Experimental evaluation of faecal *Escherichia coli* and Hepatitis E virus as biological contacts between domestic pigs and Eurasian wild boar

Short: *E. coli* and HEV as biological indicators for pig interactions

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Abstract

Domestic pigs and Eurasian wild boar (*Sus scrofa*) share several important viral and bacterial pathogens. Therefore, direct and indirect contacts between domestic pigs and wild boar present a risk of pathogen spill-over and can lead to long-term perpetuation of infection. Biological indicators could be a powerful tool to understand and characterize contacts between wild boar and domestic pigs. Here, faecal *E. coli* and Hepatitis E virus (HEV) were explored as potential biological indicators under experimental conditions. The data gained in our pilot study suggest that faecal *E. coli* can be used as biological indicator of contact between wild boar and domestic pig. For HEV, faecal transmission was also confirmed. However, molecular studies on full-genome basis did not reveal markers that would allow tracing of transmission direction. Based on these promising results, future field studies will especially target the practicability of *E. coli* microbiome molecular typing as surrogate of contacts at the wildlife-livestock interface.

Keywords: *E. coli*, Hepatitis E virus, biological contact markers, wild boar, domestic pig

1 Introduction

Contacts between domestic pigs and Eurasian wild boar (*Sus scrofa*) play a major role in the epidemiology of African swine fever and other important diseases such as classical swine fever (Fritzemeier et al., 2000), Aujezsky's disease (Boadella et al., 2012, Ruiz-Fons et al., 2008) or Brucellosis (Cvetnic et al., 2009, Wu et al., 2012). Both direct (animal to animal) and indirect (e.g. via vehicles, faeces or contaminated environment) contacts pave the way to the establishment of efficient transmission cycles. Such contacts usually occur in different backyard settings (Wu et al., 2012) but also in areas with free-ranging pigs or pigs temporarily kept on open pastures (Boadella et al., 2012).

To assess the ecology of these contacts (e.g. frequency, intensity, spatial and temporal distribution), different methodical approaches can be implemented. The use of GPS radiotracking (collars) in different interface areas (Miguel et al., 2013, Pruvot et al., 2014) or the use of camera traps (Kukielka et al., 2013) has proven to provide accurate spatial and temporal data on interactions between domestic and wildlife species. However its implementation requires long-term monitoring and resources. The implementation of questionnaires among rural stakeholders can also provide qualitative and quantitative estimates of previous contacts between domestic and wild animals based on past spatial and temporal observations (Brahmbhatt et al., 2012, Jori et al., 2011, Knust et al., 2011, Wu et al., 2012). However, these approaches may only unveil usage of a common space or environment but not transmission of biological entities. Instead, effective biological indicators are expected to provide direct evidence of transmission between two different animal populations by showing carriage or shedding of shared flora or potential pathogens.

Candidate biological indicators include faecal *Escherichia coli* (*E. coli*) as it has been observed that close contact between different host populations is mirrored by bacterial (gene)

flow detectable by in detail characterization of the *E. coli* microbiome. Recent reports confirmed an exchange of *E. coli* between humans and primates, and livestock (Rwego et al., 2008a, Rwego et al., 2008b), mongooses and humans (Pesapane et al., 2013), or between groups of large African mammals (Chiyo et al., 2014, Vanderwaal et al., 2013). To our knowledge, this method has not yet been applied to evaluate contacts between wild or domestic suids in a specific region.

To challenge the concept of using faecal *E. coli* for contact analyses in domestic pigs and wild boar, a pilot study was conducted where animals of each species were brought into contact with faecal material and tested over a period of three to five weeks. The study was meant as test and calibration phase for potential field studies. For animal welfare reasons, the pilot trial was made as a part of ongoing transmission and pathogenesis studies using wild boar derived Hepatitis E virus (genotype 3) that included transmission of faecal material from inoculated wild boar to domestic pigs (Schlosser et al., submitted). As Hepatitis E virus (HEV) itself could present a potent biological indicator that was already shown to be transmitted from Eurasian wild boar to domestic pigs (Schlosser et al., 2014), HEV was included into the assessment of contacts as potential biological indicator. Apart from faecal transmission itself, nearly full-genome sequences were targeted in search for genetic markers that would allow tracing of the transmission direction in a setting with quite high prevalence under field-conditions.

