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Identification of *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* and *Brachyspira intermedia* in commercial laying hens and commercial broiler breeders using fluorescence *in situ* hybridization (FISH) in paraffin-embedded tissues

Leonardo Gruchouskei[1] Ronaldo José Piccoli[2] Jessica Santos Streauslin[3] João Pedro Cavasin[4] Mônica Regina de Matos[5] Mayane Faccin[6] Aline de Marco Viott[7*]

[1]Curso de Medicina Veterinária, Universidade Federal da Fronteira Sul (UFFS), Realeza, PR, Brasil.
[2]Curso de Medicina Veterinária, Universidade Federal de Santa Catarina (UFSC), Curitibanos, SC, Brasil.
[3]Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, United States of America.
[4]Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, TX, United States of America.
[5]Universidade Estadual do Oeste do Paraná (Unioeste), Toledo, PR, Brasil.
[6]Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, United States of America.
[7]Curso de Medicina Veterinária, Universidade Federal do Paraná (UFPR), 85450-000, Palotina, PR, Brasil. E-mail: viott@ufpr.br.
*Corresponding author.

ABSTRACT: Bacteria of the genus *Brachyspira* can cause enteric diseases in birds; thus, this study evaluated the efficacy of the fluorescence *in situ* hybridization (FISH) technique for the identification of *B. pilosicoli*, *B. hyodysenteriae* and *B. intermedia* using cecum samples fixed in formaldehyde from laying hens and commercial broiler breeders. Samples were collected from 112 birds aged between 35 and 82 weeks that originated from commercial laying and broilers farms. For the initial evaluation, spirochaetes were isolated from the cloacal swabs. Positive samples were analysed using qPCR to identify pathogenic species. Formalized cecum segments of these same birds were then analyzed using the FISH technique with labelled probes specific to *B. pilosicoli*, *B. hyodysenteriae* and *B. intermedia*. Forty isolates characteristic of *Brachyspira* were obtained, of which 14 were identified as *B. hyodysenteriae* and seven were identified as *B. intermedia* by qPCR; two samples were positive for both species, and 21 were not characterized. Using the FISH technique, 52 samples were positive for *Brachyspira* spp., 22 were positive for *B. hyodysenteriae*, 28 were positive for *B. intermedia*, seven were positive for *B. pilosicoli*, and eight were positive for two species. The FISH technique was able to identify significantly more positive birds compared with bacterial isolation followed by qPCR. Thus, it is concluded that the FISH technique was effective for identifying the three *Brachyspira* species evaluated and can thus be used as a rapid and effective diagnostic tool.
Key words: avian intestinal spirochaetosis, broiler breeders, enteropathogens, Fluorescence *in situ* hybridization, laying hens.

Identificação de *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* e *Brachyspira intermedia* em aves de postura e matrizes comerciais pela técnica de hibridização fluorescente *in situ* (FISH) em tecidos incluídos em parafina

RESUMO: Bactérias do gênero Brachyspira podem ocasionar enfermidades entéricas em aves, assim o objetivo deste estudo foi avaliar a eficácia da técnica de hibridização fluorescente in situ (FISH) para a identificação de *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* e *Brachyspira intermedia* utilizando amostras de ceco de aves de postura e matrizes comerciais fixados em formol. Foram coletadas amostras de 112 aves entre granjas de postura comercial e granjas de matrizes de corte com idade entre 35 e 82 semanas. Para avaliação, primeiramente procedeu-se ao isolamento bacteriano a partir das fezes. As amostras positivas foram submetidas a qPCR para identificação de espécies patogênicas. Posteriormente, segmentos formolizados de ceco dessas mesmas aves, foram processados pela técnica de FISH utilizando sondas marcadas de *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* e *Brachyspira intermedia*. Das 112 amostras foram obtidos 40 isolamentos característicos de *Brachyspira* e destes, na qPCR, 14 identificadas como *B. hyodysenteriae* e sete como *B. intermedia*, duas amostras foram positivas para ambas e 21 não foram caracterizadas. Na técnica de FISH, 52 amostras foram positivas para *Brachyspira* sp., 22 para *B. hyodysenteriae*, 28 para *B. intermedia*, sete para *B. pilosicoli* e oito amostras foram positivas para duas espécies. A avaliação estatística revelou que a técnica de FISH foi capaz de indicar maior número de aves positivas quando comparada ao isolamento bacteriano seguido por qPCR, inclusive revelando aves positivas para *B. pilosicoli* que não tinham sido identificados anteriormente. Dessa forma, conclui-se que a técnica de FISH apresentou-se eficiente para identificação das *Brachyspiras* avaliadas, servindo assim como uma ferramenta rápida e eficiente para o diagnóstico.
Palavras-chave: espiroquetose intestinal aviária, matriz de corte, enteropatógenos, hibridização fluorescente *in situ*, aves de postura.

