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Prevalence of *Lawsonia intracellularis*, *Brachyspira hyodysenteriae*, and *Brachyspira pilosicoli* infection in hunted wild boars (*Sus scrofa*) in Germany

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Abstract *Lawsonia intracellularis*, *Brachyspira hyodysenteriae*, and *Brachyspira pilosicoli* are important pathogens in domestic pig production, responsible for porcine intestinal adenomatosis, swine dysentery, and porcine intestinal spirochetosis, respectively. They are widely distributed among pig-producing units around the world, and transmission is accomplished by relatively weak immunity, long shedding intervals, sequential shedding, and actual environmental survival. Little information is available on occurrence, prevalence, and quantity of these pathogens in free-ranging wild boars. The aim of the present study was to evaluate *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* infections in wild boars in Germany. Tissue samples from ileocaecal mucosa of 165 wild boars from 18 hunting grounds situated in 14 of the 16 federal states of Germany were examined by conventional PCR and quantified by multiplex real-time PCR. None of the wild boars did show any gross pathological signs of enteritis. The overall prevalence for *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* was 20.6%, 2.4%, and 12.1%, respectively. None of the three agents was detected in 68.5% of the wild boars and in 11.1% of the hunting grounds. Numbers of bacteria per sample were below the limit of quantification (100 cells/PCR reaction). This is the first study on *L. intracellularis* and *Brachyspira* spp. in free-ranging wild boars. The study revealed colonised animals without signs of disease. The meaning of these

findings remains unclear, and we do not know whether and to what extent these three pathogens are exchanged between wild boars and domestic pigs. Further research is needed to get insight into the epidemiological impact of the results.

Keywords *Lawsonia intracellularis* · *Brachyspira hyodysenteriae* · *Brachyspira pilosicoli* · Epidemiology · Wild boar

Introduction

Lawsonia intracellularis, the causative agent of porcine proliferative enteropathy, also known as ileitis, is an obligate intracellular bacterium which may be propagated in enterocytes under microaerophilic conditions (Lawson et al. 1993). The bacterium may be viable for 14 days outside the host (Collins et al. 2000), and rodents can play a role in the spread (McOrist et al. 2003), although pig to pig transmission is considered the main route of infection (Jordan et al. 2004). Transmission is accomplished by weak immunity (Guedes and Gebhart 2003), long shedding intervals (McOrist et al. 1999), and sequential shedding (Steghe et al. 2004). Infections with *L. intracellularis* have a worldwide distribution and occur commonly in all pig-producing regions and in all pig farm management systems, including outdoor enclosures. Practically all pig farms have at least a low level of infection, and some 20–40% have a notable level of subclinical and/or clinical cases (McOrist and Gebhart 2006). The disease manifests itself as diarrhoea and poor performance in growing pigs or as acute haemorrhagic enteropathy with high mortalities in gilts and finisher pigs (Jacobson et al. 2009).

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Brachyspira hyodysenteriae and *Brachyspira pilosicoli* are important swine pathogens causing swine dysentery and porcine intestinal spirochetosis, respectively (Moxley and Duhamel 1999). *B. hyodysenteriae* is relatively resistant in the environment of a pig house, particularly in faeces in moist conditions, the bacteria can survive in contaminated water up to 48 days (Chia and Taylor 1978) and up to 78 days in faecal contaminated soil (Boye et al. 2001). Although less severe in its impact on swine production, *B. pilosicoli* is more resistant to survive in the environment than *B. hyodysenteriae*, and persistence in soil for up to 210 days has been reported (Boye et al. 2001). Just like *L. intracellularis*, both pathogens can be transferred by rodents and a wide range of domestic and wild animals, birds, and even humans (Hampson et al. 2006; Hampson and Duhamel 2006; Oxberry et al. 1998). Swine dysentery is a severe enteric disease characterised by mucohaemorrhagic to fibrinonecrotic colitis and typhlitis that leads to substantial losses (Hampson and Duhamel 2006; Hampson et al. 2006). Swine dysentery is considered to be one of the most significant production-limiting porcine infections, due to mortality, decreased growth rates, poor feed conversion, and treatment expenses. Infection with *B. pilosicoli* causes a mild to moderate typhlocolitis accompanied with pulpy to watery diarrhoea, sometimes with mucus (Hampson and Duhamel 2006).

