

### Direct detection of *Campylobacter* from feces of organic and conventional pigs highlighted the presence of *Campylobacter lanienae*.

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#### Abstract

In the frame of the CORE Organic II funded European project SafeOrganic, fecal samples from 31 organic pig herds and 31 conventional pig herds were sampled in a slaughterhouse. Samples were highly positive in *Campylobacter coli* but also in another *Campylobacter* species not described at that time in France.

Identification by MALDI-TOF mass spectrometry and PCR 16S allowed us to confirm that 85 isolates were *C. Lanienae*; 56 from conventional pigs and 29 from organic pigs. Individual occurrence in *Campylobacter* spp. was thus re-estimated to 91.1 % (51/56) and 96.3 % (52/54) for conventional and organic pigs, respectively. A total of 55 isolates of *C. Lanienae* were studied for their resistance to 7 antibiotics. Only one was pansusceptible. Natural resistance to Nalidixic acid was confirmed. Resistance to Tetracycline was significantly different ( $p < 0,001$ ) between the two productions: 88 % of isolates from conventional pigs were resistant against 14% of isolates from organic pigs. Moreover, isolates from conventional pigs were mostly multiresistant (73%) whereas only 5% of strains isolated in organic pigs were multiresistant. The *C. lanienae* isolates were typed by PFGE using *KpnI* and *SmaI* enzymes. The genetic diversity was very high, whatever the enzyme used. No link between PFGE profile and isolate origin or antibiotic resistance pattern was evidenced. This study allowed us to demonstrate for the first time in France that pigs, known to be a reservoir for *C. coli* may also carry in their feces a species rarely highlighted: *C. lanienae*. The species was present in fecal samples from conventional and organic pigs. The lower level of antibiotic resistance and multiresistance of *C. Lanienae* strains for organic pigs may be related to the restricted use of antibiotics in this production.

#### Introduction

*Campylobacter* is the leading cause of bacterial zoonotic gastroenteritis in Europe (EFSA & ECDC, 2015) with *C. jejuni* the most important species found in human campylobacteriosis. However, the role of *C. coli* cannot be ignored because many studies have emphasized the importance of its multiple antibiotic resistances and its ability to also cause foodborne enteric infections. Within the EU, there are large differences between countries as well as between farms regarding usage of antimicrobials for prevention and treatment of diseases in pigs. In the frame of a CORE Organic II European project (SafeOrganic), we were interested to compare the occurrence of antibiotic resistance in slaughtered pigs from conventional and organic production, in four European countries. In France, we looked at *Campylobacter* in this project as we known that pigs were an important reservoir of *C. coli* (Denis et al., 2011). *Campylobacter* species identification allowed us to discover another species, than *C. coli*, never described in France: *Campylobacter lanienae*. Specific identifications have been conducted and, as for other strains isolated during SafeOrganic project, the occurrence of antimicrobial resistance of *C. lanienae* was determined as well as the genetic diversity of the isolates by RLFP-PFGE.

#### Material and Methods

##### Sampling and *Campylobacter* collection

Colon contents of pigs were collected in one slaughterhouse from 31 organic herds and 31 conventional herds. A total of 58 conventional pigs and 56 organic pigs were considered (1 to 2 pigs per herd).

Direct detection of *Campylobacter* was done on Karmali plate. Plates were incubated at 37°C in a microaerobic atmosphere for 48 h. Characteristic colonies were then sub-cultured on blood agar plates for 24h at 37°C for species identification by RCR and phenotypic and genotypic characterization.

#### Species identification by PCR

After DNA extraction by blowing the cells out (95°C for 10 min), a Multiplex-PCR was used to confirm the genus of the bacterial isolates and to identify them to species level (Wang *et al.*, 2002). This multiplex-PCR allows the identification of the following five *Campylobacter* species: *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. fetus*, and *C. upsaliensis*. Five µl of DNA were used for amplification. PCR products were visualized by the electrophoresis of 10 µl aliquots of each amplification product, for 3 hours at 100 V, in a 2% agarose gel stained with gel red.

