Yersinia enterocolitica prevalence and diversity in a pig slaughterhouse

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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. 516 samples of tonsils, feces and carcasses were analyzed from 344 pigs (24 batches) in a single slaughterhouse over 23 consecutive months. Enumeration and isolation were achieved by using CIN agar and a new YeCM chromogenic agar. Strains are characterized by phenotypic and genotypic methods: species identification and bioserotypes by API 20E gallery and biochemical typing confirmed by PCR, targeting the ail virulence, the plasmid (virF), the RfbC and 16SRNA genes. The total prevalence of Yersinia enterocolitica were 26.8%, 18.3% and 0% in tonsils', feces' and carcasses' pigs respectively with 94.5% of pathogenic bioserotype 4 O:3 and 4.5 % of non pathogenic bioserotype 1A (288 typable strains). Moreover, the pigs' contamination in the slaughterhouse is higher in winter (34.3%) than in summer (9.3%) even in batches from the same breeder. 100 strains were analysed and compared by PFGE typing. Only a few different genotypes were obtained: 5 distinct profiles with ApaI, 4 with NotI and 5 combined profiles. More diversity was observed with ApaI than with NotI. The strains of bioserotype 1A have different patterns from the strains of bioserotype 4 O:3. This project will contribute to a better understanding about risks caused by Yersinia enterocolitica. It is the reason 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 with 7595 reported human cases in 2009 (EFSA, 2011). Y. enterocolitica is mainly isolated from pork (tongue and tonsils) and the most encountered bioserotypes are 4 O:3, 2 O:9 and 3 O:5,27, which are pathogenic. The International Standard Organization method for the detection of presumptive pathogenic Y. enterocolitica (ISO 10273:2003) is applied with some optimization and improvement like the use of a new chromogenic media for isolation. We also used a PCR method for the identification of Y. enterocolitica and of pathogenic bioserotypes targeting virulence genes and the 16SRNA gene. The aim of this study was to evaluate the Y. enterocolitica prevalence in the pig's tonsils faeces and carcasses in a single french slaughterhouse. The isolated strains were evaluated for their pathogenicity and genetically compared to some human strains from local clinical cases of yersiniosis.

Material and methods
Bacterial strains
Reference strains of Yersinia CIP 124 (1A), CIP 134 (4 O:3), CIP 383 (2 O:9), CIP 29228 (3 O:5,27) were used as controls for growing on the plate media, presumptive and typing tests.

Pork samples
345 tonsils, 104 faeces and 72 carcasses were analyzed, in a single slaughterhouse, on 24 batches, during 23 consecutive months. One or two batches are taken for each campaign. Tonsils (about 10 cm¬2) and about ¼ forequarter external carcasses are swabbed. About 10g of faeces are taken from intestinal tract of identified animal with r emoved 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 (tonsil or carcass), 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 immerged in 9mL ITC broth.

Enumeration and isolation

Two medium were used: CIN agar (Cefsulodine, Irgasan, Novobiocine, Biorad) and a new modified YeCM (Yersinia enterocolitica Chromogenic medium) (from Weagant, 2008). Before enrichment, 0.1 mL of mixed tonsils and faeces (with dilution 10-2 and 10-4) were 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%, VWR). 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, before one drop of ferrous chlorate.

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 (peptoned 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'-GTTTATCAATTGCGTCTG TTAATGTGTACG-3'; reverse, 5'-CTATCGAGTTTGGAGTATTCATATGAAGCG-3'), virF (forward, 5'-AAGGTTGTTGAGCATTCACAAGATGG-3'; reverse, 5'-TTTGAGTGAA ATAAGACTGACTCGAGAACC-3'), rfbC (forward, 5'-CGCATCTGGGACACTAATTCG-3'; reverse, 5'-CCACGAATTCCATCAAAACCACC-3') and 16SRNA (forward, 5'-ATACC GCATAACGTCTTCG-3'; reverse 5'-TTCTTCTGCGAGTAACGTC-3') gene sequences from Y. enterocolitica (Arnold et al, 2004, Thisted Lambertz 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). 10µL 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.

