Carriage of Brachyspira hyodysenteriae on common insect vectors

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# ABSTRACT

The interactions of likely insect and murine vectors of the causative agent of swine dysentery, Brachyspira hyodysenteriae, were investigated. Insects were collected and analysed from 3 pig farms positive for *B hyodysenteriae*. Within these farms, several *Musca domestica* and Orphyra adult fly, Blatta sp cockroach digestive tracts and hover fly (Eristalis sp) pupal form contents were positive in a standard PCR assay for *B hyodysenteriae*, whereas all other insect samples on these and case control farms were negative. In challenge exposure studies, B hyodysenteriae DNA was detected in the digestive tract of cockroaches and M domestica flies from day 1 post-inoculation with cultured *B hyodysenteriae*, for up to 5 days or 10 days respectively, while control non-inoculated insects remained negative. Isolates consistent with B hyodysenteriae were only cultured from frass samples of these inoculated cockroach and flies on days 1 to 3 post-inoculation. Isolates consistent with B hyodysenteriae were detected by analysis of agar plates exposed to live *B hyodysenteriae*-inoculated adult flies wandering and feeding on these plates for 20 min per day. In generational challenge inoculation studies, *B hyodysenteriae* was detected in the adult emergent flies, and internal components of fly pupae on days 1 to 7 of the pupation period, after being inoculated with *B hyodysenteriae* as larvae. Five-week-old conventional mice (C3H) that consumed 2 meals of B hyodysenteriaeinfected flies remained negative for *B hyodysenteriae* throughout the next 10 days. The results indicated that pathogenic Brachyspira sp have a limited ability to internally colonise likely insect vectors and do not readily transmit infection to mice. However, the insect vectors analysed were demonstrably capable of mechanical carriage and likely on-farm involvement in consequence.

Keywords: pigs, Brachyspira hyodysenteriae, vectors, fly, cockroach, mouse

# 1. Introduction

The anaerobic spirochetal genus Brachyspira consists of nine recognised species, many of which cause important intestinal (hind-gut) diseases in pigs and chickens, such as swine dysentery due to *B hyodysenteriae* (Burrough, 2017). Molecular analysis has shown that each Brachyspira species has numerous genomic variants, with considerable "micro-evolution", not only due to mutation and recombination events, but importantly through transduction via pro-phage gene transfer agents (Motro et al., 2009). Consideration of the status of strains of each pathogenic Brachyspira species in the hind-gut of animals therefore also involves the presence or absence of key virulence factors, such as haemolysin, motility factors and viscotaxin (Joerling et al., 2018). On endemically infected pig and chicken farms, pathogenic Brachyspira species, particularly B hyodysenteriae and B pilosicoli, are often transmitted by oral-faecal transmission. Several animal vectors, particularly rodents and waterbirds, are capable of persistently harbouring pathogenic *Brachyspira* spp in an internal (intestinal) biological capacity, therefore contributing infected faeces and playing an important role in the maintenance and spread of the spirochetes within and between farms (Alvarez-Ordonez et al., 2013). Mice (except BALB/c strain) are also regarded as useful challenge exposure models of *B hyodysenteriae* infection in pigs (Nibbelink and Wannamuehler, 1991).

While certain common fly species (such as *Musca domestica* and *Eristalis* spp) are suspected to play a role in transmission of other bacterial pathogens in the environment of pig and chicken farms, including *Lawsonia intracellularis*, and enterotoxigenic *Escherichia coli* (Forster et al., 2007; McOrist et al., 2011), the possible contribution of insect vectors to the epidemiology of *Brachyspira* spp infection is not clear. There are two recognised forms of carriage of pathogenic microbial infections by insect (and other animate) vectors: mechanical carriage on external surfaces of the vector, or internal biological carriage, involving multiplication of the agent in the internal organs of the insect (Fukuda et al., 2020). For anaerobic hind-gut-associated bacteria, such as *Brachyspira* spp, demonstration of biological carriage implies the examination of the gut and frass of relevant insects. The digestive tract of insects, such as flies and cockroachs, consists of fore-gut, mid-gut and hind-gut in various configurations, with a consistent gut flora and basic immune system (Engel and Moran, 2013).