INTRODUCTION

Bacteria of the genus *Brachyspira* can cause enteric diseases, particularly in poultry and pigs. Four *Brachyspira* species are considered pathogenic to birds: *B. intermedia, B. pilosicoli*, *B. alvinipulli* and *B. hyodysenteriae*; one or more species may be concomitantly involved (SONG & HAMPSON,

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2009; MAPPLEY et al., 2014). The transmission of *Brachyspira* spp. between species has been observed among chickens, pigs and rodents, and all of these species can be colonized from a common environmental source; thus, rats and mice are potentially involved in the transmission of *B. pilosicoli* and *B. hyodysenteriae* by maintaining the bacteria between lots on the farms (BACKHANS et al., 2011).

In poultry, *Brachyspira* spp. are associated with a clinical condition known as avian intestinal spirochaetosis (AIS), which can cause chronic diarrhea, weight loss, low egg production and eggs dirty with faecal matter (MEDHANIE et al., 2013). The chickens can present lymphoplasmacytic typhlitis, crypt hyperplasia, epithelial erosion and an increased number of goblet cells (SHIVAPRASAD & DUHAMEL, 2005; FEBERWEE et al., 2008). The main causal agents of AIS are *B. intermedia* and *B. pilosicoli* (STEPHENS & HAMPSON, 1999; BANO et al., 2008) and, less frequently, *B. alvinipulli* (PHILLIPS et al., 2006; FEBERWEE et al., 2008).

The rate of colonization by *Brachyspira* spp. increases significantly as flock age increases, suggesting that after an initial infection, the bacterial population increases over time. Thus, routine tests for early detection could be used for diagnosis and the necessary measures for control of the agent on farms could be taken to minimize future economic losses (PHILLIPS et al., 2005; MEDHANIE et al., 2013).

Several poultry-producing countries have reported the occurrence of *Brachyspira* spp. on farms. In Italy, the incidence of *Brachyspira* was 72.4% on laying farms, and 31% of these samples were pathogenic (BANO et al., 2008). In Australia, the prevalence was 42% in broilers and 68% in laying hens (STEPHENS & HAMPSON, 1999), whereas in Argentina, positivity rates of 50 to 65% were recently found on the laying farms evaluated (ILLANES et al., 2016).

The traditional laboratory diagnosis of *Brachyspira* spp. is performed through selective anaerobic culture and biochemical tests. However, these methods are labour intensive and time consuming, and it may take up to 40 days to obtain results; in addition, species identification using biochemical parameters may be hampered by the weak growth of pure colonies or by accompanying microbiota (BOYE et al., 1998; RÅSBÄCK et al., 2006). In some cases, it is not possible to identify the species in positive samples (ILLANES et al., 2016). Thus, alternative methods for *Brachyspira* spp. diagnosis have been developed and tested (BOYE et al., 1998; JENSEN et al., 2000; PHILLIPS et al., 2005; PHILLIPS et al., 2006; RÅSBÄCK et al., 2006; SCHMIEDEL et al., 2009; SONG & HAMPSON, 2009; JENSEN et al., 2010; BURROUGH et al., 2013; WILBERTS et al., 2015).

