Campylobacter in organic and conventional pig production in France: antibiotic resistance, genetic diversity and virulence.

Denis, M.1,2*, Chidaine, B.1,2, Rose, V.1,2, Bourgouin, K.1,2, Cutimbo, M.1,2, Kerouanton, A.1,2

Abstract

Campylobacter coli strains from 56 organic and 58 conventional pig colon contents were characterized to evaluate impact of these two productions on antibiotic resistance, genetic diversity and virulence of these strains. 76.8% of organic pigs and 74.0% of conventional pigs were positive in Campylobacter. A total of 262 strains were tested for their resistance to 7 antibiotics. Significant differences were observed for 4 antibiotics (tetracycline, erythromycin, nalidixic acid, ciprofloxacin) between the two productions with higher resistance for conventional pig Campylobacter. Multiresistance was more frequently observed for conventional pig strains (54.8%) than for organic pig strains (26.8%). Strains were typed by PFGE (262 strains) and MLST (120 strains). Genetic diversity was very high for both productions with both typing methods. Strains were distributed in 60 PFGE genotypes and in 51 Sequence Types. Ten PFGE clusters (34% of the strains) and nine ST (41.6% of the strains) were common between the two productions. Presence of 9 virulence genes was checked (120 strains) by PCR. All the strains carried the cuE, iam, ciaB and flaA genes and more than 95% of the strains carried the cadF and cdtABC genes. The virb11 gene on plasmid was detected only for 13 organic pig strains. Capacity of adhesion and invasion of 61 strains were tested on Caco-2 cells. No link between virulence profile and strain origin was observed. However strains with the virb11 gene had higher invasive capacity. In conclusion, no impact of the type of production was observed on the genetic diversity and virulence of Campylobacter strains. The lower level of antibiotic resistance and multiresistance of C. coli strains for organic pigs may be related to the restricted use of antibiotics in this production and / or colonization of organic pigs with susceptible environmental strains.

Introduction

Most studies interested in organic animal production focuses on antibiotic resistance of bacteria. Indeed, antibiotic resistance is considered one of the major global threats for the future and the use of antimicrobials in animal production is contributing to the growing problem with resistant bacteria. Within organic animal production there is generally a more restricted usage of antimicrobials, which likely influences the level of antimicrobial resistance in these herds (Young et al., 2009). Moreover, animals from organic production have access to an open area leading opportunity to encounter wild type Campylobacter strains. Such situations, restricted usage of antimicrobials and access to an open area, offer the possibility that organic pigs are colonized by another population of Campylobacter that of conventional pigs. These campylobacters can come from wild animals or pets (Mohan, 2015). It was thus interesting to characterize more precisely these Campylobacter strains to see if the management of the pig herds, organic / conventional, could influence the type of strains found in pigs. In addition to antibiotic resistance; we looked at the genetic diversity of the strains and their virulence.

Material and Methods

Sampling and Campylobacter collection

Colon contents of 56 organic and 58 conventional pigs were sampled from April to October 2012 at slaughterhouse. They were streaked on Karmali plate. After 48h at 37°C in a microaerobic atmosphere, typical colonies were sub-cultured on blood agar plates for 24h at 37°C for phenotypic and genotypic characterization as described after. A total of 262 strains were kept; 138 from organic pigs and 124 for conventional pigs. All the Campylobacter were Campylobacter coli.
The strains (262) were genotyped by PFGE. DNA preparation, Kpn1 restriction endonuclease digestion and PFGE were carried out as described by the Campynet protocol. Electrophoretic patterns were compared using BioNumerics® and strains were clustered by the unweighted pair-group method using the arithmetic mean (UPGMA) (Struelens et al., 1996). The Simpson’s index was calculated (Hunter, 1990) to assess the genetic diversity of the Campylobacter populations.

**Multi-Locus Sequence Typing (MLST)**

Strains (58 from conventional pigs and 62 from organic pigs) were genotyped by MLST as described by Miller et al., [2005]. Amplification and sequencing of 7 housekeeping genes: aspA (aspartase A), glnA (glutamine synthetase), gltA (citrate synthase), gvaA (serine hydroxymethyltransferase), pgm (phosphoglucomutase), tkt (transketolase) and uncA (ATP synthase) were achieved. Each sequence was submitted to the MLST website (http://pubmlst.org/) in order to have the allele number of the 7 genes for each strain. A sequence type (ST) and clonal complex (CC) were then determined according to the allelic profile.