Detection of *Brachyspira* in domestic pigs is most often accomplished by culturing methods and biochemical differentiation (Rasbäck et al. 2006). However, *Brachyspira* species share many biochemical properties rendering it very difficult to differentiate between them (Feberwee et al. 2008). Moreover, *Brachyspira* species have a low tenacity, and therefore samples which are not processed in due time may contain non-viable bacteria which will be false-negative in culture.

Because of difficulties in culturing *Brachyspira* species and due to the obligate intracellular parasitism of *L. intracellularis*, diagnostics of these bacteria primarily relies on the detection of genomic sequences (La et al. 2006; Rasbäck et al. 2006). These organisms were detected through multiplex PCR (Su and Song 2005; Nathues et al. 2007; Song and Hampson 2009; Willems and Reiner 2010).

Little information is available on the prevalence of *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* in wild boars (*Sus scrofa*). Clinical disease caused by one of the three agents or by a mixed infection has never been reported in wild boars. However, *Brachyspira* spp. as well as *L. intracellularis* were well detected in deer species in Japan (Shibahara et al. 2000) and Canada (Drolet et al. 1996), respectively as the causative agent of enteritis. Infections with *L. intracellularis* have been demonstrated in a commercial wild boar herd from Brazil (Zlotowski et al. 2008). Jacobson et al. (2005) did not find *Brachyspira*

spp. or *L. intracellularis* in 48 Swedish wild boar samples. *L. intracellularis* was detected in 29.6% and 9.1% of free-ranging and enclosed wild boars from Czech Republic, respectively (Tomanova et al. 2002; Dezorzo-Tomanova et al. 2006). All positive samples were from the small intestinal mucosa. The authors did not find *L. intracellularis* DNA in wild boar faeces. A group of 222 feral pigs from Western Australia (Phillips et al. 2009) had a prevalence of 18.9%, 8.1%, and 0.45%, respectively for *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* (faeces or colonic content).

Because of the wide distribution of *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* among domestic pig herds and the well-recognised interaction between domestic pigs and wild boars (Albina et al. 2000; Elbers et al. 2000; Laddomada 2000), the aim of the present study was to determine the prevalence of the three pathogens in German wild boars.

Materials and methods

Sample collection

Actual numbers of wild boars in Germany are unknown, but during the hunting season 2005/2006, on average, 400,000 wild boars were shot (DJV 2009). Samples for the present study were collected between latitudes 48.1° and 54.5° N and longitudes 6.1° and 14.0° E.

Intestine samples with ileocaecal valve of 165 wild boars were collected during the hunting season 2005/2006 from 18 hunting grounds situated in 14 of the 16 federal states of Germany. Only the town states Hamburg and Bremen were not sampled. Wild boars were hunted by chance. They were gathered after hunting at central places, numbered, and rated according to age (Stubbe 2001) and weight. Of the samples, 47% were from male individuals. The wild boars were on average 12 months (1 to 84 months) old, with an average carcass weight of 34 kg (6 to 110 kg).

Samples were taken half an hour to 2 h after death with sterile disposable gloves and scalpels and stored at −20°C until further usage.

DNA extraction

DNA was extracted with the Qiagen Viral RNA Mini kit (Qiagen, Hilden, Germany), normally specified for viral RNA extraction from cell-free body fluids. Nevertheless, we thoroughly tested this kit on many different sample materials including tissue samples. Compared to other commercially available kits and in-house methods, this kit gave the best results. For DNA extraction, 25–50 mg of tissue sample was added to 560 µl of AVL lysis buffer.

Tissue cells were disrupted in a ball mill with 5 mm grinding balls at a frequency of 25 Hz for 2 min. The suspension was incubated for at least 10 min at room temperature. After centrifugation (3 min., 16,000×g), the supernatants were applied to spin columns. Further steps were performed as described by the manufacturer. Resulting DNA was eluted in 60 µl of elution buffer and stored at –20°C.