The fluorescence *in situ* hybridization (FISH) technique is rapid and sensitive and allows the identification and localization of the targeted agent. In addition to identifying species, FISH allows an evaluation of the number and morphology of bacterial cells using tissues fixed in formaldehyde. This technique detects the presence of a specific cell among 10^6 non-target cells. The FISH technique detects nucleic acid sequences using a probe labelled with a fluorescent agent that specifically hybridizes with its complementary target sequence within the intact cell. In microbiology, the more commonly used target regions are the 16S rRNA gene portions because of their conservation and genetic stability. The tested probes are produced from unique sequences that allow the identification of a specific genus or species or even identification at the intraspecific level (AMANN et al., 1995; BOYE et al., 1998; MOTER & GÖBEL, 2000; SCHMIEDEL et al., 2009).

The detection levels of *Brachyspira* spp. in feces are similar when comparing qPCR using fresh feces and FISH using formaldehyde-fixed feces. The FISH technique failed when using fresh feces because the formaldehyde used for fixation prevented the degradation of the nucleic acids targeted by the technique; in turn, the qPCR test, when performed with samples fixed in formaldehyde, was rarely positive because the formaldehyde inhibited the PCR. Thus, formaldehyde-fixed samples are more suitable for use in FISH (WILBERTS et al., 2015).

This study evaluated the efficacy of the FISH technique in formaldehyde-fixed cecum samples from commercial laying hens for the detection of *B. pilosicoli, B. intermedia* and *B. hyodysenteriae*, in comparison with cultures of cloacal swabs with subsequent species confirmation through qPCR.

MATERIALS AND METHODS

Samples

A total of 112 samples were collected, of which 42 were from commercial laying farms and 70 were from broiler breeder farms. The age of the chickens ranged from 35 to 82 weeks. Cloacal swabs were collected from birds chosen randomly within the poultry house and included birds with and without diarrhea or a history of egg drop syndrome. The collected swabs were immediately stored in an insulated box with ice and sent to the laboratory for bacterial isolation. Birds which cloacal swabs were

collected were euthanized and necropsied. Cecum fragments were collected and fixed in 10% buffered formaldehyde, and subsequently processed by the routine paraffin embedding technique.

Bacterial isolation

The protocol for bacterial isolation followed the recommendations by SATO (2022). Samples were seeded in a selective medium for *Brachyspira* spp. composed of Anaerobiosis agar (Neogen Co, MI, USA), 5% sheep blood, 6.25mg/µl rifampicin (Sigma-Aldrich Co, MO, USA), 800mg/µl of spectinomycin (Sigma-Aldrich Co, MO, USA), 25mg/µl of vancomycin (Sigma-Aldrich Co, MO, USA), 25mg/µl of colistin (Sigma-Aldrich Co, MO, USA). They were incubated in anaerobiosis jar with an anaerobic atmosphere generated with anaerobiosis media (Anaerobac®, Probac of Brazil, São Paulo, Brazil) at 42°C for three days or until evidence of hemolysis. The anaerobic environment was confirmed by an anaerobiosis indicator strip (Oxoid Anaerobic Indicator®, Thermo Fisher, MA, USA). Growth was considered positive when areas of strong and weak plaque hemolysis were evidenced; it was sometimes accompanied by white millimeter colonies, suggesting *Brachyspira* sp. These areas and/or colonies were carefully picked using a calibrated loop on anaerobic isolation agar plates (Neogen Co, MI, USA) containing 5% sheep blood and incubated anaerobically for three days at 37°C. The colonies were collected with a calibrated loop and resuspended in cryotubes containing 1.5mL foetal bovine serum and held at -20°C for subsequent phenotyping by qPCR.

qPCR (real-time polymerase chain reaction)

