Tetracycline-resistant *Chlamydia suis* in cases of reproductive failure on Belgian, Cypriote and Israeli pig production farms

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Similar cases of severe reproductive failure associated with the presence of *Chlamydia suis* in two Belgian, one Cypriote and one Israeli pig farrowing to slaughter farms are presented. Vaginal and rectal swabs from 39 sows were examined by culture and DNA microarray. Nineteen of 23 (83 %) *C. suis*-positive sows were infected with tetracycline-resistant *C. suis* strains, as determined by MIC tests. Furthermore, boar semen from a German artificial insemination centre, intended for export, was positive for *C. suis*. Emergence of tetracycline-resistant *C. suis* strains was confirmed.

Case report

In 2009, four pig breeder-fattener farms, one located in Israel (>10 000 pigs, 5000 sows), one in Cyprus (>1000 pigs, 500 sows) and two in Belgium (both >1000 pigs, 500 sows), reported severe reproductive failure, causing important economic losses.

Sows were normally inseminated a minimum of twice a year. Oestrus synchronization was not applied. Farmers operated a twice-per-day heat detection regime. Two inseminations per oestrus were carried out. All affected farms were the top pork producers. Sows were handled gently and quietly while being brought to the insemination pen. The sows were always stimulated during insemination with flank rubbing and back pressure and they were allowed to rest for 15 min after insemination. Regrouping of sows was achieved within 24 h of the end of oestrus, in order to promote optimal implantation of the embryos. The mean gestation period was 114 days, and sows were removed to the farrowing pen 4–5 days prior to farrowing. For the Israeli farm, boar semen was imported from a German pig insemination centre. The Belgian and Cypriote farms had their own sperm donors on site. Inferior semen quality in boars was noticed.

The following was observed on all farms. Before the problems occurred, the conception rate on all farms was 90 %, as demonstrated by ultrasound scanning at 25–28 days, was 90 %. However, the second ultrasound scanning performed at 50 days post-artificial insemination revealed a drop to <65 %. Thus, early embryonic death probably occurred in inseminated sows. Ten to twenty per cent of sows that delivered produced only two to five piglets of a non-uniform weight (between 1 and 1.5 kg).

Sows that lost their embryos secreted a white to yellow vulval liquid (non-smelling) indicative of endometritis and/or bacterial infection of the vulva and/or vagina. The abnormal secretion lasted for about 1 week. Litter production was abnormal during this period: it was extremely low or highly variable. Sows irregularly returned to oestrus. Clinical disease could not be improved by doxycycline [400 g (ton feed)−1] plus a combination of trimethoprim [120 g (ton feed)−1] and sulfamethoxazole [600 g (ton feed)−1] for 10–14 days during gestation and half a dose during lactation. Diagnostic tests for *Leptospira* species, *Mycoplasma* species, *Brucella suis*, *Mycobacterium* species, porcine reproductive and respiratory syndrome virus, Aujeszky’s disease, swine influenza, porcine circovirus 2, porcine enteroviruses, porcine encephalomyocarditis virus and porcine parvovirus were negative.

Vaginal and rectal swabs taken from 17 Belgian (9 sows from the first Belgian farm and 8 from the second Belgian farm), 12 Cypriote and 10 Israeli clinically affected sows were placed in 2-SP (2-sucrose phosphate buffer) *Chlamydia* transport medium or DNA/RNA stabilization
DNA extraction was performed as described by Wilson et al. (1996). Specimens were centrifuged (13,000 g) and suspended in 198 μl STD buffer [0.01 M Tris/HCl (pH 8.3), 0.05 M KCl, 0.0025 M MgCl₂·6H₂O, 0.5% Tween 20] and 2 μl proteinase K (20 mg ml⁻¹ stock solution; Sigma). The specimens were incubated at 56 °C for 1 h and subsequently heated at 100 °C for 10 min. DNA samples were further purified by extracting them twice with 200 μl phenol:chloroform (1 : 1). Precipitation was performed (1 h, 80 °C) by adding 20 μl sodium acetate (3 M) and 400 μl 100% ethanol. The pellets obtained following centrifugation (20 min, 4 °C, 16,060 g) were washed for 5 min with 500 μl 70% ethanol (4 °C, 16,060 g) and finally suspended in 30 μl sterile MilliQ water.