PCR

DNA extracted from ileocaecal samples was subjected to conventional PCR. Primers were designed with OLIGO 4.0 and directed against the *nox* gene of *Brachyspira* spp. and the *aspA* gene of *L. intracellularis*, respectively (Willems and Reiner 2010), whereas detection of *B. pilosicoli* was performed in a uniplex PCR, *B. hyodysenteriae* and *L. intracellularis* were detected in a multiplex PCR reaction. The total PCR volume for both PCR assays was 20 µl consisting of 10 µl of QIAamp Multiplex Master Mix (Qiagen, Hilden, Germany), 2 µl of each primer (final concentration 0.2 µM), and 2 µl of extracted DNA. PCR was performed as Touch-Down PCR on T_{personal} and T_{GRADIENT} thermocyclers (Biometra, Göttingen, Germany) with the following cycling conditions: DNA polymerase activation for 15 min at 95°C followed by ten cycles of denaturation at 95°C for 30 s, annealing of primers at 65°C for 90 s, and extension at 72°C for 30 s with a decrease in annealing temperature of 1°C at each cycle. Remaining 30 cycles were performed at a denaturation temperature of 95°C for 30 s, an annealing temperature of 55°C for 90 s, and an extension at 72°C for 30 s.

PCR products were analysed on 1.5% agarose gels stained with ethidium bromide (0.4 µg/ml), photographed, and stored as digital image.

Multiplex real-time PCR

Positive samples from conventional PCR were quantified by multiplex real-time PCR. Primers and probes for real-time PCR were designed with the Primer Express software (Applied Biosystems) targeting the *nox* gene of *Brachyspira* spp. and the *aspA* gene of *L. intracellularis*. For this purpose *nox* gene sequences were retrieved from the Genbank database and aligned using ClustalX. Conserved regions within species and variable regions between species were used to design primers and probes. All primers and probes were tested in silico for specificity against sequences deposited in the Genbank database using BLAST. A description of primers has been published previously (Willems and Reiner 2010).

The total reaction volume was 25 µl consisting of 12.5 µl of Quantitect Multiplex Mastermix (Qiagen, Hilden, Germany), 0.1 µl of each primer and probe (final

concentration 0.2 µM each), 9.1 µl of water, and 2.5 µl of extracted DNA. Real-time PCR was performed on a 7,300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) using the following cycling conditions: DNA polymerase activation at 95°C for 15 min, 40 cycles with a denaturation at 95°C for 60 s, and an annealing and extension at 60°C for 90 s.

Standards for real-time PCR were prepared by amplification of genomic sequences which encompass the targeted region of the real-time PCR amplicon. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and copy numbers were calculated from the concentrations of the purified amplicons.

The analytical sensitivity for the real-time PCR assay, expressed as limit of detection was below 10, 14, and 26 DNA copies for *L. intracellularis*, *B. pilosicoli*, and *B. hyodysenteriae*, respectively. The limit of quantitation was set to 100 DNA copies per PCR reaction for all species.

Sequencing

Some of the PCR amplicons were selected by chance and sequenced. Homology with *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* sequences deposited in the GenBank database was determined by BLAST analysis, respectively.

