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Short communication

First screening for *Brachyspira hampsonii* in Swiss pigs applying a new high resolution melting assay

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ABSTRACT

A new High Resolution Melting (HRM) assay was developed for the rapid detection of *Brachyspira (B.) hampsonii*. *B. hampsonii* occurs in different European countries, however, until today it has not been encountered in Switzerland.

Four *B. hampsonii* reference strains were used to develop the HRM assay: *B. hampsonii* clade I ATCC BAA2463 and clade II ATCC BAA2464 strain, as well as two isolated strains P280/1 from the UK and the German isolate 5369-1x/12. A conserved region of the *nox* gene was used to design *B. hampsonii*-specific primers. The HRM melting curves for the four reference strains showed reproducible difference graphs with distinct differences between the four strains based on a slight variation between the four amplicon sequences. In addition, DNA from 22 *B. hampsonii* strains representing four genetic *B. hampsonii* groups was used to validate the method. Melting temperatures in the interval between 73.1 and 74 °C were obtained for all *B. hampsonii* strains and allow differentiating *B. hampsonii* from other *Brachyspira* species.

In total 897 Swiss porcine fecal *Brachyspira* isolates, cultured between 2009 and 2015, were analysed by the HRM protocol. *B. hampsonii* was not detected among these Swiss *Brachyspira* isolates. In conclusion, the rapid and low-cost HRM approach allows a sensitive and specific identification of *B. hampsonii*.

1. Introduction

In the beginning of the 2010s new outbreaks of bloody diarrhea in pigs with swine dysentery (SD) similar clinic, associated with strongly beta-hemolytic *Brachyspira,* were reported in North America (Chander et al., 2012). However, the samples were negative in *Brachyspira* species-specific PCR analysis for *Brachyspira* (*B.*) *hyodysenteriae,* the causative agent for SD. Through sequencing of *nox* and 16S rDNA genes these newly discovered isolates were shown to be distinct from all other *Brachyspira* spp. and were given the provisional name *B. hampsonii*. This novel species is differentiated from other *Brachyspira* spp. by *nox* and 16S rDNA gene sequencing. On the basis of the *nox* gene, *B. hampsonii* shares 83–93% homology with all other known *Brachyspira* spp., whereas the homology within the 16S rRNA gene comes up to 96–99.4% (Chander et al., 2012). Although being genetically different from *B. hyodysenteriae,* clinical studies of experimentally infected pigs with *B. hampsonii*, both clade I and II, showed to give rise to clinical symptoms indistinguishable from those of SD (Burrough et al., 2012; Rubin et al., 2013a).

To date, *B. hampsonii* has not only been isolated from pigs and lesser snow geese in Canada and USA (Chander et al., 2012; Rubin et al., 2013b), it has also been reported in commercial pigs and migratory wild birds in Europe (Mahu et al., 2014; Martinez-Lobo et al., 2013; Rohde et al., 2014). The fact that *B. hampsonii* has been found in wild birds greatly increases the risk of spreading the agent between countries. Switzerland was free from SD until 2008 when the first cases of *B. hyodysenteriae* infected pigs were reported (Speiser et al., 2011). Since then the agent spread throughout Switzerland and currently *B. hyodysenteriae* is diagnosed in nearly 2% of all pig herds (Borgström et al., 2016; Figi et al., 2014).

To analyse the potential spreading of *B. hampsonii* in Switzerland a new High Resolution Melting (HRM) assay was developed for screening of *B. hampsonii* in large sample groups. All *Brachyspira* isolates screened in this study were previously analysed with a multiplex real-time PCR (Borgström et al., 2016) and the species distribution was determined (Table 1). Testing the *B. hampsonii* reference strains with the multiplex real-time PCR gave a clear signal for the apathogenic *Brachyspira* spp. triplet (*B. intermedia, B. innocence* or *B. murdochii*) for strains clade I ATCC BAA2463, German isolate 5369-1x/12 and isolate P280/1 from UK

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Table 1
All 897 porcine fecal *Brachyspira* isolates were analysed with a *Brachyspira* multiplex real-time PCR for species specification prior to the *B. hampsonii* screening. Apathogen = *B. murdochii, B. innocens* or *B. intermedia.*

Screened *Brachyspira* isolates (%)	
B. hyodysenteriae	131 (14.6%)
B. pilosicoli	97 (10.8%)
apathogen	385 (42.9%)
mixes	
B. hyodysenteriae + apathogen	85 (9.5%)
B. pilosicoli + apathogen	161 (18%)
B. pilosicoli + *B. hyodysenteriae*	20 (2.2%)
B. hyodysenteriae + *B. pilosicoli* + apathogen	6 (0.7%)
negative	12 (1.3%)
total isolates	897

as a result of the high similarity of the 23S rDNA between *B. hampsonii* and the apathogenic *Brachyspira* spp. triplet. However, clade II ATCC BAA2464 resulted in a signal for *B. hyodysenteriae*.

In this study 897 porcine fecal *Brachyspira* isolates sampled between 2009 and 2015 were analysed for *B. hampsonii* with a novel HRM assay.

2. Material and methods

2.1. Bacterial reference strains and isolates

Four *Brachyspira* reference strains were used for assay development and as positive controls in each experiment. Two strains were obtained from the ATTC strain collection: *B. hampsonii* clade I ATCC BAA2463 and *B. hampsonii* clade II ATCC BAA2464. P280/1, a UK isolate from the 1980s was kindly provided by David Hampson at Murdoch University, Australia, whereas the German *B. hampsonii* isolate 5369-1x/12 was contributed by Judith Rhode at the University of Veterinary Medicine Hannover, Foundation.

