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A simplified method for detecting pathogenic *Yersinia enterocolitica* in slaughtered pig tonsils

Marc Fondrevez [a], Annie Labbé [a], Emmanuelle Houard [a], Philippe Fravalo [a], François Madec [b], Martine Denis [a,*]

[a] Anses (Agence nationale de sécurité sanitaire), unité HQPAP, BP53, 22440 Ploufragan, France
[b] Anses (Agence nationale de sécurité sanitaire), unité EBEP, BP53, 22440 Ploufragan, France

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ABSTRACT

The aim of this study was to collect preliminary data on the carriage of pathogenic *Yersinia enterocolitica* in slaughtered pigs in France and to test a simplified method for detecting these strains from tonsils. From January to March 2009, 900 tonsil swabs were taken from pigs at one slaughterhouse in Brittany, France. The swabs were vortexed in 10 ml PSB broth, then 1 ml was added to 9 ml ITC broth. The media were incubated for 48 h at 25 °C. The PSB enrichment broth was streaked on CIN plates and the ITC enrichment broth on SSDC plates. In addition to the ISO 10273 method, we also streaked ITC enrichment broth on CIN plates. The plates were incubated for 24 h at 30 °C, and we then streaked a maximum of four typical colonies per plate onto a plate containing chromogenic medium (YeCM), for the isolation of pathogenic *Y. enterocolitica* isolates. In parallel, biochemical assays were carried out to confirm the identification of the isolates as *Yersinia* and to determine biotype.

After passage on a YeCM plate and biochemical tests, 380 strains were confirmed to be pathogenic *Y. enterocolitica*. Finally, with the ISO 10273 method, 9.1% ($CI_{95\%}$ [5.8–12.4]) of tonsil swabs and 60% ($CI_{95\%}$ [45.4–74.6]) of the batches were positive. With the ITC-CIN method, 14.0% ($CI_{95\%}$ [10.7–17.3]) of the tonsil swabs and 68.9% ($CI_{95\%}$ [54.3–83.5]) of the batches were positive. Identification as pathogenic *Y. enterocolitica* was confirmed for 97.0% of the typical colonies obtained on the chromogenic medium, YeCM. The most prevalent biotype was biotype 4 (80.5% of the isolates), followed by biotype 3.

This study demonstrates that the ITC-CIN method, followed by streaking on YeCM, may be an effective approach to the isolation of pathogenic *Y. enterocolitica* from tonsil swabs and the recovery of positive samples. This method is less time-consuming than the ISO 10273 method and reduces the number of biochemical tests required for the confirmation of *Yersinia* identification, through the use of YeCM.

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1. Introduction

In 2007, there were 8,792 reported cases of human yersiniosis in the European Union, making *Yersinia* the third most important zoonotic agent implicated in human enteritis, in terms of the number of cases, after *Campylobacter* and *Salmonella* (EFSA (European Food Safery Authority), 2009). *Yersinia enterocolitica* was the most common species identified in human cases and was isolated from 93.8% of all confirmed cases. In France, the incidence was about 16 confirmed cases per 100,000 inhabitants in 2003 (Leclercq and Carniel, 2004). Human pathogenic *Y. enterocolitica* strains can be classified into five biotypes: 1B, 2, 3, 4 and 5. Biotype 1A strains are generally considered to be non-pathogenic and widespread in the environment (Bottone, 1999). In France, biotype 4 is the most prevalent biotype among the strains isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (1%) (Savin and Carniel, 2008).