The aim of this study was to determine whether insects likely to be dominant on pig farms could act as mechanical or biological vectors for *Brachyspira hyodysenteriae* and whether transmission is feasible between likely vector groups, namely common insects and mice.

### 2. Methods

#### 2.1. Invertebrate collections and analysis for Brachyspira hyodysenteriae

Three pig farms (100 to 300 breeding females) in the United Kingdom known to be positive for *B hodysenteriae* (swine dysentery) and 10 case control farms were subjected to identical insect and pig sample processing. Invertebrate traps (aerial sticky traps and walk-in floor traps) were placed throughout the summer months of April to November. Within-farm trap locations at each site included feed storage areas, weaner and finisher pig areas and breeder pig areas. The number and identity of all invertebrates on these entire trap devices was annotated via standard taxonomic methods. In conjunction, fresh faeces samples were collected on 3 occasions from cross-sectional samples of 10 pigs from each age group (breeder, weaner, finisher) on each farm. Blood tests are not considered reliable for Brachyspira testing. DNA was extracted from each faecal sample by routine commercial kit methods (QIAamp® DNA Stool kit, Qiagen, Germany). To achieve adequate invertebrate DNA amounts, each sample extraction incorporated the whole body of 20 flies or the internal contents of each pupa or larva; or the dissected digestive tracts of 2 cockroaches. It was aimed to analyze the digestive tracts of these samples for carriage, rather than the outer surfaces. Each cockroach, pupa or larva form was surface-washed and sterilized for 10 minutes with 70% ethanol prior to their internal contents being dissected for DNA extraction. It was not feasible to surface-sterilize the body of adult flies, due to crushing in the trapping devices. Each internal content, digestive tract or fly sample was frozen with liquid nitrogen and crushed into a fine powder, then DNA extracted by routine commercial kit methods (QIAamp® DNeasy Blood and tissue kit). Each extracted pig faecal or insect-derived DNA sample was then analyzed by PCR, incorporating oligo-primers specific for the NADH gene of Brachyspira hyodysentyeriae DNA, as described previously (La et al., 2003). Control DNA derived from *B hyodysenteriae* ATCC strain 27164 was used as the positive control.

#### 2.2. Challenge exposure inoculations of insects with Brachyspira hyodysenteriae

Challenge cultures of pathogenic *Brachyspira hyodysenteriae* (isolates P4412/1 and P4418/1) were originally isolated from pigs affected with muco-haemorrhagic colitis, typical of swine dysentery. The isolates were identified as anaerobic, indole-positive, hippurate- and ribose-negative, diffusely spreading, strongly β-haemolytic colonies on blood agar. Isolates were resuscitated and incubated anaerobically at 42 °C for 5 days and propagated anaerobically in brain heart infusion (BHI) broth (Oxoid, UK), supplemented with 5.0 % rabbit serum. All anaerobic procedures were conducted in a purpose-built anaerobic handling facility (Bactron II Anaerobic Chamber system, Sheldon Inc, USA). Recent low-passage isolates were used for challenge exposure studies, in preference to stock collection strains, to preserve potential virulence determinants.

Adult Oriental cockroaches (Blatta orientalis) and house flies (Musca domestica) were housed in separate purpose-built colonies and fed sterile sugar-water sponge material. Groups of three of each insect were either left un-inoculated or starved of food and water for 48 h, then fed once either 100 µl or 3 µl respectively of fresh BHI broth containing each isolate of *B hyodysenteriae* ( $10^9$  per ml), then normal feeding behaviour resumed for 10 days. Inoculated and control cockroaches and flies were killed daily, externally sterilised, then prepared individually for the *B hyodysenteriae* PCR analysis, as described above. Insect frass was also collected daily from freshly-soiled filter papers at the base of each housing, daily for 10 days post-inoculation. The frass from each collection was inoculated onto Brachyspira selective medium (BSM) agar plates and incubated anaerobically at 42°C for 5 days; BSM was prepared in 0.5 L batches as described previously (Carranza et al., 2021), consisting of Columbia Agar Base No. 2 (Oxoid, U.K.) supplemented with 5.0 % sheep blood and 10 ml of a stock antibiotics preparation consisting of spectinomycin dihydrochloride, vancomycin hydrochloride, colistin methane sulphonate at 2.0, 0.125 and 0.125 g respectively, in 100 ml distilled water. Samples taken from any areas of apparent lysis within the test agar plates were analysed by the B hyodysenteriae PCR as described above. Control DNA derived from *B hyodysenteriae* ATCC strain 27164 was used as positive control.

