Genetic characterization of Yersinia enterocolitica collected from tonsils of slaughtered pigs

Denis, M.
Fondrevez, M., Labbé, A., Houdayer, C.*

Anses, Unité Hygiène et Qualité des Produits Avicoles et Porcins, BP 53, 22440 Ploufragan, France.

e-mail : marc.FONDREVEZ@anses.fr fax : 0(33)296018538

Abstract

From January to March 2009, detection of pathogenic Yersinia enterocolitica was done from 900 tonsils swabs collected from 45 pig batches in one slaughterhouse. 316 Y. enterocolitica isolates were collected and confirmed as pathogenic biotype by biochemical tests. For this study, these strains were genetically characterized on the basis of their virulence genes and their PFGE profiles.

Real Time PCR was used to evaluate the presence of genes ail, myfA, and ystA on the genome and the gene yadA on the pYV plasmid. PFGE analysis using XbaI enzyme was also realised.

Most of the isolates belonged to biotype 4 (85.1 % of the isolates) and 14.9% of the isolates were from biotype 3. All the isolates carried the 3 chromosomal genes, ail, myfA and ystA genes, except one for which ystA was not detected. Among these strains, 278 isolates carried the pYV plasmid (88.0%); 233 from biotype 4 and 45 from biotype 3. All the 31 positive pig batches in pathogenic Y.enterocolitica have at least one isolate which exhibits the virulence plasmid.

The PFGE revealed 5 Xba1 genetic profiles coded G1 to G5. Forty-nine % of the isolates highlighted the same major PFGE patterns, pattern G4; followed by pattern G5 (16.7% of the isolates), pattern G2 (14.8% of the isolates), pattern G1 (12.3%) and pattern G3 (7.0%). Among the patterns, patterns G2 and G3 were only associated to biotype 4 while patterns G1, G4 and G5 were found for the biotype 4 and 3. On the 31 positive pig batches, one to three different PFGE patterns could be found in one batch, with several possible combinations.

The study on this collection of isolates showed that all the pathogenic isolates have virulence genes. This indicates that identification of pathogenic biotype can be realised by detection of the ail and myfA genes through PCR method instead of biochemical tests. PFGE analysis using XbaI enzyme showed that Y. enterocolitica is very clonal with a major PFGE patterns but this method do not allowed the differentiation of biotype 4 from biotype 3.

Introduction

In 2009, yersiniosis was, for the sixth consecutive year, the third most frequently reported human zoonosis in the Europe, with a total of 7,595 confirmed cases (EFSA, 2011). The species is divided into six biotypes. Y enterocolitica can be classified into biotype 1A, generally regarded as nonpathogenic, and the pathogenic biotypes 1B, 2, 3, 4, 5 (Wauters et al, 1987). In France and most other countries worldwide, biotype 4 is the most prevalent biotype isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (Savin and Carniel, 2008). Pigs are considered the principal reservoir for the types of Y. enterocolitica pathogenic to humans. Pigs do not develop clinical signs, but they do carry Y. enterocolitica in their oral cavity, on tongues and tonsils, and in lymph nodes, and excrete this bacterium in their feces (Thibodeau et al., 1999).

Identification of biotype lies on panel of biochemical tests as described in the ISO 10273-2003 method allowing differentiation of pathogenic biotypes from the non-pathogenic biotype. Moreover, strains of biotypes 1B and 2 - 5 possess chromosomal virulence genes: the ail gene product is a small outer membrane protein, which promotes bacterial adhesion to, and invasion of, cultured epithelial cells; ystA, which is responsible for the production of thermostable enterotoxin, which facilitates the invasion of the Y. enterocolitica into tissues by damaging the intestinal epithelium; and the myfA gene, which encodes the major subunit of antigen Myf. This fibrillar structure has been found to promote the colonization of the intestine (Revell and Miller 2001; Tennant et al., 2003). Virulent Yersinia strains carry an approximately 70 kb plasmid termed pYV. The outer membrane protein YadA (Yersinia adhesin A) encoded by genes on the pYV plasmid has been found to play multiple functions in pathogenesis. YadA protects yersiniae against the antibacterial activity of complement and defensin (Roggenkamp et al. 2003) and mediates specific binding of Y. enterocolitica to collagen, laminin and cellular fibronectin (Heesemann et al., 2006).