Additional 22 *B. hampsonii* strains, representing all four genetic groups (Mirajkar et al., 2015), were kindly provided by several research groups in USA, Spain and Canada (Supplementary Table 1). A total of 897 procine fecal *Brachyspira* isolates, originated from rectal swabs of diseased or healthy animals, collected during a Swiss SD monitoring programme between August 2009 and September 2015, were included in this study. All 897 isolates were analysed with a species specific multiplex real-time PCR before the study begin to make sure that the samples displayed a good and diverse distribution of *Brachyspira* spp. (Table 1, (Borgström et al., 2016)). This multiplex real-time PCR is aiming at the target gene 23S rDNA with three specific probes identifying the following species*: B. hyodysenteriae, B. pilosicoli* and the so- called "apathogen" probe detecting *B. murdochii, B. innocens* and *B. intermedia.*

2.2. Bacterial culture

Rectal swabs in Amies medium were collected from 897 feeder pigs on 375 farms from 18 different Swiss Cantons. Isolates from the fecal swabs were cultured anaerobically for *Brachyspira* growth as described elsewhere (Dünser et al., 1997; Prohaska et al., 2014). Areas of weak or strong beta-hemolysis on TSA plates (trypticase soy agar supplemented with 5% cattle blood and colistin, vancomycin, spectinomycin, spiramycin and rifampicin) were examined for spirochetal growth by dark-field microscopy of surface scrapings resuspended in a small volume of 0.15 M NaCl. Spirochetal isolates were subcultured on Colombia sheep blood agar plates (Oxoid, Basel, Switzerland). For DNA analyses, spirochetes were harvested with 1 ml distilled water by scraping the bacteria lawn from the agar surface with the aid of a drigalski spatula.

2.3. DNA extraction

DNA was extracted from fecal isolates with InstaGene Matrix (Bio Rad, Cressier, Switzerland) according to the manufacturer's instructions. The DNA concentrations were measured by reading the absorbance at 260 nm using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Reinach, Switzerland) and diluted to a concentration of 20 pg/μl DNA.

2.4. HRM

A primer pair was designed to specifically bind a conserved region for *B. hampsonii* on the *nox* gene. The forward 5′-CTCCTCCTATAGAAGGATTAAAACAGGA-3′ and reverse 5′-TTCAT-TAATAATATCCTGTCCTTGTTGGAA-3′ primers flank a 104 base pair (bp) amplicon. The chosen primer region is conserved for *B. hampsonii* strains of all four genetic groups with maximally one point mutation. In contrast, other *Brachyspira* family members have a greater variability in the primer region as well as in the whole 104 bp amplicon (Fig. 1).

The assay was set up with the aid of QIAgility (Qiagen, Hilden, Germany), an automated and rapid pipetting system in a 100-sample-format using a Rotor-Disc 100 (Qiagen). The real-time PCR assay was performed on Rotor-Gene 6000 system (Qiagen) with the Type-it HRM PCR Kit (Qiagen). 2 μl bacterial genomic DNA template (20 pg/μl) was added to a reaction mixture consisting of 7.5 μl 2× Type-it HRM Master Mix (Qiagen) containing EvaGreen DNA-binding dye, 0.7 μM final concentration of each primer (Microsynth AG, Switzerland), and ultrapure water to a final volume of 15 μl. The PCR thermocycling parameters were as follows: initial denaturation at 95 °C for 5 min, 40 cycles with denaturation at 95 °C for 10 s and annealing/extension at 55 °C for 30 s, followed by HRM ramping from 67 to 80 °C. Fluorescence data was acquired at 0.1 °C increments every 2 s in order to generate specific melting curves. For each experiment, the four reference strains were included as melting curve standards and positive controls. To exclude contaminations in the reaction mixture, a no template control was added to each experiment. Data analysis was performed using the Rotor-Gene Q Software 2.3.1 (Qiagen). Some *Brachyspira* spp. isolates were sequenced on the *nox* gene (Rohde et al., 2002).

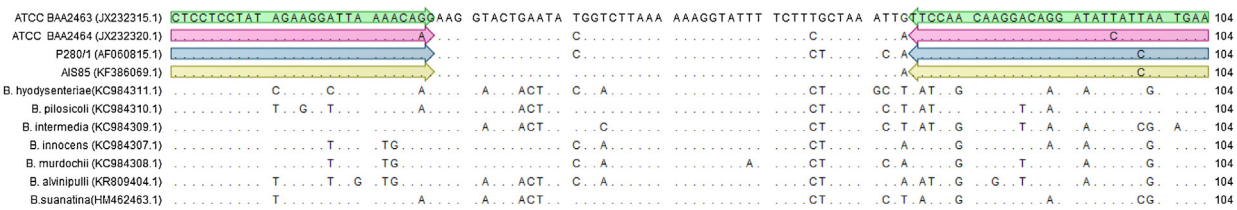

Fig. 1. Sequence alignment of the amplicon generated by the real-time PCR of the HRM. Primer regions of *B. hampsonii* isolates are indicated with colored arrows and matching residues are marked as dots. Each color represents a different genetic group of *B. hampsonii*: green for genetic group I, pink for genetic group II, blue for genetic group III and yellow for genetic group IV. *B. hampsonii* primer regions are conserved except for single point mutations. Accession numbers of GenBank are added in parenthesis.