For the microarray, DNA was amplified and biotin-labelled during 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, using primers U23F-19 [5’-ATT GA(c/a) AGG CGA (a/t)GA AGG A-3’] and 23R-22 [5’-biotin-GC(t/c) TAC TAA GAT GTT TCA GTT C-3’] targeting the 23S rRNA, generating an amplicon of 273 bp. Species-specific hybridization and signal measurements (ATR-03 array tube reader and Iconoclust software, version 2.3; Alere Technologies) were performed as described by Sachse et al. (2005).

The in vitro activity (MIC value) of doxycycline (Sigma) against Chlamydia suis strains carrying the tet(C) gene was determined as described by Butaye et al. (1997), using twofold doxycycline dilutions ranging from 0.05 μg ml⁻¹ to 12.8 μg ml⁻¹. The MIC of doxycycline was defined as the lowest concentration preventing the detection of more than 90% of the chlamydial inclusions compared with a drug-free control. In addition, fresh diluted semen samples from five boars in a German artificial insemination centre were examined in the same way as the swabs. The semen samples originated from five boars which provided semen for export to the Israeli farm.

Culture and microarray results for all four farms are presented in Table 1. Nine sows were examined on the first Belgian farm, two of which (22%) were positive. The tet(C) gene was present in one vaginal C. suis isolate. The MIC of doxycycline was 1.6 μg ml⁻¹, as compared to 0.05 μg ml⁻¹ for a C. suis control strain without the tet(C) gene. The latter was isolated from the lung of a deceased fattening pig (45 kg) showing post-weaning multi-systemic wasting syndrome and porcine dermatitis and nephropathy syndrome (unpublished results). Eight sows were examined on the second Belgian farm, seven of which (87.5%) were positive. The tet(C) gene was detected in one rectal and six vaginal C. suis isolates. The MIC of doxycycline varied from 1.6 μg ml⁻¹ to 3.2 μg ml⁻¹. Twelve sows were sampled on the Cypriote farm, 11 of which (92%) sows were positive. The tet(C) gene was present in 8 of 13 (61.5%) C. suis isolates. The MIC of doxycycline was 3.2 μg ml⁻¹. Ten sows were examined on the Israeli farm, three of which (30%) were positive. The tet(C) gene was discovered in all C. suis isolates. The MIC for doxycycline was 3.2 μg ml⁻¹.

C. suis was also isolated from four of five (80%) boar semen samples from the German artificial insemination centre. Semen appeared to be negative when examined by the microarray. However, the microarray identified three of four (75%) chlamydia isolates as C. suis. One of them contained the tet(C) gene, leading to a MIC value of 6.4 μg ml⁻¹.

Most of the time, C. suis species identification by use of the microarray was only possible on chlamydia isolates. Other Chlamydiaceae species, reluctant to grow, could have been present in the field samples. We therefore examined all field samples as well as cell culture harvest of all first cell culture passages (stored in sucrose phosphate glutamate buffer at −80 °C) retrospectively by a Chlamydia psittaci-specific real-time PCR (Geens et al., 2005), a real-time PCR for Chlamydia abortus (Livingstone et al., 2009), a real-time PCR for Chlamydia pecorum (Wan et al., 2011) and two real-time PCR assays for Chlamydia-like organisms (Casson et al., 2008; Goy et al., 2009). We could not detect C. pecorum, C. psittaci, C. abortus, Waddlia or Parachlamydia DNA.