This bacterium has been detected in pigs and pork meat (Bhaduri et al., 2009; Hudson et al, 2008; Von Altrock et al., 2006; Boyapalle et al., 2001; Lambertz et al., 2007). Pigs thus constitute a potential reservoir of *Y. enterocolitica* strains pathogenic for humans. Pigs develop no clinical signs, but they carry *Y. enterocolitica* in the oral cavity, particularly on the tongue and tonsils and in the lymph nodes, and they excrete the bacterium in their faeces (Thibodeau et al., 1999; Nesbakken et al., 2003). *Y. enterocolitica* is found in the tonsils of pigs, even at slaughter. In general, it is easier to recovery larger amounts of *Y. enterocolitica* from tonsils than from tongues (Nesbakken, 1985). Nesbakken et al. (2003) found that the rate of pathogenic *Yersinia* species detection in tonsils was six times that in faeces. Fredriksson-Ahomaa et al. (2007) and Gürtler et al. (2005) also reported a significantly higher frequency of pathogenic *Yersinia* isolation from tonsils than from faeces at slaughter. The reported prevalence of *Y. enterocolitica* carriage on pig tonsils, based

* Corresponding author. Tel.: +33 2 96 01 62 31; fax: +33 2 96 01 85 38.
E-mail address: martine.DENIS@anses.fr (M. Denis).

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on culture methods, generally exceeds 30% (e.g. 52% in Finland in 2007 (EFSA, 2007); 34% in Switzerland (Fredriksson-Ahomaa et al., 2007)).

The incidence of human cases of yersiniosis attributed to the consumption of pork has recently been estimated at 2.8 cases per 100,000 inhabitants per year in Europe (Fosse et al., 2008). This bacterium is thus the second most frequent contaminant of pig, lying after *Salmonella* (3.3) but ahead of *Campylobacter* (2.1).

In France, few data concerning the carriage of *Y. enterocolitica* in pig have been published. In the study by Magras et al. (2008), *Y. enterocolitica* was detected in 11% of pig tonsils, but the authors reported difficulties in the isolation of the bacterium by the ISO 10273-2003 method (ISO, 10273, 2003). This method is recommended for use with both food and pig tonsils (EFSA, 2007), but involves time-consuming enrichment steps followed by plating on selective media (de Boer, 1992).

Van Damme et al. (2010) recently showed that the use of a two-day incubation period at 25 °C, instead of five days for the PSB (peptone sorbitol bile) broth resulted in a significantly higher recovery rate (94.2%) of *Yersinia*. In addition, Weagant (2008) has developed a chromogenic medium for the specific detection of pathogenic *Y. enterocolitica*.

The aim of this study was to collect preliminary data concerning the presence of pathogenic *Y. enterocolitica* on the tonsils of slaughtered pigs in France. We also compared the performance of a simplified method for isolating pathogenic *Y. enterocolitica* from pig tonsils with that of the ISO 10273:2003 method.

2. Materials and methods

2.1. Tonsil swabs

From January to March 2009, we tested 45 batches of pigs (5 batches per week) for *Y. enterocolitica*. We sampled 20 pigs per batch. The head of the pig was removed from the carcass just before the chilling step and the tonsils were swabbed with sterile cotton wool. We collected 900 tonsil swabs in total. Sampling was carried out at a single slaughterhouse in Brittany, France.

2.2. Detection (Fig. 1)

Each swab was placed in a tube containing 10 ml of peptone sorbitol bile (PSB) broth (prepared in the laboratory, as described in the ISO 10273:2003 method). The tube was vortexed, and 1 ml of the suspension was removed and added to 9 ml irgasan–ticarcillin–potassium chlorate (ITC) broth (Bio-Rad, Marnes La Coquette, France).

The PSB enrichment broth was incubated for 48 h at 25 °C, and 10 μl was then streaked on cefsulodin–irgasan–novobiocin (CIN) agar plates (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK) and 10 μl of ITC enrichment broth was streaked on *Salmonella-Shigella*-sodium deoxycholate-calcium chloride (SSDC) agar plates (*Salmonella-Shigella* Agar base; Oxoid, Basingstoke, UK; sodium deoxycholate; Sigma-Aldrich, Saint-Louis, USA, calcium chloride; Merck, Darmstadt, Germany).

We decreased the incubation time in PSB broth to two days, as suggested by Van Damme et al. (2010). In addition to the ISO 10273:2003 method, we also tested a method in which 10 μl ITC enrichment broth was also streaked on CIN plates. All the plates were incubated at 30 °C for 24 h.