2 Material and Methods

2.1 Trial design

The trial comprised a total of 4 experimental groups. Prior to enrolment into the study, all animals were tested negative for HEV genome in faeces by real-time RT-PCR (Schlosser et al., 2014), and antibodies against HEV by antibody ELISA (PrioCHECK HEV Ab porcine ELISA kit, Prionics). Groups 1a and 1b consisted of young wild boar (n = 4 each; group 1b included one additional animal as uninfected control [referred to as animal 1b_c]) in two separated pens. Both wild boar groups (with the exception of the control animal) were infected intravenously using a genotype 3 HEV strain obtained in a previous experiment from a liver sample (Schlosser et al., 2014). Groups 2a and 2b comprised four domestic pigs (two month of age) each and were housed together in one common pen. To assess the influence of the immune status on infection dynamics and shedding (within the transmission trial), animals of groups 1a and 2a were immunocompromised by dexamethasone treatment (0.5 mg/kg) 1.5 weeks prior and after infection (in total nine intramuscular injections). From the 1st day post HEV infection (1 dpi) of wild boar, collective faeces of group 1b (without dexamethasone treatment) were transferred to both together housed groups of domestic pigs (referred to as group 2) using a shovel. Blood sampling was conducted for all animals at time points 0, 1, 4, 6, 9, 13, 15, 18, 21, 24, and 28 dpi and additionally, at 30 dpi in groups 1a and 1b (wild boar) and at 31, 36, and 41 dpi in groups 2a and 2b (domestic pigs), respectively. At the end of the trial (day 30 for wild boar, day 41 for domestic pigs) all animals were subjected to post-mortem examinations and organ sampling. The experimental protocol was reviewed by an independent animal welfare and ethics committee and was approved by the competent authority (State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Western Pomerania, Rostock, Germany, reference number LALLF M-V/TSD/7221.3-1.1-022/13).

Faecal samples for *E. coli* characterisation were collected before, at 0 dpi (all animals), and after HEV infection, at 21 dpi (control piglet 1b_c), from 24 through 28 dpi (wild boar), and
from 36 through 38 dpi (domestic pigs), respectively. For HEV detection and characterization, blood, liver, and gall samples were collected at necropsy. These samples were compared with the inoculum used for infection at 0 dpi. Moreover, transmission of HEV was confirmed by real-time RT-PCR using blood and organ samples.

2.2 Isolation of coliform bacteria

Collected faeces (0.1 g) were suspended in 900 µl phosphate buffered saline (PBS) and a log10 serial dilution suspension was made in PBS. Of each dilution step (10^{-1}-10^{-5}), 100 µl were plated on MacConkey agar (Sifin Diagnostics). Of the first dilution (10^{-1}) additional 100 µl were streaked on Gassner agar (Sifin Diagnostics). Following incubation at 37 °C for 18-20 h, up to ten single coliform colonies were randomly selected and each transferred to 500 µl lysogeny broth (LB) and incubated (18-20 h, 37 °C). After adding glycerine (30 %), bacterial stocks were stored at -80 °C. A sub-cultivation step on blood agar for proof of purity of the stocks was done before further characterisation of the respective isolate.

Up to ten putative *E. coli* isolates were isolated from each faecal sample according to the colony morphology. Due to the detection limit of 100 cfu/g faeces, the number of isolates per faecal sample varied from 2 up to 10. Overall, 300 *E. coli* single isolates were isolated and analysed. In detail, 67 isolates were analysed for animals of group 1a, 76 isolates of group 1b, 10 isolates of the control animal 1b_c, 70 isolates of group 2a, and 77 isolates of group 2b.

2.3 Analysis of PFGE patterns of *Xba*I restricted DNA from *E. coli* isolates

Contour-clamped homogeneous electric field-pulsed-field gel electrophoresis (CHEF PFGE) and cluster analysis was performed as previously described (Geue et al., 2010). In brief,