**Discussion**

Members of the Chlamydiaceae are Gram-negative, obligately intracellular bacteria infecting a broad range of animals and humans. Pigs can become infected by Chlamydia pecorum, Chlamydia abortus, Chlamydia psittaci and Chlamydia suis (Teankum et al., 2006; Kauffold et al., 2006a, b; Schautteet & Vanrompay, 2011; Salinas et al., 2012). Currently, the pig is the only known natural host for C. suis. C. suis causes conjunctivitis, enteritis and pneumonia, as demonstrated by experimental reproduction of clinical infections in gnotobiotic pigs (Rogers & Andersen, 1996, 1999; Rogers et al., 1996). In addition, C. suis is associated with MMA syndrome (mastitis, metritis and agalactia), reproductive failure and asymptomatic infections (Schautteet & Vanrompay, 2011).

The type strain of C. suis, S45, was isolated in Europe in the late 1960s from faeces of an asymptomatic Austrian pig. The strain is tetracycline-sensitive (TcS), as are other chlamydia species. However, tetracycline-resistant C. suis strains have been isolated on swine farms in Iowa, Nebraska, Italy and Switzerland (Andersen & Rogers, 1998; Di Francesco et al., 2008). The C. suis tetracycline-resistant phenotype (TcR) is manifested through the
tetracycline-resistance gene \textit{tet}(C), which is integrated into the chlamydial chromosome (Dugan et al., 2004). More recently, Borel et al. (2012) described the occurrence of \textit{C. suis} in a Swiss pig farm with an outbreak of conjunctivitis and diarrhoea. After tetracycline treatment, strains harbouring the \textit{tet}(C) gene-coding region were predominant, indicating selection under tetracycline treatment conditions. According to Borel et al. (2012), the adaptive acquisition of the \textit{tet}(C)-resistance gene could have far-reaching consequences for the emergence of antibiotic resistance in chlamydiae and could pose a potential threat for food safety.

To date, TcR \textit{C. suis} strains have been isolated from eyes (H5 and R22), the intestine (R27, R19, 130 and 132) and nasal mucosa (R24) (http://www.ncbi.nlm.nih.gov/nuccore). Furthermore, the presence of the tetracycline-resistance gene \textit{tet}(C), as demonstrated in American and Italian \textit{C. suis} strains, is always associated with a tetracycline-resistant phenotype, characterized by a MIC value of 4 \(\mu\)g ml\(^{-1}\) or higher. In our study, the MIC value of doxycycline ranged from 1.6 \(\mu\)g ml\(^{-1}\) to 6.4 \(\mu\)g ml\(^{-1}\). The \textit{tet}(C) gene was present in several \textit{C. suis} isolates originating from different geographical locations. The Israeli farm most likely became infected with \textit{C. suis} through using contaminated German boar sperm. For the other farms, the origin of the infection was not detected.

Reproductive disorders were completely resolved by using enrofloxacin (fluoroquinolone, Baytril 5%; Bayer Healthcare). Enrofloxacin was added to the sperm diluter [2 ml (1 diluter) \(^{-1}\)] and it was also used to rinse the sow’s reproductive tract (Baytril 5%, 5 ml+95 ml distilled water) immediately before artificial insemination.

The microarray was often negative when performed on clinical samples, as probe hybridization failed. Nevertheless, a biotinylated PCR product could be generated in some cases, as visualized by agarose gel electrophoresis. According to Ehrich et al. (2007), amplification of \textit{Escherichia coli} DNA present in commercial DNA polymerases can take place during PCR or microarrays. However, we could not visualize a biotinylated PCR product on the gel when evaluating the amplification results of our non-template controls. The biotinylated PCR product was only present when using DNA extracted from field samples, and the excised bands that we used for sequence analyses indeed contained a fragment of \textit{E. coli} 23S rDNA.

In conclusion, we demonstrated emerging \textit{tet}(C)-positive \textit{C. suis} strains in pigs. Research on the development of preventive measures, such as probiotics or vaccines, should be promoted. The spread of tetracycline-resistant \textit{C. suis} strains, for instance through international trade of boars and sperm, should be monitored more carefully.

Acknowledgements

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (convention RF 10/6234). We gratefully thank D. Beeckman, T. Van Vooren, Y. Jönsson and A. Dumont for their assistance during diagnosis.

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