The 3 detection ways (PSB-CIN, ITC-SSDC, and ITC-CIN) are described in Fig. 1 and coded respectively to method 1, 2 and 4. The method 3 is the combination of method 1 and method 2 and corresponds to the two detection ways used in the ISO 10273:2003 method.

We checked for the presence of typical colonies on CIN and SSDC plates. As indicated in the ISO 10273:2003 method, *Y. enterocolitica* colonies on CIN agar are typically small and smooth, with a red centre and a translucent rim and, when examined with obliquely transmitted light, they are non-iridescent and finely granular. On SSDC agar,

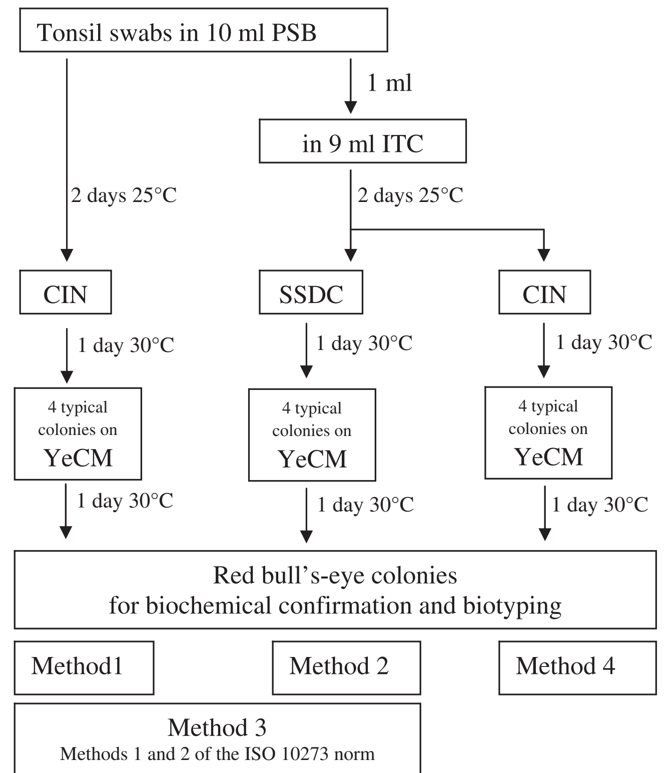

Fig. 1. Overview of the methods used to isolate pathogenic *Yersinia enterocolitica* from pig tonsil swabs in this study.

Y. enterocolitica colonies are typically small and grey, with an indistinct rim, and are non-iridescent and very finely granular when examined with obliquely transmitted light.

We then streaked a maximum of four characteristic colonies per plate (CIN and SSDC) on plates containing the *Y. enterocolitica* chromogenic medium (YeCM) (prepared in the laboratory as described by Weagant, 2008), for the presumptive selection of pathogenic *Y. enterocolitica* isolates (red "bull's-eye" colonies). Each isolate was subcultured on Plate Count Agar (PCA) plates (AES, Bruz, France) and incubated at 30 °C for 24 h for biochemical assays. Strains were stored in peptone glycerol broth, at −80 °C.

2.3. Biochemical confirmation and biotyping of the isolates

The biochemical assays used to confirm that the isolates were *Yersinia* were carried out as described in the ISO 10273:2003 method (degradation of glucose and no degradation of lactose on Kligler media (AES, Bruz, France), degradation of urea and absence of tryptophan deaminase). Colonies displaying typical reaction patterns were biotyped further, as described in the ISO 10273:2003 method, with the following tests: esculin hydrolysis, indole production, and fermentation of xylose and trehalose. Strains of biotype 1A (IP124), biotype 4/0:3 (IP134), biotype 3/0:5,27 (IP29228) and biotype 2/0:9 (IP383), purchased from the Pasteur Institute (Paris, France), were used as controls.

3. Results

We retained 440 typical colonies from CIN and SSDC plates (Table 1): 179 (40.7%) with the ISO 10273 method and 261 (59.3%) with the CIN after ITC method (method 4). For the two methods outlined in the ISO 10273 norm, we collected 71 typical colonies from CIN plates after enrichment in PSB (method 1) and 108 from SSDC plates after enrichment in ITC (method 2).