E. coli bacteria were grown in LB broth and adjusted to an optical density at 600 nm of 1.0. Thereof 1.5 ml bacterial suspension was centrifuged, washed twice in 0.89 % NaCl solution and the bacteria embedded in 1.2 % InCert agarose (Biozym Scientific) in Tris-borate-EDTA (1 x TBE) buffer. The agar plugs were incubated for 24 h with proteinase K (Roche Diagnostics) and washed with Tris-EDTA (TE) buffer. Subsequently, DNA in the agarose plugs was digested overnight with 20 U *Xba*I (New England Biolabs) at 37 °C, and the resulting fragments separated in 1.0 % Seakem Gold agarose (Biozym Scientific) gels in 0.5 x TBE at 10 °C in a CHEF Mapper XA system. The pulse times for the *Xba*I digests were increased from 5 to 50 s (gradient of 6 V/cm) during 25 h at a constant angle of 120°. After electrophoresis, gels were stained in ethidium bromide solution (50 µg/ml), and banding patterns recorded under UV illumination. Interpretation of PFGE patterns was performed by visual inspection and computer analysis with Bionumerics (version 6.6, Applied Maths NV, Sint-Martens-Latem, Belgium). Distance matrices were calculated by pairwise comparisons of the fragment patterns produced by the restriction endonucleases used for the PFGE analysis including DNA fragments between 49 and 630 kb length (cluster analysis: Dice with 2.5 % tolerance; un-weighted pair group method with arithmetic mean, UPGMA).

2.4 Detection and sequencing of HEV

For diagnostic purposes, viral RNA was extracted from blood and organ samples using the QIAamp® Viral RNA Mini Kit or the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN). Subsequently, HEV detection was done by real-time RT-PCR as previously described (Schlosser et al., 2014).

For the generation of full genome sequences, nucleic acids were extracted from gall samples using Trizol Reagent (Life Technologies) in combination with the RNeasy Mini Kit (QIAGEN) and DNase digestion on the spin column. The RNA was subsequently

concentrated using the Agencourt RNAClean XP beads (Beckman Coulter) according to the manufacturer's instructions. Thereafter, RNA was reversely transcribed into cDNA with a second strand synthesis using the Roche cDNA-Synthesis System (Roche). The generated cDNA was purified with Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions.

Purified cDNA was then fragmented to a length of approximately 300-400 bp with the M220 Focused-ultrasonicator™ (Covaris). Sequencing library preparation of each fragmented DNA was done using the SPRIworks Fragment Library Cartridges II (Beckman Coulter) with the SPRI-TETM Nucleic Acid Extractor (Beckman Coulter) machine. During library preparation NEXTflex Adapters (Illumina) were used for later sequence identification during data analyses. To decrease the amount of sequences with unsuitable size, selection for 350 bp inserts was done with Agencourt AMPure XP beads (Beckman Coulter) as recommended by Illumina. Quality of the libraries was checked on a Bioanalyzer 2100 (Agilent Technologies) using the High Sensitivity DNA Kit (Agilent Technologies). Final quantification of each library was done by qPCR with a KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems). Sequencing was done using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 (Illumina). Raw sequence data were analysed and assembled using the Genome Sequencer software suite v. 2.6 (Roche). Sequences were analysed using Geneious Software v6.1.6 (Biomatters Ltd).

2.5 Statistics

Significant differences in the mean numbers of *E. coli* isolates and identified PFGE clones were calculated by one-way ANOVA with Bonferroni post-hoc-analysis using IBM SPSS Statistics (version 19.0.0.2, IBM Deutschland GmbH, Ehningen).

3 Results

3.1 Isolation of *E. coli*

By cultivation of faecal samples on Gassner and MacConkey agar coliform bacteria were detected in each sample. The number of coliform bacteria [cfu/g faeces] ranged from 1×10^2 to 1×10^6 for wild boar and from 2×10^2 to 6×10^5 for domesticated pigs (Table 1). Nevertheless, animal groups did not significantly differ in the coliform bacterial load (ANOVA, p = 0.788). Neither the host species (wild boar *vs.* domestic pig) nor the immune status (with *vs.* without dexamethasone treatment) significantly impacted on the number of *E. coli* isolates picked from each sample (ANOVA, p = 0.176 and p = 0.533) (Table 1).