# 2.3.Mechanical carriage and generational studies of insects inoculated with Brachyspira hyodysenteriae

Further groups of housed adult *Musca domestica* flies (n = 30) were starved for 12 h then fed 3 µl of the BHI broth inoculated with *B hyodysenteriae* (10<sup>9</sup> per ml), as described above. The following day and each day for 2 further days, individual flies were released onto fresh BSM agar plates and observed to wander and feed for 30 min before being removed. Fresh fly frass material was also collected daily from the inoculated flies and placed directly onto fresh BSM agar plates. These fly- or frass-exposed plates were then incubated anaerobically for 5 days and tested as described above. Remaining inoculated flies were also sampled and analysed daily, as described above.

Generational studies of bacterial carriage in flies were conducted following procedures described previously (Pava-Ripoll et al., 2015). In brief, second instar fly larvae (n = 20) of bronze bottle fly (*Lucilia cuprina*) were released for feeding onto BSM agar plates containing either lawns of *B hyodysenteriae* or an uninoculated surface for 24 h. The larvae were then removed, placed in a sterile dry area at 20°C and provided with the sterile food. The fed larvae proceeded to pupation (larval period 5 days, pupation period 7 days). At each day of pupal growth, and at adult fly emergence, sub-sets of the *B hyodysenteriae*-inoculated and control groups were removed. The pupae samples were surface-sterilised by immersion in 0.26 % sodium hypochlorite preceded and followed by washes in distilled water. The pupae samples were dissected and internal organs removed for testing. These larval and pupal organs and adult flies were processed for *B hyodysenteriae* by anaerobic culture on BSM agar plates and the *B hyodysenteriae* PCR method (on direct samples and BSM agar growth) as described above.

#### 2.4. Challenge inoculations of mice with insects and Brachyspira hyodysenteriae

Three further groups of housed adult *Musca domestica* flies (n = 30 per group) were starved for 24 h then fed twice 3 µl (24 h apart) with BHI broth containing challenge culture of the *Brachyspira hyodysenteriae* isolates (P4412/1 or P4418/1, 10<sup>9</sup> per ml) or left uninoculated, then normal feeding behaviour resumed, as described above. Entire adult fly

samples and frass materials were collected daily for 5 days post-inoculation and tested for *B hyodysenteriae* by anaerobic culture and PCR methods as described above.

Ten flies were collected at both days 4 and 5 post-inoculation and placed into mouse feeding bowls on each occasion to healthy 5 weeks-old outbred conventional male C3H mice (Charles River). The mice were housed in an animal house with controlled temperature and provided ad libitum feed and water throughout. The 3 groups of challenge- or controlexposed mice (7 mice per group) were thus exposed twice 24 h apart; all mice were observed to eat fully the fly meals offered. Further cohort groups of control positive and strict negative mice were challenged separately once orally with 0.25 ml of *B hyodysenteriae* isolate ATCC 27164 (10<sup>9</sup> per ml) culture in BHI broth or distilled water respectively. Faecal pellet samples ( $n \ge 3$ ) were collected from each mouse daily for 10 days post-challenge or control inoculation. The pellets were homogenised in 0.5 ml of normal saline and processed for *B hyodysenteriae* culture and PCR as described above for pig faecal samples.