Table 1
Number of pathogenic *Y. enterocolitica* isolates, positive tonsil swabs and positive pig batches, by detection method. Method 3: combination of methods 1 and 2 of the ISO 10273 norm. All methods: combination of the ISO 10273 method and ITC-CIN method. Percentage of positive swabs and positive batches are indicated in bracket.

Method	ISO 10273-YeCM		ISO 10273 YeCM Method 3	ITC-CIN YeCM Method 4	All methods
	PSB-CIN Method 1	ITC-SSDC Method 2			
Number of typical colonies from CIN and/or SSDC plates	71	108	179	261	440
Number of pathogenic YE after YeCM and biochemical assays	52	104	156	224	380
Number of positive swabs	47 (5.2%)	39 (4.3%)	82 (9.1%)	126 (14.0%)	178 (19.8%)
Number of positive batches	19 (42.2%)	16 (35.5%)	27 (60.0%)	31 (68.9%)	36 (80.0%)

These typical colonies were streaked on YeCM plates for the selection of presumptive pathogenic *Y. enterocolitica* isolates (red bull's-eye colonies) and tested in biochemical assays to confirm that they belonged to the genus *Yersinia*. After growth on YeCM and confirmation that the colonies did indeed belong to the genus *Yersinia*, 86.4% of the isolates (380 on 440) were confirmed to be pathogenic *Y. enterocolitica*: 156 of the 179 (87.1%) isolates analysed by the ISO 10273 method and 224 of the 261 (85.8%) isolates plated on CIN after enrichment in ITC (method 4) (Table 1). For the two methods outlined in the ISO 10273 norm, 52 of the 71 isolates (73.2%) analysed by method 1 were confirmed to be pathogenic *Y. enterocolitica,* versus 104 of the 108 isolates (96.2%) for method 2.

The performance of the broths for the detection of pathogenic *Y. enterocolitica* from pig tonsils was evaluated by comparing the numbers of typical colonies obtained on CIN that were confirmed to be pathogenic *Y. enterocolitica* when grown on YeCM. ITC broth significantly outperformed PSB broth for the detection of pathogenic *Y. enterocolitica* (χ^2-test, $p<0.05$).

The performance of the agar plates for detecting pathogenic *Y. enterocolitica* from pig tonsils was evaluated by comparing the number of typical colonies obtained on CIN and on SSDC plates after enrichment in ITC broth; growth on YeCM confirmed that these colonies were pathogenic *Y. enterocolitica*. CIN agar plates significantly outperformed SSDC agar plates for the detection of pathogenic *Y. enterocolitica* (χ^2-test, $p<0.05$).

Only 13 of the 440 typical colonies retained from CIN and SSDC plates (5 on CIN after PSB enrichment, 3 on SSDC after ITC enrichment and 5 on CIN after ITC enrichment) had a characteristic appearance on YeCM (red bull's-eye) but were not confirmed to be *Yersinia* in biochemical assays. These isolates were identified the API20E panel (Biomérieux SA, Lyon, France) as *Rahnella aquatilis, Pantoea* spp., *Citrobacter freundii*, *Vibrio metschnikovii* and *Morganella morganii.* Finally, 97.0% of the isolates giving red bull's-eye colonies on YeCM were positively identified as pathogenic *Y. enterocolitica.*

In total, 178 of the 900 tonsil swabs (19.8%; $CI_{95\%}$ [16.5–23.1]) were positive in pathogenic *Y. enterocolitica.* A comparison of the different methods used showed that method 4 (ITC-CIN-YeCM) resulted in the recovery of a larger number of positive samples (126) than method 3 (ISO 10273-YeCM), for which 82 positive samples were obtained. The difference between these methods was statistically significant (χ^2-test, $p<0.05$). Thirty of the 178 samples tested positive with both methods.

For the two methods outlined in the ISO 10273 norm, more positive samples (47 in total) were recovered with method 1 (PSB-CIN-YeCM) than with method 2 (ITC-SSDC-YeCM), for which 39 positive samples were recovered. However, this difference between the methods was not statistically significant (χ^2-test, $p>0.05$).

