Yersinia enterocolitica prevalence in a French slaughterhouse: first results

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Abstract

Yersinia enterocolitica is involved in human foodborne infections. Pigs are considered as a major reservoir in many countries. The aim of the study was to contribute to the evaluation of the prevalence of Y. enterocolitica in France in pigs at the slaughterhouse level with optimized detection methods based on ISO 10273-2003. Several samples of tonsils over nine consecutive months were analyzed in a single slaughterhouse. Enumeration and isolation were achieved by using CIN agar and YeCM chromogenic medium (modified from the Wcagant medium, 2008). Two enrichment media were used: a peptone, sorbitol and biliary salts broth (PSB) and the Irgasan, Ticarcillin and potassium chlorate broth (ITC). The identification was performed on lactose negative, oxidase negative, urea positive, and TDA negative strains using API 20E gallery, and biotypes were identified by biochemical typing. We determined bio- serotypes most frequently encountered in Europe (i.e. 1A, 4 O:3, 2 O:9 and 3 O:5.27) by PCR, targeting the ail virulence gene, the plasmid (virF), the RfbC and 16S RNA genes. The total prevalence of Yersinia enterocolitica at slaughterhouse, was 25% on 236 tonsils’ samples, and 18.8% on 32 faeces’ samples, with a large majority of bio- serotype 4 O:3. Several samples showed the presence of the bacteria only on the modified YeCM medium. Besides, the number of CFU was higher compared to CIN agar. Best results were also obtained with ITC than PSB broth enrichment. This project will contribute to a better understanding about risks of food infections caused by Yersinia enterocolitica. That’s why it is important to develop more efficient protocols using classical microbiology and molecular biology methods.

Introduction

Yersinia enterocolitica in human infections is increasing, and its prevalence comes just after Salmonella and Campylobacter in Europe (Fredriksson-Ahoma and Korkeala, 2003, Thibodeau et al, 1999). Y. enterocolitica is mainly isolated from pork (tongue and tonsils) and the most encountered bio- serotypes are 4 O:3, 2 O:9 and 3 O:5.27, which are pathogenic. Therefore, the International Standard Organization method for the detection of presumptive pathogenic Y. enterocolitica (ISO 10273:2003) is applied. However, this standardized protocol describes enrichment and isolation procedures, which are not enough selective for Y. enterocolitica because many members of the Enterobacteriaceae family can grow on described media like CIN and SSDC. This leads to difficulties in the isolation from foods containing contaminants. Improvements of these methods are necessary. The aim of our study was to optimize and improve the method ISO 10273 and to develop a DNA-based method for the identification of Y. enterocolitica and of pathogenic bio- serotypes. The PCR used in this study targets the virulence genes and the 16SRNA gene. These optimized techniques were used to evaluate the Y. enterocolitica prevalence in the pig’s tonsils and feces in a single French slaughterhouse, and also to appreciate the isolated strains’ pathogenicity.

Material and methods

Bacterial strains. Reference strains of Yersinia are provided by the Pasteur Institute: CIP 124 (1A), CIP 134 (4 O:3), CIP 383 (2 O:9), CIP 29228 (3 O:5.27). They were used as controls to assess the growing on the plate media, presumptive tests and biotyping tests.

Pork samples. 236 tonsils and 32 faeces were sampled and analyzed, in a single slaughterhouse, on 10 batches, during 9 months. One or two batches are taken for each sampling day. Tonsils are first swabbed (10cm²) and partly or entirely excised (10g). About 10g of faeces are taken from intestinal tract of identified animal with removed tonsils.
Enrichment. 10g from each fecal sample and tonsil were added in a stomacher bag, suspended in 90mL of peptone water (faeces) or PSB (Peptones Sorbitol, Biliary salts) broth (tonsils), and then mixed in a stomacher for 30 seconds. One milliliter of the suspension was added to 9mL of ITC (Biorad) broth and was incubated at 25°C for 48h. Swabs are also immersed in 9mL ITC broth. The tonsils in PSB broth were further incubated at 25°C during 5 days.