The aim of this study was the confirmation of the pathogenicity of Y. enterocolitica strains isolated from pigs by using Real-Time PCR assays for the detection of plasmid- and chromosome-borne virulence genes. Moreover, the analysis of the occurrence of virulence genes in different biotypes and PFGE Xba1-patterns was conducted.

Material and Methods

Origin of the strains

A total of 316 isolates of pathogenic Y. enterocolitica were collected from tonsils swabs of 31 pig batches (on the 45 studied) in one slaughterhouse between January and March 2009 (Fondrevez et al., 2010). Biotyping was realised by biochemical tests 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.

DNA extraction and Real-Time PCR for detection of virulence genes

Real Time PCR was used to evaluate the presence of virulence genes ail, myfA, and ystA carried by the genome and the gene yadA carried by the pYV plasmid.

Strains were sub-cultured on PCA at 30°C for 24h. DNA was extracted from some colonies with QIAamp DNA mini kit (Qiagen, USA) following the manufacturer's instructions. The 4 PCRs were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California), in a final volume of 25µl with the Sybr® Green JumpstartTM Taq ReadyMix TM (Sigma-Aldrich, Saint Louis, Missouri).) at 1X. The genes ail, myfA, yadA (Kot et al, 2010) and ystA (Ibrahim et al, 1997) were detected with specific primers as indicated in table 2. The final concentration of primers in the PCR reaction was 0.3 µM for ail and myfA, 0.2µM for yadA and ystA. The amplification conditions for each gene are detailed in table 1.

Table 1: Primer sequences and PCR conditions for detection of virulence genes by Real-Time PCR

Genes	Primer sequence	First step	Cycle of amplification	Melt Curve	Size in bp	Expected Tm
ail	F - TGG TTA TGC GCA AAG CCA TGT R - TGG AAG TGG GTT GAA TTG CA	94°C 2 min	35 cycles of Denaturation 91°C 30 sec Annealing 57°C 30 sec Extension 72°C 60 sec	55°C -> 89°C, increment of 0.5°C for 5 sec	356	82.5°C
myfA	F - CAG ATA CAC CTG CCT TCC ATC T R - CTC GAC ATA TTC CTC AAC ACG C	94°C 2 min	35 cycles of Denaturation 94°C 60 sec Annealing 58°C 90 sec Extension 72°C 90 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	272	84.5°C
yadA	F - TAA GAT CAG TGT CTC TGC GGC A R - TAG TTA TTT GCG ATC CCT AGC AC	94°C 2 min	35 cycles of Denaturation 94°C 60sec Annealing 58°C 60sec Extension 72°C 60sec	55°C -> 95°C, increment of 0.5°C for 5 sec	747	86°C
ystA	F - AAT GCT GTC TTC ATT TGG AGC R - ATC CCA ATC ACT ACT GAC TTC	94°C 2 min	35 cycles of Denaturation 92°C 10sec Annealing 55°C 25 sec Elongation 72°C 20 sec	54°C -> 90°C, increment of 0.5°C for 5 sec	145	83.5°C

RFLP/PFGE for genotyping

Strains were sub-cultured on PCA at 30°C for 24h. Bacterial suspension in TN were adjusted to an optical density (600 nm) of 1.5 and mixed with 1% agarose for the plug preparation. Plugs were incubated for 24h at 50°C in lysis solution (Na2EDTA 0,5M, pH9, N-lauryl-Sarcosyl 1% (p/v), protéinase K 1 mg/ml). A total of six washes (0.01 M Tris-EDTA buffer, pH 8.0) were used to remove excess reagents and DNA was then digested with 40U of XbaI 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.