The samples were analysed in a private laboratory. For the amplification of *B. hyodysenteriae*, the primer pair H1 (5’-ACTAAAGATCCTGATGTATTTG-3’) and H2 (5’-CTAATAAACGTCTGCTGC-3’) was utilized, targeting a 354-bp region of the NADH oxidase (nox) gene, the primer pair P1 (5’-AGAGGAAAGTTTTTTCGCTTC-3’) and P2 (5’-GCACCTATGTTAAACGTCCTTG-3’) was used for *B. pilosicoli*, focusing on an 823-bp region of the 16S rDNA, the primers employed by LA et al. (2003). For *B. intermedia*, the primer pair Int1 (5’-AGAGTTTGATGATAATTATGAC-3’) and Int2 (5’-ATAAACATCAGGATCTTTGC-3’), targeting a 567-bp region from base position 517 to 1083 on the NADH oxidase (nox) gene, was employed based on the sequence described by PHILLIPS et al. (2005). The protocol recommended by SONG & HAMPSON (2009) was used.

Fluorescent in situ hybridization (FISH)

To perform the FISH technique, four probes were used that were individually tested in each sample; one probe was specific for the genus *Brachyspira*, and the other three were commercially synthesized species-specific probes (Invitrogen). The sequences used were those described by SCHMIEDEL et al. (2009) for the *Brachyspira* spp. 16S rRNA target region (ATTAGTCCATGTTTCCAT); by BOYE et al. (1998) for the *B. hyodysenteriae* 23S rRNA region (CTCACGATGAACCTTCGAC) and the *B. pilosicoli* 16S rRNA region (GCTCATCGTGAAGCGAAA); and by PHILLIPS et al. (2006) for the *B. intermedia nox* gene region (ATAAACATCAGGATCTTTGC). All probes were labelled with Alexa Fluor 555 (Thermo Fisher) and purified by HPLC. Histological sections (5 µm) from silanized slides were deparaffinized in two xylene baths for 10 minutes each and then rehydrated for 5 minutes in each of the following solutions: absolute ethanol, 90% ethanol, 80% ethanol, 70% ethanol and distilled water. The slides were dried at room temperature and then mounted on coverplates (Thermo Fisher, cat # 72110017, Waltham, MA, USA) and placed in a hybridization chamber (coverplate slide rack, Thermo Fisher, cat # 73310017, Waltham, MA, USA). Each slide was incubated with 99 µl of hybridization buffer (100 mM Tris, pH 7.2; 0.9 M NaCl; 0.1% sodium dodecyl sulfate) and 1 µl of a solution containing 100 ng of probe at 45°C for 16 hours. After this period, the slides were washed three times with hybridization buffer pre-heated to 45°C, washed three times with a washing solution pre-heated to 45°C (100 mM Tris, pH 7.2 and 0.9 M NaCl) and finally washed in ultrapure water for 2 minutes and dried in an oven at 45°C. The slides were mounted using the mounting medium for fluorescence microscopy (Fluoroshield, Sigma-Aldrich, F6182) and were immediately read under a fluorescence microscope (Olympus, FSX 100, Japan) with a filter suitable for the fluorochrome at 40x magnification.

Statistical analysis

The results were compared for four groups: bacterial isolation against the FISH *Brachyspira* spp. technique and the specific qPCR and FISH analyses for the three species (*B. hyodysenteriae, B. intermedia* and *B. pilosicoli*) using positive samples from the bacterial isolation step. A chi-squared test was performed with a significance level of $P < 0.05$ using SAS 9.0 software.

RESULTS AND DISCUSSION

A comparison between FISH and qPCR using the positive samples from the bacterial isolation is shown in table 1. The results showed that the FISH technique was statistically superior for the identification of the species *B. pilosicoli* and *B. intermedia*; there was no significant difference between the techniques for *B. hyodysenteriae* and *Brachyspira* spp.

Among the samples analysed by isolation/ qPCR, two chickens had mixed infections, and it was not possible to identify which of the three species was present in 21 individuals. Among the pathogenic species described in birds, the frequency of *B. alvinipulli* in the field is low with few isolations or outbreaks reported in the literature (PHILLIPS et al., 2005; FEBERWEE et al., 2008), and thus, it was not a target species in this study. Nevertheless, a large number of apathogenic *Brachyspira* species have been identified in the intestines of birds, including *B. innocens, B. murdochii* and *B. pulli* (FEBERWEE et al., 2008), which explains the presence of non-characterized samples in this study.