**Antimicrobial susceptibility testing**

Minimal inhibitory concentrations of antimicrobials were determined for all strains using the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) document M31-A3 with Sensititre® plates (Biocentric, Bandol, France) and were interpreted according to the cut-off values recommended by the EU Reference Laboratory for Antimicrobial Resistance. The antimicrobials tested included gentamicin (GEN), streptomycin (STR), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), erythromycin (ERY) and chloramphenicol (CHL). All cut-off values used in the interpretation of the Campylobacter MIC results have been developed by EUCAST (www.eucast.org).

**Virulence**

Presence of 9 virulence genes was checked (120 strains) by Real Time PCR SYBR® Green developed for *Campylobacter* populations.

Antigenic acquisition of iron (a) in the chromosome and one on the plasmid (virB11), the acquisition of iron (ceuE) and in the production of toxine CDT (Cytotoxlethal Distenting Toxin) (cdtA, cdtB, cdtC).

Capacity of adhesion and invasion of the strains (61 strains) was tested in vitro assays on Caco-2 human intestinal epithelial cells as described by Guyard-Nicodeme et al., [2013]. The capacity of adhesion and invasion were expressed as percentage of bacteria which adhere to- and invade in- Caco-2 cells from a starting inoculum of 10^7 UFC Campylobacter.

**Results**

**Genetic diversity of the isolates**

Typing by PFGE (262 strains) and MLST (120 strains) allowed to see that genetic diversity was very high for both productions with both typing methods. The index of diversity was slightly higher for organic strains (0.96) but not significantly different from those of conventional strains (0.93). Isolates were distributed in 60 PFGE genotypes and in 51 Sequence Types. 91% of the strains were from Clonal Complex ST-828. Ten PFGE genotypes (34% of the strains) and nine ST (41.6% of the strains) were common between the two productions with a dominant ST854 (18.3% of the strains).

**Antimicrobial susceptibility.**

Only 10 strains (3.8%) among the 262 strains were pansusceptible to the five family of antimicrobials tested for both productions with both typing methods. The index of diversity was slightly higher for organic strains (0.96) but not significantly different from those of conventional strains (0.93). Isolates were distributed in 60 PFGE genotypes and in 51 Sequence Types. 91% of the strains were from Clonal Complex ST-828. Ten PFGE genotypes (34% of the strains) and nine ST (41.6% of the strains) were common between the two productions with a dominant ST854 (18.3% of the strains).

The most frequent resistance profile was resistance to tetracycline with streptomycin; 33.3% of the organic pig strains from and 24.2% of conventional pig strains. Multiresistance (resistance to 3 or more antimicrobial families) were more frequently observed for strains from conventional pigs (54.8% of the isolates) than for strains from organic pigs (26.8% of the strains) (χ^2, p<0.01)

**Virulence**

All the 120 strains tested carried the *ceuE, iam, ciaB* and *flaA* genes and more than 95% of the strains carried the *cadF* and *cadABC* genes. The *virB11* gene on plasmid was detected in 13 strains from organic pigs. There was no significant difference between organic and conventional strains (61 isolates) for adhesion (χ^2, p=0.523) and for invasion (χ^2, p=0.590) on Caco-2 cells But strains with the *virB11* gene have significantly a higher invasive capacity (χ^2, p = 0.040) (Figure 2 A and B).

**Discussion**

The greatest genetic diversity in organic pigs may be related to the access of pigs to an open area leading the opportunities to encounter Campylobacter from wildlife. Wild animals can excrete Campylobacter and birds play an important role in terms of prevalence and transmission of this bacterium (Greig et al., 2015). Common genotypes between organic and conventional could indicate that these genotypes are adapted to pigs; the other
Pulsed-field gel electrophoresis (PFGE)

The strains (262) were genotyped by PFGE. DNA preparation, Kpn1 restriction endonuclease digestion and PFGE were carried out as described by the Campynet protocol. Electrophoretic patterns were compared using BioNumerics® and strains were clustered by the unweighted pair-group method using the arithmetic mean (UPGMA) (Struelens et al., 1996). The Simpson’s index was calculated (Hunter, 1990) to assess the genetic diversity of the Campylobacter populations.