3.2 Genetic relatedness of the *E. coli* isolates

Eighty-eight individual *E. coli* clones were detected by *Xba*I restriction and PFGE analysis (≥ 90 % similarity or ≤ 3 different fragments, respectively; Tenover et al. (1995)). Mean numbers of identified clones did not differ significantly between groups (ANOVA, p = 0.804) (Table 1). Selecting only one out of up to nine isolates representing a distinct clone from an individual faecal sample, 123 *E. coli* isolates were further analysed (Figure 1). Overall, 17 different clones were found in several animals of a group (1a, 1b or 2) or both samples from one animal (grey arrows in Figure 1). Additionally, five clones were detected in group 1b as well as in contact group 2 (black arrows in Figure 1). One of these clones was found in three piglets of group 1b and one piglet of group 2. No clones from group 1a, faeces of which was not transferred to group 2, were found in animals of group 2.

3.3 Detection and characterization of HEV

Irrespective of whether or not animals received dexamethasone treatment, an efficient HEV replication was shown in HEV-inoculated wild boar, and HEV was successfully transmitted to

Table 1

Cultivated *E. coli* isolates and identified PFGE clones. (SD = standard deviation; SEM = standard error of the mean)

animals		**number of pigs / sampling times** [total no. of samples]	**Coliform bacteria cfu/g faeces** [mean ± SEM (min - max)]	***E. coli* isolates tested / sample** [mean ± SD]	**mean number of *Xba*I-PFGE clones / sample** [mean ± SD]
1a	wild boar with dexamethasone treatment	4 / 2 [8]	$1.9\times10^5 \pm 1.3\times10^5$ (2×10^2 - 1×10^6)	8.25 ± 3.0	3.63 ± 1.8
1b	wild boar without dexamethasone treatment	4 / 2 [8]	$1.8\times10^5 \pm 1.1\times10^5$ (1×10^2 - 8×10^5)	9.25 ± 1.4	3.00 ± 1.3
1b_c	control piglet	1 / 2 [2]	$2.5\times10^2 \pm 1.5\times10^2$ (1×10^2 - 4×10^2)	5.00 ± 4.2	4.00 ± 2.8
2a	domestic pigs with dexamethasone treatment	4 / 2 [8]	$7.5\times10^4 \pm 4.1\times10^4$ (2×10^2 - 3×10^5)	8.75 ± 2.1	3.50 ± 2.0
2b	domestic pigs without dexamethasone treatment	4 / 2 [8]	$1.8\times10^5 \pm 8.8\times10^4$ (3×10^2 - 6×10^5)	9.63 ± 0.7	4.25 ± 2.0