The four FISH probes were individually tested on each of the 112 samples, and the results were as follows: 52 samples were positive for the probe specific to *Brachyspira* spp, 22 samples were positive for the probe specific for *B. hyodysenteriae*, seven samples were positive for the probe specific for *B. pilosicoli*, and 28 samples were positive for the probe specific for *B. intermedia*. Forty-four samples were negative for all probes, and 11 samples were positive only for the probe specific for *Brachyspira* spp. Among the samples with mixed infections, five contained *B. hyodysenteriae* and *B. intermedia,* one contained *B. intermedia* and *B. pilosicoli*, and two contained *B. hyodysenteriae* and *B. pilosicoli*. HESS et al. (2017) reported that chickens can become infected with different *Brachyspira* species during their lifetime, with the species changing over time. This occurs as a result of the introduction of different bacterial species to the environment, leading to the continuous reinfection of the chickens, where one strain does not interfere with heterologous infection.

The number of cases identified by FISH with the probe for *Brachyspira* spp. (52 samples) was two more than those identified by bacterial isolation (50 samples). However, there was no significant difference between the two techniques. This difference may be because the number of bacteria present in the sample can limit bacterial detection in culture (RÅSBÄCK et al., 2006; MAPPLEY et al., 2014). This limitation was also observed when using the qPCR technique alone. According to PHILIPS et al. (2006), the limit of detection for the D-PCR technique on DNA extracted from seeded washed chicken feces is 10^6 cells/g of feces for both *B. pilosicoli* and *B. intermedia*. When bacterial isolation is combined with 2S-N-D-PCR, the limit decreases to 10^3 cells/g for both species. For the fecal culture, the detection limits, as reported by RÅSBÄCK et al. (2006), were 10^3 CFU/ml of pig feces for *B. hyodysenteriae* and 10^2 CFU/ml of pig feces for *B. pilosicoli*.

Bacterial isolation can be influenced by the medium used, and the implementation of pre-treatment steps can assist in the recovery of the agent. The use of spiramycin and vancomycin in the culture medium has a negative impact, inhibiting the growth of *B. pilosicoli* and, to a lesser extent, *B. hyodysenteriae* (CALDERARO et al., 2005). The lack of pre-treatment and the use of a medium containing vancomycin may have compromised the isolation in the present study.

Low numbers of bacterial cells can be maintained in the intestines of chickens without the occurrence of AIS, which may manifest after the birds experience a stressful situation (MAPPLEY et

Table 1 - Comparison of species identification of *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia* using FISH in formalin fixed and paraffin block sections of cecum and qPCR in culture samples positive for *Brachyspira* spp. from commercial laying and broiler farms (N = 40).

FISH*	---------qPCR**---------	-------*B. hyodysenteriae*------	------*B. pilosicoli*-----	------*B. intermedia*-----
Positive	Positive	10	0	6
Positive	Negative	3[a]	4[a]	9[a]
Negative	Positive	4[a]	0[b]	1[b]
Negative	Negative	23	36	24

*Fluorescent *in situ* hybridization; **qPCR, Real-time Polymerase Chain Reaction. Different lowercase letters in the columns indicate significant differences based on the chi-squared test ($P < 0.05$).

al., 2012 and 2014). As the chickens in this study did not show clinical signs of AIS, the number of excreted bacteria could be below the limit of detection of the culture method; however, the FISH technique is able to detect even a single bacterial cell, provided that it is present in the histological section visualized (BOYE et al., 1998).

The FISH-positive samples showed a strong bright signal with typical spirochaete shape, this signal was identified (Figure 1) and stood out from the autofluorescence of the tissue. The same results were observed by ROJAS et al. (2017), who performed FISH using a probe for the genus *Brachyspira* in human intestine samples. However, BOYE et al. (1998) had difficulty visualizing *B. pilosicoli* when performing FISH on pig intestines due to the weak signal of the probe in this species. To circumvent this difficulty when the identification was questionable, the filter was replaced with a non-specific filter for the tested probe, thus preventing the probe from emitting its signal. The seven cases positive for *B. pilosicoli* in this study were not obfuscated, nor did they have a weak signal.