Gel images were captured on a gel doc 1000 imaging system (Bio-Rad, Hercules, California) and analysed with BioNumerics software (Applied Math, Sint-Martens-Latem, Belgium).

Results

Most of the isolates belonged to biotype 4 (85.1 % of the isolates) and 14.9% of the isolates were from biotype 3 (table 2).

Table 2: distribution of the isolates according their biotype, virulence genes and PFGE patterns

		PFGE patterns					
Biotype	Presence of virulence gene	G1	G2	G3	G4	G5	total
Biotype 4	ail+, myfA+, ystA+, yadA+	7	45	21	122	38	233
	ail+, myfA+, ystA+, yadA-	5	2	1	14	13	35
	ail+, myfA+, ystA-, yadA-	0	0	0	1	0	1
Biotype 3	ail+, myfA+, ystA+, yadA+	27	0	0	18	0	45
	ail+, myfA+, ystA+, yadA-	0	0	0	0	2	2
	Total	39	47	22	155	53	316

All the isolates carried the 3 chromosomal genes, ail, myfA and ystA genes, except one for which ystA was not detected. Among these strains, 278 isolates carried the pYV plasmid (88.0%); 233 from biotype 4 and 45 from biotype 3. Only one biotype 4 isolate didn't carry the ystA gene but has the ail and myfA genes. All the 31 positive pig batches in pathogenic Y. enterocolitica have at least one isolate carrying the virulence plasmid. In 12 batches among the 31, isolates without plasmide were detected.

The PFGE revealed 5 Xba1 PFGE patterns coded G1 to G5 (table 2). Forty-nine % of the isolates highlighted the same major PFGE patterns, pattern G4; followed by pattern G5 (16.7% of the isolates), pattern G2 (14.8% of the isolates), pattern G1 (12.3%) and pattern G3 (7.0%). Among the patterns, patterns G2 and G3 were only associated to biotype 4 while patterns G1, G4 and G5 were found for the biotype 4 and 3.

On the 31 positive pig batches, 11 batches have isolates which belong to one PFGE pattern and 20 batches present a combination of two to three different patterns. Several combinations were found between the five patterns. Patterns 1 and 3 were never found alone in a batch, they were always associated with another pattern.

Discussion

Genotype ail+ myfA+ystA+yadA was predominant in our strain collection whatever the biotype. This is consistent with the result of Kot et al., (2010) for which all the biotype 4;0:3 had all these virulence genes. Results confirming the presence of the ail and ystA genes in biotype 1B.,2, 3 and 4 were also collected by Thoerner et al. (2003). In our study, 12% of the isolates did not carry the pYV plasmid. It is known that strains subjected to long-term storage and repeated passaging at 37°C lost their virulence plasmid, but in our survey the DNA of isolates was extracted shortly after their isolation, and no step was made at 37°C after the swabbing. So it can be assumed that strains were without pYV plasmid on our pig tonsils. Therefore, the assessment of the indicators of Yersinia enterocolitica virulence need not to be restricted to the detection of plasmid-localized genes of virulence, but requires at least one chromosomal gene of virulence be present so that errors are avoided in the estimation of strain virulence.

All our isolates could be biotyped by biochemical tests and detection of the virulence genes confirmed their pathogenicity. However, authors (Kote et al., 2010) indicated that some strains are not typeable according to the scheme of Wauters et al., (1987). Consequently, detection of the virulence genes can be useful to separate pathogenic strains from non-pathogenic strains as it is known that the ystB gene is in nearly all the pig-derived strains of biotype 1A (Platt-Samoraj et al., 2006) and that the ail gene (Wannet et al., 2001; Kote et al ., 2010) and the myfA gene (Kote et al., 2010) were in nearly all the pig- and human-derived strains of pathogenic biotype.