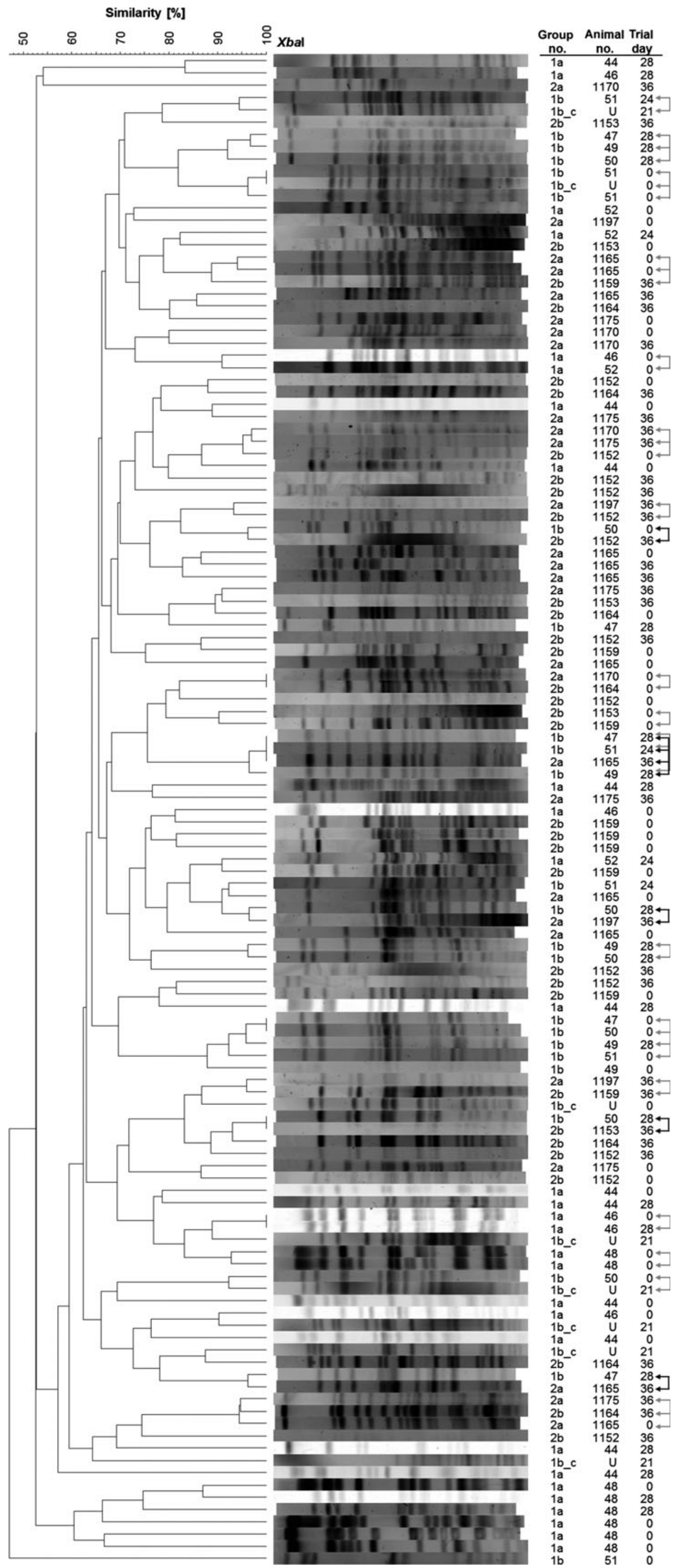

Fig. 1. Dendrogram of analysis of genomic *Xba*I-restricted DNA of *Escherichia coli* isolated from porcine faecal samples. Animal groups: 1 = wild boar with (a) and without (b) dexamethasone treatment or (b_c) untreated control pig, 2 = domestic pigs with (a) and without (b) dexamethasone treatment. Faecal samples were collected before (0 dpi) and after (from 21 to 36 dpi) HEV infection. Dendrogram (Dice coefficient, UPGMA) embracing 123 individual clones. Black arrows mark identical *E. coli* clones across groups; grey arrows mark identical *E. coli* clones within groups.

domestic pigs by the faecal-oral route. Transmission was confirmed for all animals (data not further shown). Viral loads (viral RNA) in serum, faeces, bile, and different tissues were comparable among the inoculated groups, and liver and bile samples of all wild boar and almost all domestic pigs tested positive for HEV RNA (Schlosser et al., submitted for publication). Sequence analyses of the nearly full-length HEV genomes in different groups revealed high overall variability (synonymous and non-synonymous substitutions, small deletions) that was however not consistent with group allocation and transmission direction (data not shown).

4 Discussion

Direct and indirect contacts between domestic pigs and wild boar present a risk of pathogen spill-over (Meng et al., 2009) and can lead to long-term perpetuation of infection (Artois et al., 2002). The growing population of wild boar across Europe (Massei et al., 2015), and the increased trend to keep domestic pigs outdoors facilitates these contacts (Wu et al., 2012). Examples are classical swine fever, where contacts with infected wild boar were held responsible for almost two thirds of primary infections in German domestic pigs (Fritzemeier et al., 2000), and African swine fever (Food and Agriculture Organization. United Nations, 2013), where cohabitation of wild boar and free-ranging domestic pigs was shown to be crucial for virus maintenance on Sardinia (Laddomada et al., 1994). To understand extent and dynamics of domestic pig / wild boar interactions, biological indicators could be a most useful tool. In the presented study, HEV and faecal *E. coli* were assessed for their biological indicator potential in an experimental pilot trial.

With regard to HEV, transmission from wild boar to domestic pigs through faecal contact was confirmed. Thus, HEV transmission could be a potential biological indicator for pig contacts

provided that the domestic pig population is HEV free prior to contact with infected wild boar, and that other HEV sources such as newly introduced animals or environmental contamination do not play a role. Given the fact, that HEV (genotype 3) is wide-spread among domestic pigs and Eurasian wild boar, the applicability of this approach might however be limited. In the presented study, genetic pattern upon transmission were investigated as a second aspect that could help to trace transmission dynamics. Here, whole-genome sequencing revealed a high degree of overall variability that was however not clearly linked to groups or the direction of transmission. Thus, genetic variability in itself may not be the primary target as biological indicator. Field approaches are currently underway that will address the question of practicability and suitability.