We found that 36 of the 45 pig batches, (80%; $CI_{95\%}$ [65.4–94.6]) included at least one pig contaminated with pathogenic *Y. enterocolitica*: 27 (60%) detected by the ISO 10273-YeCM method and 31 (68.9%) detected by ITC-CIN-YeCM method (Table 1). Twenty-two of these 36 pig batches tested positive with both methods.

Analysis of the number of positive swabs per batch of pigs (Figs. 2 and 3) showed that the ITC-CIN-YeCM method led to the detection of a larger number of positive batches than the ISO 10273-YeCM method.

Regardless of the detection method used, the most prevalent biotype was biotype 4 (Table 2; 80.2% of all isolates), followed by biotype 3 (19.4% of all isolates). In total, 140 tonsil swabs were contaminated with

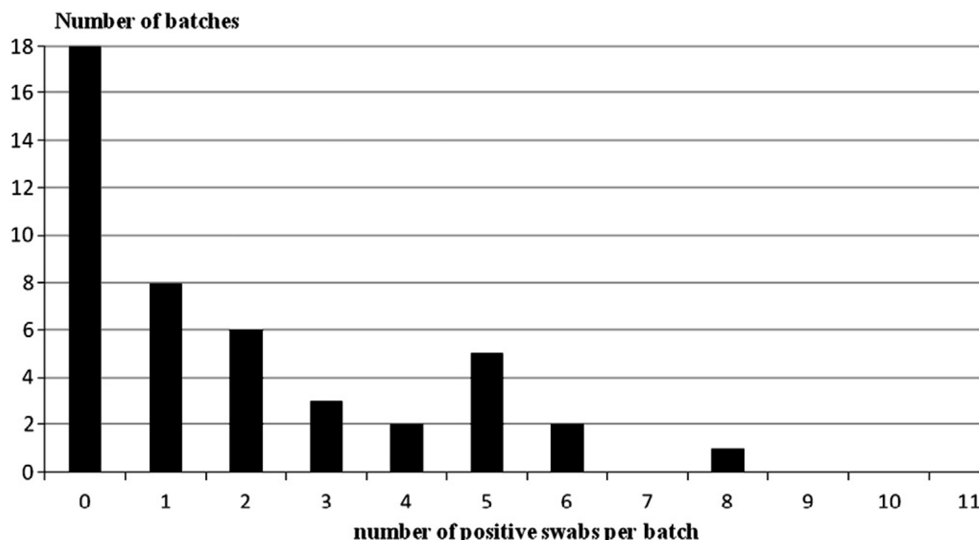

Fig. 2. Distribution of the number of positive swabs per batch of pigs, as determined with the ISO 10273-YeCM method.

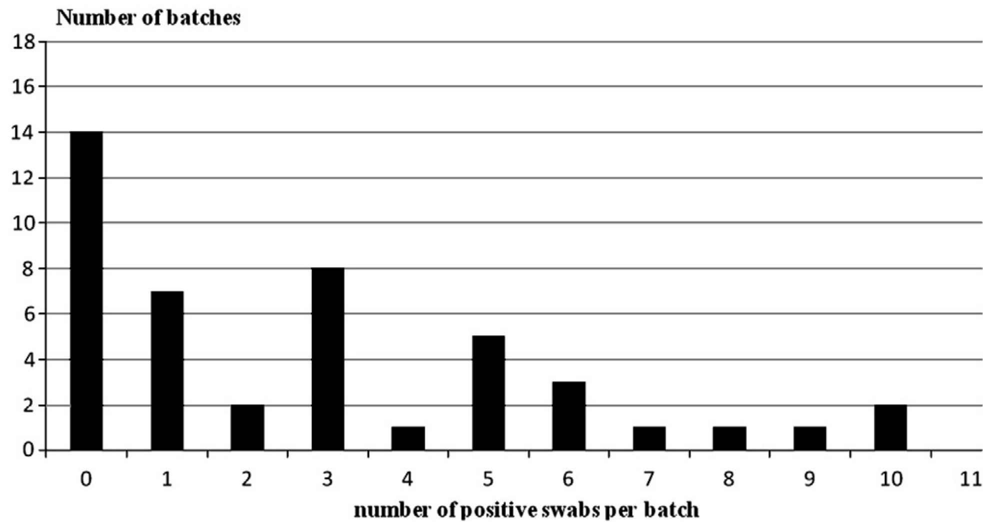

Fig. 3. Distribution of the number of positive swabs per batch of pigs, as determined with the ITC-CIN-YeCM method.