Multi-Locus Sequence Typing (MLST)

Strains (58 from conventional pigs and 62 from organic pigs) were genotyped by MLST as described by Miller et al., (2005). Amplification and sequencing of 7 housekeeping genes: aspA (aspartase A), glnA (glutamine synthetase), g3P (citrate synthase), gylA (serine hydroxymethyltransferase), pgm (phosphoglucosumase), tkt (transketolase) and uncA (ATP synthase) were achieved. Each sequence was submitted to the MLST website (http://pubmlst.org/) in order to have the allele number of the 7 genes for each strain. A sequence type (ST) and clonal complex (CC) were then determined according to the allelic profile.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations of antimicrobials were determined for all strains using the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) document M31-A3 with Sensititre® plates (Biocentric, Bandol, France) and were interpreted according to the cut-off values recommended by the EU Reference Laboratory for Antimicrobial Resistance. The antimicrobials tested included gentamicin (GEN), streptomycin (STR), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), erythromycin (ERY) and chloramphenicol (CHL). All cut-off values used in the interpretation of the Campylobacter MIC results have been developed by EUCAST (www.eucast.org).

Virulence

Presence of 9 virulence genes was checked (120 strains) by Real Time PCR SYBR® Green developed for this study using published primers or primers designed for this study. Heights of the genes were localized on the chromosome and one on the plasmid (virB11). The genes are involved in adhesion/invasion of epithelial cell (fpaA, ciaB, cadF, iam, virB11), in the acquisition of iron (ceuE) and in the production of toxin CDT (Cytolethal Distending Toxin) (cdtA, cdtB, cdtC).

Capacity of adhesion and invasion of the strains (61 strains) was tested in vitro assays on Caco-2 human intestinal epithelial cells as described by Guyard-Nicodème et al., (2013). The capacity of adhesion and invasion were expressed as percentage of bacteria which adhere to- and invade in- Caco-2 cells from a starting inoculum of $10^5$ UFC Campylobacter.

Results

Genetic diversity of the isolates

Typing by PFGE (262 strains) and MLST (120 strains) allowed to see that genetic diversity was very high for both productions with both typing methods. The index of diversity was slightly higher for organic strains (0.96) but not significantly different from those of conventional strains (0.93). Isolates were distributed in 60 PFGE genotypes and in 51 Sequence Types. 91% of the strains were from Clonal Complex ST-828. Ten PFGE genotypes (34% of the strains) and nine ST (41.6% of the strains) were common between the two productions with a dominant ST854 (18.3% of the strains).

Antimicrobial susceptibility

Only 10 strains (3.8%) among the 262 strains were pansusceptible to the five family of antimicrobials tested (8 from organic pig and 2 from conventional pigs). None strain was resistant to CHL and only one strain from conventional pig was resistant to GEN. The percentage of strains resistant to STR was high and similar in the two productions; 75.4% of the strains for organic pigs and 73.4% for conventional pigs had this resistance (Figure 1). We observed significant difference for resistance to NAL, CIP, ERY and TET ($\chi^2$, p<0.05) between the two productions. For these four antibiotics, resistance was higher for Campylobacter isolated from conventional pigs.

Discussion

The greatest genetic diversity in organic pigs may be related to the access of pigs to an open area leading the opportunities to encounter Campylobacter from wildlife. Wild animals can excrete Campylobacter and birds play an important role in terms of prevalence and transmission of this bacterium (Greig et al., 2015). Common genotypes between organic and conventional could indicate that these genotypes are adapted to pigs; the other
The prevalence of virulence genes was already described in other works on C. coli (Wieczorek & Osek, 2013), although clast gene was found in only 20% of the C. coli strains of Ack et al., (2013). Only 7% of our strains have the virB11 gene as described before (Ack et al., 2013, Wieczorek & Osek, 2013). This gene is involved in the invasion (Bacon et al. 2002) and we observed that our strains with this gene had a greater invasive capacity than other.

**Conclusion**

No impact of the type of production was observed on the genetic diversity and virulence of our Campylobacter strains. The lower level of antibiotic resistance was observed in conventional strains for organic pigs. One of the reasons may be related to the restricted use of antibiotics in this production and / or colonization of organic pigs with susceptible environmental strains. However, although significantly different or not between the two productions, the percentage of strains with resistance in organic pigs remain in some cases relatively high. These pigs are therefore able to bring in some strains. However, although significantly different or not between the two productions, the percentage of strains with resistance in organic pigs remain in some cases relatively high. These pigs are therefore able to bring in some strains.

**Acknowledgements**

This work was funded by a European (Eranet Core Organic II) and French (FranceAgrimer) fundings.

**References**

• Guyard-Nicodeme et al. (2013) Characterization of Campylobacter spp. transferred from naturally contaminated chicken legs to cooked chicken slices via a cutting board. JFM, 164:7-14.

**Results**

Mean residual detection after one year survey was 10% (240/2,496 samples analyzed). The prevalence of L. mono over time revealed that, whatever the season considered, the residual contamination detection