# 3. Results

#### 3.1. Invertebrate collections and analysis for Brachyspira hyodysenteriae

On the 3 case study pig farms (#1, #2, #3), the pig-associated fly community was dominated by *Musca domestica* (house fly; farms#1, #2), except farm #3, where *Ophyra* sp (black garbage fly) was dominant. Also noted in lesser populations were *Muscina stabulans* (false stable fly), *Stomoxys calcitrans* (stable fly), and *Eristalis* species (hover flies); *Blatta orientalis* Oriental cockroach populations were only noted on one farm (#1).

On these 3 farms, 23 to 36 percent of faecal samples from 16-week-old and 25 week-old finisher and breeder pigs were positive in the PCR assay for *B hyodysenteriae*. One of 12 (8.3 %) *Musca domestica* adult fly samples (pools of 20 flies) from farms #1 and #2, 5 of 15 (33 %) cockroach digestive tracts (pools of 5) from farm #1, one of 7 (14 %) black garbage adult fly samples (pools of 20 flies) from farm #3, and 4 of 5 (80 %) hover fly pupal form contents from farm #2 were positive in the PCR assay for *B hyodysenteriae*. All other insect samples tested on case study and case control farms were negative in the PCR assay for *B hyodysenteriae*.

#### 3.2. Challenge exposure inoculations of insects with Brachyspira hyodysenteriae

*B hyodysenteriae* DNA was detected in the digestive tract of inoculated cockroaches and from whole *M domestica* flies each day from day 1 post-inoculation, from 1 up to 5 days or 10 days respectively, while the control non-inoculated insect samples remained negative throughout. Isolates consistent with *B hyodysenteriae* were cultured on BSM agar from samples of both cockroach and fly frass on days 1, 2 and 3 post-inoculation, but samples taken thereafter were negative. All these carriage results were identical for the 2 challenge isolates tested in parallel. Growth consistent with *B hyodysenteriae* was confirmed by PCR test results in positive-culture agar samples.

# 3.3. Mechanical carriage and generational studies of insects inoculated with Brachyspira hyodysenteriae

Entire inoculated flies remained positive for *B hyodysenteriae* PCR throughout the 5-day study period. Isolates consistent with *B hyodysenteriae* were detected by PCR and culture analysis of BSM agar plates exposed to the live *B hyodysenteriae*-inoculated adult flies wandering and feeding for 20 min per day, and from their frass collected daily. These PCR-positive isolates were noted on the agar plates of wandering flies or frass placed on day 1, 2 and 3 post-inoculation, but were negative thereafter. Control flies (not fed *B hyodysenteriae*) remained negative throughout.

Isolates and PCR assay were positive for *B hyodysenteriae* in the direct samples of internal components of pupae on days 1 through to day 7 of the pupation period (exposed to *B hyodysenteriae* as larvae). These assays were also positive in samples of contiguous adult flies, taken on day 1 after emergence. Control negative larvae, pupae (internal contents) and adult flies remained negative for *Brachyspira* spp culture and PCR.

#### 3.4. Challenge inoculations of mice with insects and Brachyspira hyodysenteriae

Adult fly and frass samples collected from all groups of flies challenge-inoculated with *B hyodysenteriae* isolates were positive at days 2 and 3 post-inoculation, while control negative flies remained negative.

All housed mice that consumed these *B hyodysenteriae*-infected flies remained negative for faecal *B hyodysenteriae* throughout the 10-day study period in repeated PCR and culture assays. Control positive mice inoculated with cultured *B hyodysenteriae* alone consistently developed shedding of *B hyodysenteriae* in their faeces starting 2 days after inoculation (50 to 70 % of faecal samples, collected daily for 10 days post-inoculation), while the control negative challenge (control negative flies) and strict control (distilled water) mice remained negative throughout. All the mice remained healthy during the challenge studies, with normal faecal consistency.