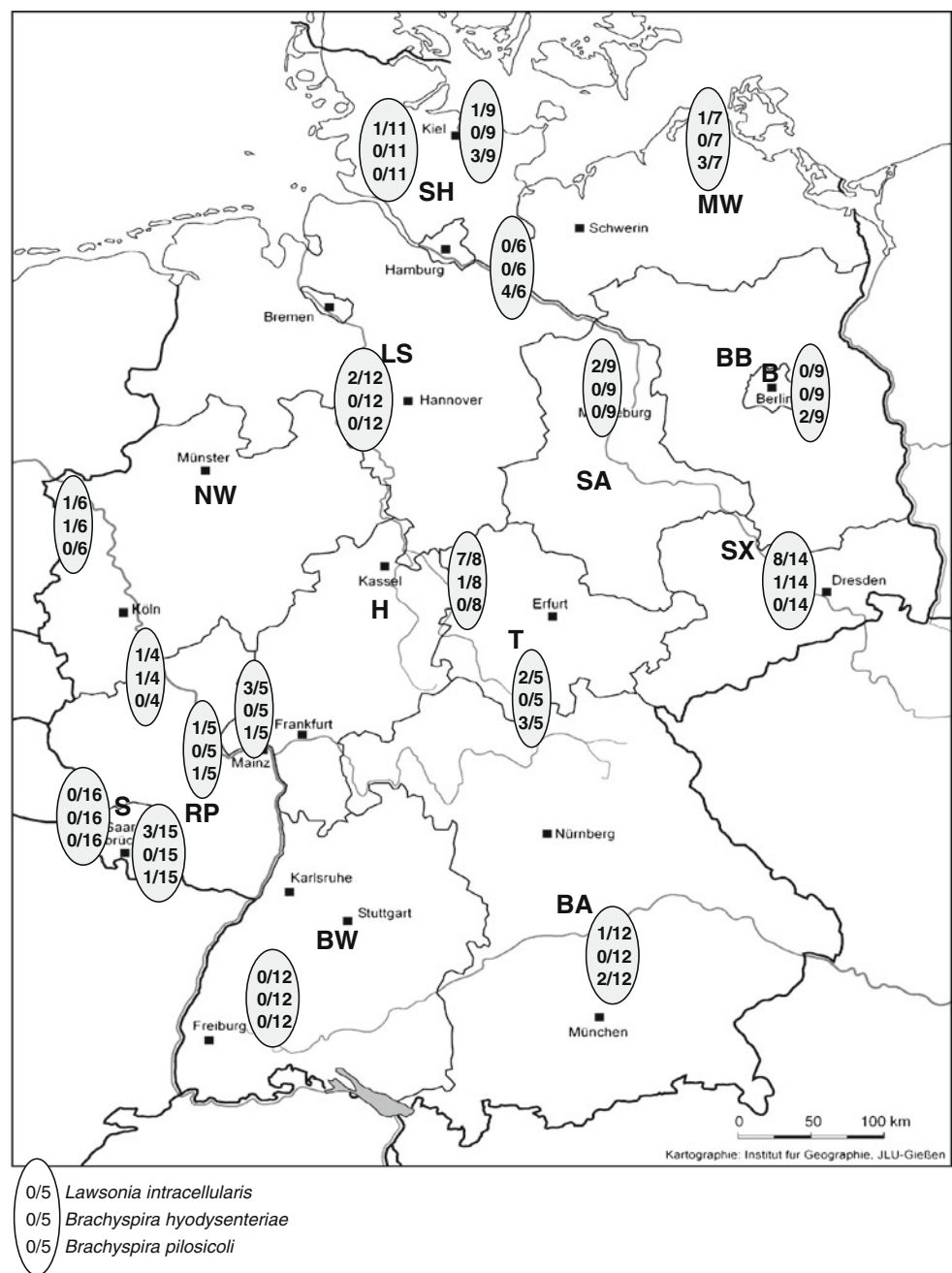
Statistical methods

Descriptive statistics were calculated with the “Statistical Package for Social Sciences” (SPSS, version 15.0). Effects of the sex were tested with chi-square test. Effects of latitude, longitude, age, and weight on prevalence were tested with Spearman correlation analysis.

Results

Figure 1 shows the geographical situation of the 18 hunting grounds, including the numbers of positive and the numbers of investigated wild boars for *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli*. The overall prevalence for *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* was 20.6%, 2.4%, and 12.1%, respectively. None of the three organisms was detected in 68.5% of the wild boar samples; 17.0%, 1.2%, and 9.7% of the animals were monoinfected with *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli*, respectively; 2.4% and 1.2% of the wild boars had a mixed infection with *L. intracellularis*/*B. pilosicoli* and *L. intracellularis*/*B. hyodysenteriae*, respectively, whereas no mixed infection with *B. pilosicoli*/*B. hyodysenteriae* was detected. Of the hunting grounds, 88.9% were found positive for at least one of the three