Credible biological indicators for monitoring contacts between members of different host species require a host range overlapping in the host species of interest. *E. coli* bacteria dominate the aerobe microflora in the gastrointestinal tract of several vertebrates (Schierack et al., 2007, Gordon and Cowling, 2003, Hartl and Dykhuizen, 1984) and have already been used as biological indicators of natural contacts between different host species (Pesapane et al., 2013, Rwego et al., 2008a, Rwego et al., 2008b). Under experimental conditions, indirect transmission of enterohemorrhagic *E. coli* O157:H7 between pigs, even when held in separated pens, occurs at high frequency (Cornick and VuKhac, 2008). Only little is known about transmission efficacy of commensal *E. coli* in groups of pigs in general and between wild boar and domesticated pigs in particular. Heterogeneity of *E. coli* clones in domestic pigs as well as in wild boar seems to be quite high. For this reason, other authors recommend to test three (Lidin-Janson et al., 1978), 2-14 (Döpfer et al., 2008) or even 28 (Schlager et al., 2002) isolates per sample to assess the coliform microflora's heterogeneity. Because those recommendations apply to other hosts (humans, cattle, sheep) and other aims (e.g., identification of specific *E. coli* pathotypes), we decided to analyse the majority of strains and

included 10 randomly picked *E. coli* isolates per sample according to the suggestion by Hartley et al. (1977). *E. coli* variability in domesticated pigs and wild boar was strikingly similar under the conditions of our study. By having isolated 3.6 different clones on average per faecal sample and considering that *E. coli* clones from colon and faeces are highly related (Dixit et al., 2004); our results match results by Schierack and colleagues. These authors detected up to 10 different clones (on average 4.7) in colon samples from pigs and up to 9 (on average 3.0) in colon samples from wild boar (Schierack et al., 2007, Schierack et al., 2009). These findings strongly argue in favour of the *E. coli* microbiome being heterogenic and conserved enough between individuals and within host species, respectively, to be utilized as biological indicator indicating contact and transmission between wild and domestic pigs.

Similar to published data, resident clones (same PFGE pattern in first and second sample) were found within one animal and/or within one pen but the majority of clones was detected only once. Detection of resident clones during experimental infections of piglets normally requires a high inoculation dose (10^7 to 10^{10} cfu/dose) to make faecal excretion traceable, e.g., over at least five weeks for the probiotic *E. coli* strain Nissle (Barth et al., 2009) or even two month for some *E. coli* pathotypes (STEC, ETEC, EPEC) (Booher et al., 2002). In the current study, the inoculation dose of a single strain is defined by the amount of faecal material and the (unspecified) number of bacteria belonging to a certain clone therein taken up by individual piglets but can be suspected to be much lower. This observation suggests that transmission of clones between groups by plain contact to faecal material was sufficient to initiate intestinal colonisation of domesticated pigs and wild boar qualifying the *E. coli* microbiome as robust biological indicator of former contact even if the contact event and, even more importantly, the transmission event did not occur immediately prior to sampling.

In order to unequivocally prove identity of individual *E. coli* isolates, a highly discriminating typing method is needed. The use of antibiotic resistances profiles, PCRs targeting repetitive elements (rep-PCRs) or multi-locus sequence typing (MLST) (Pesapane et al., 2013, Rwego et al., 2008b, Rwego et al., 2008a) failed to be as sensitive as PFGE analysis used in the current study (Jonas et al., 2003, McLellan et al., 2003). Although much more time and cost consuming than other methods, PFGE analysis' reproducibility is higher than that of other tests, e.g., rep-PCR (Foley et al., 2004). Consequently, PFGE tests are recommended for tracing back studies to identify the source of human infections with pathogenic *E. coli* strains (Laidler et al., 2013), environmental *E. coli* contamination (Jay et al., 2007, McLellan et al., 2003) or occurrence of single *E. coli* clones in bovine (Geue et al., 2010) or porcine (Schierack et al., 2009, Schierack et al., 2007) herds. Indeed, PFGE analyses of faecal *E. coli* showed promising results in this limited pilot trial as biological indicator for contacts between domestic pigs and wild boar. Further studies, including samples from pigs and wild boar under field conditions, are needed to estimate the robustness and applicability of the method as an indicator of direct and/or indirect interactions.

Conflict of interest

The authors declare that no conflicts of interest exist.

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