biotype 4, and 32 tonsil swabs were contaminated with biotype 3. Isolates of both these biotypes were detected together in six tonsil swabs.

4. Discussion

In this study, pathogenic *Y. enterocolitica* was isolated from 19.8% of the samples tested from a single slaughterhouse. These findings confirm that French pigs carry this pathogen on their tonsils at the time of slaughter. The reported incidence of pathogenic *Y. enterocolitica* on pig tonsils varies greatly between studies: 10% in Poland and the Netherlands (Kot et al., 2007; de Boer et al., 2008), 34% in Switzerland (Fredriksson-Ahomaa et al., 2007), 37.4% in Belgium (Van Damme et al., 2010), 52% in Finland (EFSA, 2007) and 89% in Estonia (Martínez et al., 2009). However, these percentages are not necessarily comparable, due to the different methods used for *Y. enterocolitica* detection in these studies. The low prevalence at this particular slaughterhouse may also reflect the slaughtering process, in which the pig heads are removed before the carcass is split. As the tonsils are kept intact within the head, this slaughtering technique helps prevent cross-contamination between carcasses, as demonstrated by Christensen and Lüthje (1994). Fredriksson-Ahomaa et al. (2000) showed that the slicing of the head resulted in the presence of *Y. enterocolitica* on the materials used for cutting. This facilitates the spread of the bacterium to subsequent tonsils, increasing the number of positive samples.

The percentage of positive samples detected in this study is similar to that reported in the study by Magras et al. (2008) for France, in which pathogenic *Y. enterocolitica* was detected in 11% of pig tonsils. The authors of this previous study used the ISO 10273-2003 method for pig tonsils, as recommended by EFSA (2007), but authors reported problems isolating the bacterium with this method, from such samples. This method is time-consuming, with long enrichment steps in PSB broth followed by plating on selective media. We therefore decided to compare a simplified method with the ISO 10273:2003 method for the isolation of pathogenic *Y. enterocolitica* from pig tonsils. Our method involved streaking the ITC broth onto a CIN agar plate, and then streaking typical colonies onto chromogenic medium, YeCM. This medium, developed by Weagant (2008), made it possible to identify pathogenic *Y. enterocolitica* as red bull's-eye-like colonies, whereas colonies of the non-pathogenic biotype 1A were blue-purple. Our method was carried out in parallel to the ISO method, and included a reduction of the duration of incubation in PSB broth to two days, as described by Van Damme et al. (2010), who reported a significantly higher rate of *Yersinia* recovery with this method than with the ISO method.

In our study, a method involving streaking from ITC broth onto a CIN agar plate, followed by the streaking of typical colonies onto the chromogenic medium, YeCM, recovered a larger number of positive samples than the modified ISO method: 14.0% of tonsils tested positive with the new method, versus only 9.1% with the modified ISO method. This was due to the combination of culture in ITC followed by streaking on CIN. We found that the ITC broth/CIN plate combination significantly outperformed the PSB broth/CIN plate combination for the detection of pathogenic *Y. enterocolitica*. With the addition of an additional step, involving streaking on YeCM, we found that the percentage of non-pathogenic *Y. enterocolitica* isolated on CIN after enrichment in PSB broth (26.8%) was higher than that isolated on CIN after enrichment in ITC broth (14.2%). This result is consistent with the findings of Wauters et al. (1988), indicating that enrichment in PSB broth gave better results for non-pathogenic strains, whereas enrichment in ITC broth gave better results for pathogenic strains.