Numeration and isolation. Two medium were used: CIN agar (Cefsulodine, Irgasan, Novobiocine, Biorad) and modified YeCM (Versinia enterocolitica Chromogenic medium) (Weagant, 2008). Before enrichment, 0.1mL of mixed tonsils and feces (with dilution 10^2 and 10^4) are spread on and these media are incubated during 24 to 48H at 30°C. After enrichments and isolation, same incubation conditions were applied. A maximum of five colonies were subcultured on TCS (Trypton Casein Soya, Biorad) agar at 30°C for 24h.

Target tests. Y. enterocolitica formed on CIN agar small colonies with deep red center surrounded by a clear colorless zone. On modified YeCM, it formed small colorless colonies surrounded by a yellow halo at 24h. At 48h, colonies center became blue-green with colorless border. Characteristic colonies were subcultured and four presumptive tests were made: Lactose on VRBL medium (Violet crystal, neutral Red, Biliary salts, Lactose, Biorad), oxidase (solution at 1%, WVR). Urea and TDA tests were both carried out on the same plate of 96 wells: after the reading of the urea test, one drop of HCl 1N is added in the wells, then one drop of ferrous chiorure.

Identification. The lactose negative, oxidase negative, urea positive and TDA negative strains were identified by API 20E gallery (Biomérieux).

Biotyping. The biotypes were defined by using 6 tests: esculine hydrolysis (ROSCO discs), pyrazinamidase (pyrazinamide medium, ISO 10273), tween-esterase (TWEEN-ESTERASE medium, ISO 10273), indole (peptone water, and revelation with Kovacs reagent, Biorad), xylose and trehalose fermentation (carbohydrate fermentation media at 1% with bromocresol purple).

DNA extraction. DNA extraction was performed by boiling at 95°C during 10 minutes some colonies suspended in 200µL of TE 1X (10mM Tris-Hcl-1mM EDTA, Euromedex). After centrifugation at 5000 rpm during 3 minutes, the supernatant was transferred in a new tube.

PCR assay. The PCR contains primers targeting ail (forward, 5'-GTTTAGATATTTGCGTCTG TTAATGTGTACC-3'; reverse, 5'-CTATGGTTGGAGATTTATGATGACG-3'), virF (forward, 5'-AAGGTGTTGAGACTTCAAGATGG-3'; reverse, 5'-TGGTGTGACTTGAGACG-3'), rbcC (forward, 5'-CGCATCTGGGACGACTAATTCC-3'; reverse, 5'-CCGCGATTCCTCAAAAAGCCACC-3') and 16S rRNA (forward, 5'-ATACCGATAATTTTAATGTCA-3'; reverse 5'-TTGCTGGGAAGCTTACC-3') gene sequences from Y. enterocolitica (Arnold et al., 2004, Thisted Lambert et Danielsson-Tham, 2005). The sequences were synthesized (Invitrogen) and amplified respectively 454bp, 700bp, 405bp and 345bp DNA fragments. The PCR mixture contained 1X PCR buffer (New England Biolabs), 2mM MgCl2 (New England Biolabs), 0.25mM dNTPs (Invitrogen), respectively 10µM, 15µM, 20µM and 6.4µM of primers, 1U of Taq polymerase (New England Biolabs), and 5µL of DNA template. Thermal cycling conditions were as follows: 94°C for 3min, followed by 30 cycles of 94°C for 30s, 55°C for 1min, and 72°C for 1min. PCR was performed in microtubes, using a thermal cycler (Applied Biosystems). Ten µL of PCR products were migrated in a 1.5% agarose gel, at 110V during 1h30. After migration, the gel is stained in an ethidium bromide bath (0.2 µg/mL) during 20 minutes and then rinsed in clear water.