#### Identification with Bruker MALDI Biotyper™

For presumed *Campylobacter* isolates not identified by multiplex PCR, species identification was carried out with a MALDI Biotyper system (Bruker Daltonics, Germany), according to the manufacturer's instructions, as explained by He *et al.* (He *et al.*, 2010). Isolates were grown on blood agar plates for 24 h at 37°C, and one colony per isolate was then individually picked and transferred to one of the 96 wells of a MALDI-TOF plate. The bacteria were then recovered by 1 µl of matrix solution (5 mg of HCCA in 1 ml of a solution of 50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid). The matrix was allowed to crystallize for 5 minutes at room temperature. The microplate was then placed in the Maldi Biotyper for genus and species identification.

#### Specific 16S *Laniinae* PCR and sequencing

After DNA extraction using InstaGene matrix (Bio Rad), a 16S rRNA PCR for *Campylobacter lanienae* was performed as previously described (Logan *et al.*, 2000, Inglis & Kalischuk, 2003) using the following primers, CLAN 76F and CLAN5521021R. The conditions used for amplification were: a denaturation cycle at 95°C for 5 min, followed by 35 cycles of 5 s at 95°C, 5 s at 55°C, and 60 s at 68°C, ending with an extension cycle of 1 min at 72°C.

Before sequencing, PCR products were purified using ExoSAP-IT kit (GE Healthcare). The PCR product were submitted for sequencing using a BigDye® Terminator v3.1 cycle sequence kit (Applied bio systems, Forester City, CA, USA) following manufacturer instructions. Then, sequenced products were run on the ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences obtained were compared to the one of *C. lanienae* NCTC 13004 strain using BioNumerics® software (Applied Maths, Sint-Martens-Latem, Belgium).

#### Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) of antimicrobials were determined for all strains using broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) document M31-A3 with Sensititre® plates (Biocentric, Bandol, France). The antimicrobials tested included gentamicin (GEN), streptomycin (STR), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), erythromycin (ERY) and chloramphenicol (CHL).

ECOFFs for *Campylobacter* resistance were >2 µg/ml for gentamicin (GEN), >4 µg/ml for streptomycin (STR), >0.5 µg/ml for ciprofloxacin (CIP), >16 µg/ml for nalidixic acid (NAL), >2 µg/ml for tetracycline (TET), >8 µg/ml for erythromycin (ERY) and >16 µg/ml for chloramphenicol (CHL).

### Pulsed-field gel electrophoresis (PFGE) and analysis of electrophoretic profiles

DNA preparation, restriction endonuclease digestion and PFGE were carried out as described by the Campynet protocol (Rivoal *et al.*, 2005). DNA macrorestriction was performed with *KpnI* and *SmaI* enzymes. Electrophoretic patterns were compared using BioNumerics®. Simpson's index (D) was used to assess the genetic diversity of the *Campylobacter* populations (Hunter & Gaston, 1988).

### Results

#### Species identification

Identification by Maldi-Tof mass spectrometry, and sequencing of 16S allowed us to confirm that a total of 85 isolates out of 118 were *C. Lanienae*. They originated from conventional pigs (n=56) as well as from organic pigs (n=29).

#### Antimicrobial susceptibility testing

A total of 55 isolates of *C. Lanienae* were studied for their resistance to antimicrobials. All isolates were susceptible to Chloramphenicol. Only one isolate was pansusceptible. A high level of resistance to Nalidixic acid (93 %) was observed. Resistance to Tetracycline and Ciprofloxacin was significantly different between the two productions (figure 1.). For tetracycline, 88 % of isolates from conventional pigs were resistant against 14% of isolates from organic pigs. Moreover, the analysis of resistance pattern showed that isolates from conventional pigs were mostly multiresistant (73%) whereas only 5% of strains isolated in organic pigs were multiresistant.