We used biochemical tests to confirm the identification of pathogenic *Y. enterocolitica* on YeCM plates, to ensure that this new medium was selective. Only 2.9% of the typical colonies collected from

Table 2
Number of isolates and swabs for each biotype of *Y. enterocolitica*, by method.

Biotype	Method 1		Method 2		Method 3 (1 and 2)		Method 4		All methods	
	Number of isolates	Number of swabs	Number of isolates	Number of swabs	Number of isolates	Number of swabs	Number of isolates	Number of swabs	Number of isolates	Number of swabs
4	42	38	80	27	122	62	184	98	306	140
3	10	9	24	6	34	14	40	25	74	32
3 and 4	-	0	-	6	-	6	-	3	-	6
Total	52	47	104	39	156	82	224	126	380	178

CIN and SSDC had a typical phenotype on YeCM (red bulls-eye) but not confirmed to be *Yersinia* in biochemical tests. Thus, this chromogenic medium is highly selective for pathogenic *Y. enterocolitica.* The use of this medium alone for the detection of pathogenic isolates would be less time-consuming, because it would overcome the need to carry out additional biochemical tests for confirmation. Moreover, as growth on YeCM facilitates the discarding of non-pathogenic isolates, this method also decreases the number of biotyping tests to be carried out.

In our study, the most prevalent biotype was biotype 4 (80.2% of all isolates), followed by biotype 3. Bioserotype 4/0:3 is generally the predominant bioserotype in pig production systems (Skjerve et al., 1998; Fredriksson-Ahomaa et al., 2000; Bonardi et al., 2003; Gürtler et al., 2005; Kechagia et al., 2007; Laukkanen et al., 2009; Van Damme et al., 2010), but a recent study identified bioserotypes 2/0:5 and 2/0:9 as the most prevalent on English pig farms (Martínez et al., 2010). The distribution of biotypes observed in our investigation resembles that for human yersiniosis in France: 78.8% of the human isolates were of biotype 4, and the next most frequent biotypes were biotypes 2 and 3 (21.2% of the isolates; Carniel et al., 2007). Further studies are required to assess the potential role of pigs as a reservoir of *Y. enterocolitica* strains pathogenic to humans in France.

No biotype 2 strains were detected in this study, probably because ITC broth and CIN plates both favour the growth of biotype 4 (De Zutter et al., 1994; Schiemann, 1982). In any case, this biotype does not seem to be particularly frequent in European pigs (Verhaegen et al., 1998; Bonardi et al., 2003; Korte et al., 2004; Fredriksson-Ahomaa et al., 2007). The cases of biotype 2 infections in humans in France may have a source other than pork, such as the consumption of beef, for example (Gourdon et al., 1999).

In conclusion, we have described a simplified method that efficiently detects pathogenic *Y. enterocolitica* in pig tonsils. This method is less time-consuming than the ISO 10273 method, and, with the use of YeCM, decreases the need for biochemical tests for confirmation and biotyping. Other alternative methods using PCR (Lambertz et al., 2008; Fredriksson-Ahomaa et al., 2009) for detecting *Y. enterocolitica* from food or tonsil have been published . In the study of Fredriksson-Ahomaa et al., 2009, prevalence of enteropathogenic *Y. enterocolitica* in individual pigs was significantly lower by culturing (9%) compared to PCR (35%). The authors explained the results by the low sensitivity of culture method compared to PCR. However, this difference could be explained by the capacity of the PCR method to detect also death cells. Moreover, while PCR can be useful to quickly detect suspected positive samples, only culture method enable to recover isolates. Realizing streaking on CIN after enrichment in ITC only for positive PCR samples could be a good compromise for future standard method. We are actually working in this aim.

These preliminary data indicate that pathogenic *Y. yenterocolitica* may be carried on the tonsils of close to 20% of pigs in France. However, it is difficult to make sweeping statements concerning the situation nationwide, because we studied only one slaughterhouse, over a period of only three months. We have now initiated a larger study, in which more than 3000 tonsil swabs will be collected from 16 French slaughterhouses over the course of one year, to investigate the carriage of this bacterium in more detail.

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