# 4. Discussion

We analysed on-farm insects and established that likely vectors such as domestic and garbage flies, hover fly larvae and cockroachs were capable of carriage of *B hyodysenteriae*, where this spirochete was widely present in the on-farm pig population. While numerous permutations of insect challenge/interactions were possible, we aimed to simply investigate possible mechanical and/or biological transmission processes among likely insect vectors for *B hyodysenteriae*. Limitations of our study included the unclear sensitivity of the detection methods used and the viability or otherwise of Brachyspiral contamination present. Our experimental challenge results indicated that inoculations of flies or cockroachs with *Brachyspira* sp led to a relatively limited ability to establish internal colonisation, with no clear ability to transmit infection to susceptible mice known to ingest infected insects. It could be extrapolated that this meant that pigs would not readily become infected by ingestion of *B hyodysenteriae*-infected insects. Nevertheless, the experimental studies also suggested that wandering flies were demonstrably capable of mechanical carriage with likely on-farm involvement in consequence.

In common with other pathogenic bacteria/fly generational studies (Pava-Ripoll et al., 2015), we found that *Brachyspira* sp was able to be transmitted across generations of fly stages (adults/larvae). The repeated experimental challenge studies we performed with

inoculation of adult flies or cockroachs with *B hyodysenteriae* led to demonstrable initial infections, confirmed by analysis of their frass and body parts. However, these infections appeared limited to several days only, which may suggest that the Brachyspira inoculum survived in the insect gut in a retention manner or with minimal reproduction. *B hyodysenteriae* is a relatively hardy spirochete capable of survival in contaminated farm surfaces and farm slurry for long periods (Boye et al., 2001). Detailed investigations of both fly and cockroach gut flora show an absence of spirochetes (Park et al., 2019; Schauer et al., 2012), suggesting some innate resistance or incompatibility for this class of bacteria within the bowels of insects. This contrasts with the known ability of rodent vector hind-guts to hold anaerobic spirochetes, such as *Brachyspira* spp, for extended periods (Joens and Kinyon, 1982; Songer et al., 1978). We therefore suggest that on-farm transmission of *Brachyspira* spp by insects may not be due to biological carriage and that rodents are more likely to actively hold the infection across or between farm pig or chicken livestock groups. Our study failed to clearly link the insect vectors and mice via the *B hyodysenteriae*-positive insect-challenged mice.

The studies of wandering flies and larvae infected with *B hyodysenteriae* suggested that mechanical carriage on their outer surfaces was likely for several days after exposure to the agent. We aimed to limit possible contamination of the test agar system by infected frass via a short 30-min surface exposure. This type of external mechanical carriage is well established for flies and cockroachs in contaminated environments (Bahrndorff et al., 2017, Moges et al., 2016). The flora carried upon the external surfaces, particularly the wings and legs, of insects is much more diverse than their internal gut flora (Junquiera et al., 2017). This type of insect carriage has been studied for numerous pathogenic bacteria, such as enterotoxigenic *Escherichia coli* (Kobayashi et al., 1999; Khamesipour et al., 2018), and whilst anaerobic *Brachyspira* sp survival may be limited in some aerobic cuticle environments, we confirm its relatively hardy nature in surface environments (Boye et al., 2001).

In terms of the relevance of these studies to the control and eradication of swine dysentery or avian intestinal spirochetosis, insect activity is greater in warm summer months, whereas Brachyspira infections are considered more active in cold winter months. The activity of insects as mechanical vectors of brachyspiral infections may therefore be self-limiting, if infections only last for several days. However, within this time frame, infected flies and larvae may be closely associated with pigs or chickens and can move considerable distances (Nazni et al., 2005; Rochon et al., 2004), creating a potential for spread of

pathogens to nearby farms or storage facilities. While the role of mice and their faeces in ongoing on-farm transmission of *Brachyspira* sp infection is well-documented, including direct mouse-into-pig oral inoculation studies (Joens 1980; Alvarez-Ordonez et al., 2013), our study failed to clearly link insect vectors and mice. It is therefore likely that both may act independently to cause transmission of *B hyodysenteriae* around livestock farms.

### **Declaration of Competing Interest**

The authors declare that they have no competing interests. All mouse studies were approved under the Institutional of Animal Care and Use Committee of the Universidad Nacional Autonoma de Mexico, México City, México.

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