Colonization by *B. intermedia* can lead to losses in egg production even in cases where macroscopic or microscopic lesions are not observed in the digestive system. In these cases, the damage is associated with the presence of spirochaetes in the lumen of the crypts. Thus, the diagnosis of AIS caused by *B. intermedia* depends on complementary methods that allow for the identification of this agent (STEPHENS & HAMPSON, 2002; HESS et al., 2017). The FISH technique was effective for the detection of *B. intermedia*, and it can be used for screening farms where the manifestation of AIS is mild.

Intestinal colonization by *B. hyodysenteriae* in pigs can cause severe diarrhea, referred to as swine dysentery, which can result in high economic impact (BOYE et al., 1998). It has already been documented that rodents present on farms can carry *Brachyspira.* spp. (BACKHANS et al., 2011). Thus, positive samples for *B. hyodysenteriae* should serve as a red flag in regions where agribusiness is prevalent due to the proximity of pig and poultry farms, because chickens can serve as a source of infection for pigs.

Despite the high efficacy of FISH, caution should be taken regarding the use of molecular diagnostic methods alone. We must note that possible genetic mutations or even some limitations in the sequence database with respect to *Brachyspir*a spp. could cause positive cases to go undetected by both FISH and qPCR. Thus, bacterial isolation is still a useful tool in some cases, particularly when subsequently testing for bacterial resistance (RÅSBÄCK et al., 2006; ROJAS et al., 2017).

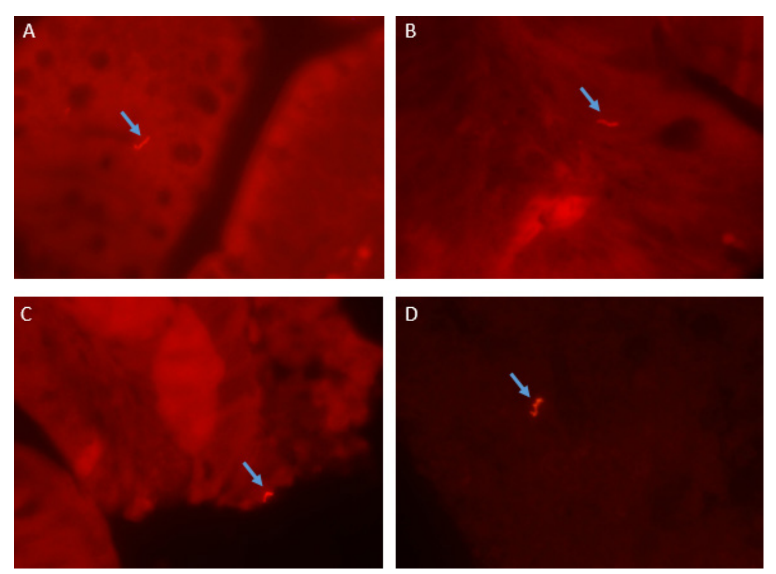

Figure 1 - Photomicrograph with positive labelling for *Brachyspira* in chicken cecum samples using the FISH technique. (A) Specific labelling for *Brachyspira* spp. (arrow); (B) specific labelling for *B. hyodysenteriae* (arrow); (C) specific labelling for *B. pilosicoli* (arrow); (D) specific labelling for *B. intermedia* (arrow).

CONCLUSION

The results showed that the FISH technique applied to formaldehyde-fixed cecum samples of chickens was effective for the identification of *Brachyspira* spp. (*B. intermedia, B pilosicoli* and *B. hyodysenteriae*) and can be used as a rapid tool for the diagnosis of the main AIS-causing *Brachyspira* species, thus reducing the time between sampling and diagnosis and allowing an earlier onset of therapeutic intervention.

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was approved by the Ethics Committee on Animal Use of the Palotina Sector of the Universidade Federal do Paraná (UFPR) under CEUA/Palotina protocol 06/2017.

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