Fig. 1 Numbers of positive/numbers of investigated wild boars of *L. intracellularis* (upper value), *B. hyodysenteriae* (middle value), and *B. pilosicoli* (lower value) by hunting grounds. *B* Berlin, *BA* Bavaria, *BB* Brandenburg, *BW* Baden-Wuerttemberg, *H* Hesse, *MW* Mecklenburg West-Pomerania, *NW* North-Rhine Westphalia, *LS* Lower Saxony, *RP* Rhineland-Palatinate, *SX* Saxony, *SA* Saxony Anhalt, *SH* Schleswig-Holstein, *S* Saarland, *T* Thuringia



agents; overall prevalences of *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* within hunting grounds were 77.8%, 22.3%, and 50.0%, respectively. *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* were detected in 16.7%, 0.0%, and 11.1% of the hunting grounds as mono-infections; 36.8% and 21.1% of the hunting grounds were positive with *L. intracellularis*/*B. pilosicoli* and *L. intracellularis*/*B. hyodysenteriae*, respectively. Numbers of bacteria per sample were consistently low with C_t values above 37, and none of the positive samples exceeded the threshold of 100 copies per real-time PCR reaction. Prevalence of the three species was neither dependent on

sex, age, or weight of the animals, nor on longitude or latitude of hunting grounds.

Discussion

This is the first study on *L. intracellularis* and *Brachyspira* spp. in free-ranging wild boars. Presented data demonstrate that there are only few hunting grounds in South-west Germany (Fig. 1), in which none of the three pathogens could be detected. Otherwise, detection rates substantiate a wide distribution, especially for *L. intracellularis*, which is

found throughout Germany. The prevalence of *L. intracellularis* corresponds well with reports from the Czech Republic (Tomanova et al. 2002; Dezorzova-Tomanova et al. 2006) and Western Australia (Phillips et al. 2009).

In contrast, *B. hyodysenteriae* was only detected in single wild boars from hunting grounds situated in a West-eastern band of central Germany (Fig. 1). Prevalence of *B. hyodysenteriae* was found to be rather low (2.4%) compared to such of Australian feral pigs (8.1%) and such observed in domestic pigs from Germany (up to 36%). Nevertheless, hunting ground prevalence of 22.3% corresponds well with that of domestic herds.

Interestingly, *B. pilosicoli* was determined only in certain regions of Germany, i.e., in the North-East, South-West, and South-East of Germany (Fig. 1). Common features of these areas are a high fragmentation into humanised and agricultural regions. Whether this maybe a reason for the restriction of wild boars infected with *B. pilosicoli* to these areas remains unclear. Prevalence of *B. pilosicoli* in wild boars was higher than those for domestic pigs (2.5%; Wendt et al. 2006) Also, *B. pilosicoli* was considerably less common in feral pigs from Western Australia (Phillips et al. 2009).

Mixed infections with *B. hyodysenteriae* and *L. intracellularis* (Herbst et al. 2004; Biksi et al. 2007), and *B. hyodysenteriae* and *B. pilosicoli*, or with all three agents (Biksi et al. 2007) have been described, with frequencies up to 35% at the herd and 19% at the individual sample level (Biksi et al. 2007). These rates are also in accordance with the present data of wild boars.

Missing of gross pathological lesions argue for a colonisation of the wild boars without exhibiting symptoms of disease. This situation might be explained to a great extent by the low number of bacteria detected in each positive sample. Guedes et al. (2003) found a close correlation between the number of bacteria and the outcome of disease, and only a huge number of bacteria was able to trigger disease. Differences in resistance of animals against the three agents or in virulence potential of pathogens derived from wild boars and domestic pigs might play a further role in the outcome of infection. It remains unclear, whether and to what extent these three agents are exchanged between wild boars and domestic pigs. Wild boars may be threatened by exposure to *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* from domestic origin. As has been demonstrated with other pathogens (Albina et al. 2000; Elbers et al. 2000; Laddomada 2000), wild boars may serve as a reservoir with impact on domestic pig production. If this is also the case for *L. intracellularis*, *B. hyodysenteriae*, and *B. pilosicoli* needs to be addressed by future studies.

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