td>
<td>39</td>
<td>0.472</td>
<td>[0.354 ; 0.591]</td>
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**Discussion**

This study showed that BT4 strains are mainly recovered in pig slaughterhouses, as in many other studies (Van Damme, Habib, and De Zutter 2010). The RFLP-PFGE analysis revealed the presence of an identical XbaI-PFGE profile over the two years. This profile is perhaps strongly associated with the French pork industry. The others PFGE profiles were different over the two years suggesting that the population colonizing the pig seems to be changing over time.

This study revealed that MLVA shows a better discriminatory power than RFLP-PFGE for the BT4 strains. This result is in accordance to those of Sihvonen et al. (2011). MLVA typing methods seem to be a better tool for discriminating of *Y. enterocolitica* BT4 than RFLP-PFGE using XbaI enzyme. For the BT3, we observed that MLVA and RFLP-PFGE had exactly the same discriminatory power. This result is not in agreement with a study of Wang et al. (2012) that revealed in MLVA a great
heterogeneity between BT3 strains isolated during a 20 year period. Our study was carried out on 39 strains isolated during the year 2009 which may explain our result.

On the other hand, we used only one enzyme (XbaI). It would be interesting to compare our MLVA results with the combination of RFLP-PFGE profiles issued from digestion with other restriction enzyme, which may give a better discriminatory power (Fredriksson-Ahomaa, Stolle, and Korkeala 2006)

**Conclusion**

This study revealed that the population of *Y. enterocolitica* colonizing the pig seems to be changing over time. It also showed that MLVA typing present a better discriminatory power than RFLP-PFGE using XbaI restriction enzyme for BT4 strains.

**Acknowledgements**

This work was conducted in the frame of a thesis founded by the agglomeration of Saint-Brieuc and the Brittany region. It responds in part to a project funded by le Compte d’Affectation Spéciale "Développement agricole et rural".

**References**