Results

According to our nine months study, the prevalence of Y. enterocolitica in the pig's tonsils, whichever breeder or whatever period, was about 25% (59/236). The detection rate varied from 0% (0/20) to 95% (19/20) depending on the batch (Table 1). The bacteria presence in the faeces was less frequent than in the tonsils: 18.8% (6/32). According to some sampling day results, the use of YeMC modified agar increased the sensitivity of the detection procedure; each positive samples with CIN was detected with modified YeCM. Some samples were also only positive with this medium for the numeration before enrichment. Besides typical colonies of Y. enterocolitica on this plate appeared more differentiable compared to those on CIN.

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98.3% (58/59) of the positive tonsils contained the bioserotype 4 O:3. One of the tonsils (1.7%) contained *Y. enterocolitica* of the both bioserotypes 1A and 4 O:3 and another one the 3 O:5,27 bioserotype. The same trend was observed for faeces with 83.3% of them (5/6) containing 4 O:3 and 16.7% (1/6) non pathogenic 1A.

<table>
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<th>Numbers of positive samples on YeCM</th>
<th>Numbers of positive samples on modified YeCM</th>
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<tr>
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<td></td>
<td>Total</td>
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*Table 1: Prevalence of *Y. enterocolitica* except October and December.*

PCR analysis was performed to complete the results. This molecular method allowed us to confirm the identification by API gallery and to determine the pathogenic strains and the serotype O:3 of those strains. Three different profiles presented in Figure 1 were obtained from the amplified fragments. The results obtained with the strains from pork confirmed that *Y. enterocolitica* belonged to the biotype 1A and to the serotype O:3. The proportion of the pathogen bioseerotype carrying the plasmid was about 88%, showing thus that this plasmid can be lost during the cultivation in laboratory.

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Figure 1: Four control strains m-PCR profiles.

M: ladder 50 bp; 1: 1A; 2: 4 O:3 (without plasmid); 3: 2 O:9 (with plasmid);
4: 3 O:5,27 (with plasmid); 5: Negative control.

Discussion
The prevalence of *Y. enterocolitica* in pig’s tonsils observed in this slaughterhouse is quite high. Detection of the bacteria is better when using swabs than analyzing the entire tonsils. The prevalence appeared lower using PSB enrichment compared to ITC use. Besides, enrichment with PSB broth showed a very important contaminant flora, due to the lack of selectivity of this medium, which was not observed with ITC broth. Because of several antibiotics and inhibitors presence in its formula, this last prevents the annex flora to grow, leading to an easier observation on plate. The rate of *Y. enterocolitica* observed in tonsils led us to search the bacteria in the faeces. However, despite faeces and tonsils were analyzed from the same animal, it seems that no correlation exists between the presence in tonsils and presence in faeces from the same pork. The predominance of pathogenic bioseerotypes shows that slaughtering must be done with precautions to avoid the carcass’ contamination. Evisceration and head removing is an important step to avoid the dispersion of *Y. enterocolitica*. These bioseerotypes which were found belong to the pathogenic 4 O:3, in accordance with the trend in Europe (Fredriksson-Ahomaa and Korkeala, 2003). However, the current method of PCR used in this study, cannot differentiate yet two of the major serogroups in Europe (2 O:9 and 3 O:5,27). Its prevalence doesn’t seem to be influenced by the season, because the observed *Y. enterocolitica* rates didn’t decrease or increase during the year. However, a difference of prevalence is noted between the batches analyzed, according to the breeder, indicating the possibility of specific farm factors. It can be possible to complete our investigation in researching the bacteria on the pigs’ carcass. PFGE (Pulsed Field Gel Electrophoresis) technique, in establishing the genetic profiles of the bacteria would allow us to determine a possible common contamination origin.

Conclusion
The study of *Y. enterocolitica* prevalence in a single pigs slaughterhouse, allowed us to establish its prevalence, which was quite important. Indeed, the found predominance of pathogenic bioseerotypes 4 O:3, follows the trend in Europe. The application of a multiplex PCR showed that it was possible to confirm the specie *Y. enterocolitica* and to determine the pathogenic strains. These results underline the importance of hygiene in the first steps of slaughtering, because this bacterium seems to take an important place among foodborne diseases, causing a public health issue.

References