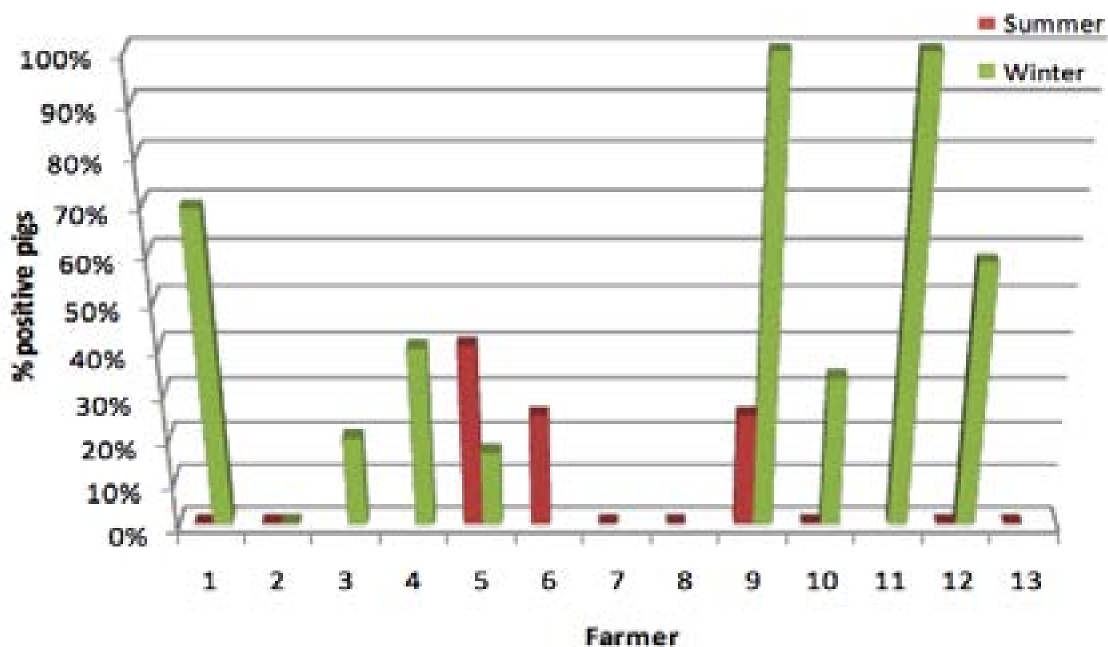
PFGE typing

The genomic DNAs were prepared in agarose plugs, using a protocol described by PulseNet for Listeria (CDC, 2004), with a 2 h at 37°C lysis (solution: 50 mM Tris, pH 8, 50 mM EDTA, pH 8, 1% sarcosyl, 500 µg proteinase K/strain) for. After washes, they were digested with 20 U ApaI or NotI (New England Biolabs) for 5 h at respectively 25°C and 37°C. Pulsed field gel electrophoresis was performed in 1% Seakem Gold agarose (Invitrogen) using a CHEF DR III system (Bio-Rad) in 0.5x TBE (Tris borate EDTA) buffer at 14°C during 18h. Gels were stained with ethidium bromide and digitalized with the Gel Doc 2000 apparatus (Bio-Rad). Salmonella ser. Braenderup strain (H9812) digested with XbaI was used as a reference standard. Similarity is based on 90% by UPGMA method.

Results

The prevalence of Y. enterocolitica in the pigs in a single slaughterhouse was about 31%. 67% of pork's batches are contaminated: the rate varies from 0% (0/20) to 100% (12/12) depending on the batch The prevalence of Y. enterocolitica in the pig's tonsils, whichever breeder or whatever period, was about 26.8% (92 /345) . The prevalence of the bacteria in the faeces was lower than in the tonsils: 18,3% (19 /104). None of the 72 carcasses was detected as contaminated. Some campaigns showed that the modified YeCM medium seemed to be sensitively better than the CIN agar. Besides typical colonies of Y. enterocolitica on this plate are more differentiable compared to those on CIN. The prevalence seemed to be higher in winter/spring than in summer. A difference of prevalence was noted between the batches analyzed according to the pigs' primary producer, indicating the possibility of specific farm factors (see figure 1).