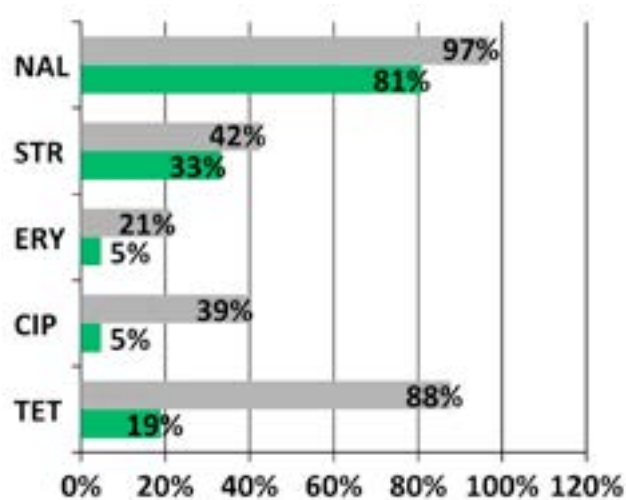


Figure 1: Percentage of resistance to 5 antimicrobials observed *C. lanienae* isolated tested for their resistance to antimicrobials (isolates from organic pigs in green and conventional pigs in grey).

### Pulsed-field gel electrophoresis (PFGE) and analysis of electrophoretic profiles

After *KpnI* restriction, 46 of the 57 isolates were typable. They were distributed in 43 *KpnI* profiles. For *SmaI* restriction, only 27 isolates were typable and highlighted 19 profiles. Index of diversity was very high in both productions whatever the enzyme used ( $D > 0.96$ ).

## Discussion

*C. lanienae* was originally isolated from abattoir workers exposed to pigs and cattle in Switzerland (Logan *et al.*, 2000), but, so far, it has not been associated to human disease. The primary source of the bacterium seems to be swine (Sasaki *et al.*, 2003) and/or cattle (Guevremont *et al.*, 2008). *C. lanienae* also has been isolated from sheep (Oporto & Hurtado, 2011). *C. lanienae* is very fastidious to grow on most commercially available media tend to select *Campylobacter*. It could be an underestimated zoonotic agent. In this study, we have been able to detect *C. lanienae* probably because of implementation of direct isolation.

Initially, in our study, individual occurrence in *Campylobacter spp* in colon content was estimated on the basis of the presence of *C. coli* (Kérouanton *et al.*, 2014). Occurrence was not significantly different between organic (76.8%) and conventional pigs (74.0%). The confirmation of the presence of another species allowed us to re-estimate the occurrence of *Campylobacter* to 91.1 % (51/56) and 96.3 % (52/54) for conventional and organic pigs, respectively.

Antimicrobial resistance testing allowed us to confirm the natural resistance to Nalidixic acid as previously described (Logan *et al.*, 2000). The significant tetracycline resistance on conventional pork (88%) and the presence of multiresistant isolates raise the question whether *C. lanienae* could play a role in the spread of resistance.

Diversity was very high whatever the enzyme used for macrorestriction and PFGE, as previously observed (Schweitzer *et al.*, 2011). *C. Lanienae* species probably have extensive genetic diversity and genome plasticity similar to other *Campylobacter* species. By PFGE, no evidence of genetic clusters specific to a production was shown. Likewise, no link was observed between PFGE profiles and resistance profiles.

## Conclusion

This study allowed us to demonstrate for the first time in France that pigs, known to be a reservoir for *C. coli*, may also carry in their feces a species rarely highlighted: *Campylobacter lanienae*. The species was present in fecal samples from conventional and organic pigs. The lower level of antibiotic resistance and multiresistance of *C. Lanienae* strains for organic pigs may be related to the restricted use of antibiotics in this production. Diversity of isolates was high, as usually observed for *Campylobacter* genus bacteria.

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