The virulence profile obtained for the biotypes and serotypes of Y.enterocolitice, based upon the selected genes of virulence, can be applied as distinguishing markers and indicators of the potential virulencq of Y.enterocoliticq strains, excluding bio-serotyping tests.

Conclusion

The study on this collection of isolates showed that all the pathogenic isolates have virulence genes. This indicates that identification of pathogenic biotype can be realised by detection of the ail and myfA genes through PCR method instead of biochemical tests. PFGE analysis using XbaI enzyme showed that Y. enterocolitica is very clonal with a major PFGE patterns but this method do not allowed the differentiation of biotype 4 from biotype 3.

Acknowledgements

This work was supported by a PhD grant from Anses and Brittany ARED and, an AAP-CBB financial support from the Regional Council of Brittany.

References

1. EFSA, 2011, The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009, EFSA Journal 2011, 9(3) : 2090 (378 pages)
2. Fondrevez M., Labbe A., Houard E., Fravalo P., Madec F., Denis M., 2010, A simplified method for detecting pathogenic Yersinia enterocolitica in slaughtered pig tonsils. J Microbiol Methods, 83(2) 244-249
3. Heesemann J., Sing A., Trulzsch K., 2006, Yersinia's stratagem: targeting innate and adaptive immune defense, Curr Opin Microbiol 9: 55-61
4. Ibrahim A., Liesack W., Griffiths M., Robins-Browne R.M., 1997, Development of a highly specific assay for rapid identification of pathogenic strains of Yersinia enterocolitica based on pcr amplification of the yersinia heat-stable enterotoxin gene (yst). J Clin Microbiol 35: 1636-1638.
5. Kot B., Piechota M., Jakubszak A., 2010, Analysis of occurrence of virulence genes among Yersinia enterocolitica isolates belonging to different biotypes and serotypes, Pol J Vet Sci, 13(1): 13-19
6. Platt-Samoraj A, Ugorski M, Szweda W, Szczerba-Turek A, Wojciech K, Procajło Z (2006) Analysis of the presence of ail,ysta andystb genes in Yersinia enterocolitica strains isolated from aborting sows and aborted fetuses. J Vet Med B 53:34i-346.
7. Revell P.A., Miller V.L., 2001, Yersinia virulence: more than a plasmid, Fems Microbiol Lett 205: 159-164.
8. Roggenkamp A., Ackermann N., Jacobi C.A., Truelzsch K., Hoffmann H., Heesemann J., 2003, Molecular analysis of transport and oligomerization of the Yersinia enterocolitica adhesin yada, J Bacteriol, 185 3735-3744.
9. Savin C., Carniel E., 2008, Les diarrhées d'origine bactérienne : le cas de Yersinia enterocolitica, revue francophone des laboratoires - mars 2008 - n°400 49-58revue francophone.
10. Tennant SM, Grant TH, Robins-Browne R.M., 2003, Pathogenicity of yersinia enterocolitica biotype 1a. Fems Immunol Med Microbiol 38l. i27-137.
11. Thibodeau V, Frost E.H., Chenier S., Quessy S., 1999, Presence of yersinia enterocolitica in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter, Can J Vet Res 1999; 63: 96-100
12. Thoerner P, Bin Kingombe CI, Bógli-Stuber K, Bissig-Choisat B, Wassenaar TM, Frey J, Jemmi T (2003) PCR detection of virulence genes in yersinia enterocolitica and, yersinia pseudotuberculosis and investigation of viru]ence gene distribution. Appl Environ Microbiol 69: 1810-1816.
13. Wannet WJ, Reessink M, Brunings LIA, Maas HM (2001) Detection of pathogenic Yersinia enterocolitica by a rapid and sensitive duplex pcr assay. J Clin Microbiol.39: 4483-4486.
14. Wauters G., Kandolo K., Janssens M., 1987. Revised biogrouping scheme of Yersinia enterocolitica. Contrib. Microbiol. Immunol. 9:14-21.