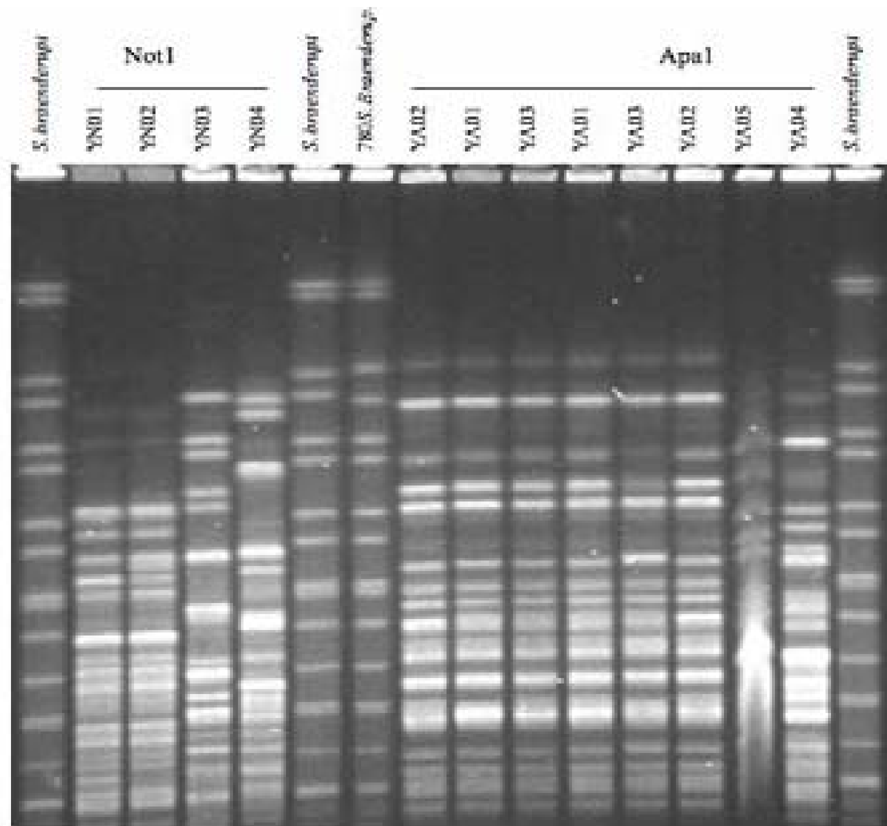
Figure 1: Y. enterocolitica pigs' contamination according to farm origin and season (% positive)



PCR allowed us to confirm the species identified by API gallery and to determine the pathogenic strains and the serotype O:3 of those strains. 94.5% of the 288 analysed strains from pork confirmed that the Y. enterocolitica belonged to the bioserotype 4 O:3 and 4.5 % to the non pathogenic biotype 1A,1% of the positive tonsils contain the bioserotype 4 O:3. One of the tonsils (1.9%) contains Y. enterocolitica of the both bioserotypes 1A and 4 O:3 and another one the 3 O:5,27 bioserotype. The same tendency is observed for faeces with 88.5% of them containing 4 O:3 and 11.5% non pathogenic 1A.

Only a few different genotypes were obtained by PFGE analysis of the 100 isolates of Y. enterocolitica from pork origin (100 with NotI; 69 with ApaI ; 63 with combined profile): 4 distinct NotI patterns; 5 distinct ApaI patterns; 5 distinct combined NotI ApaI profiles) (see figure 2). 53% of pigs' strains showed the same NotI pattern and 45% of pigs' strains showed the same ApaI pattern. The 4 strains of bioserotype 1A present 2 distinct profiles (YN03 and YN04 for NotI; YA04 and YA05 for ApaI) which were clearly different from the 96 strains of bioserotype 4 O:3.

back to index



Discussion

The prevalence of Y. enterocolitica in pig's tonsils observed in this slaughterhouse is quite important. The rate of Y. enterocolitica observed in tonsils led us to search the bacteria in the faeces. However, because faeces and tonsils were analyzed on the same animal, it seems that no correlation exists between the presence in tonsils and presence in faeces from the same pork. No carcass was detected positive. The predominance of pathogenic bioserotypes 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 bioserotypes 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). The prevalence seems to be infuenced by the season, because the observed Y. enterocolitica rates more important in winter than in summer kike observed by Milnes et al (2007). However, a difference of prevalence is noted between the batches analyzed, according to the breeder, indicating the possibility of specific farm factors. Characterisation of pork isolates of Y. enterocolitica by PFGE analysis revealed a low genetic diversity, lower than those observed by Fredriksson-Ahomaa and Korkeala (2003). This could be explained by the pork origin: a single slaughterhouse. More diversity was observed with ApaI than with NotI. The strains of bioserotype 1A present clearly different patterns from the strains of bioserotype 4 O:3.

Conclusion

The study of Y. enterocolitica prevalence in a single pigs slaughterhouse, allowed us to establish its prevalence, which was quite important. Indeed, the predominance of pathogenic bioserotypes 4 O:3 found, 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 with this technique. The contamination is more important in winter than in summer. 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.

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