Non-cultured faecal and gastrointestinal seed samples fail to detect Trichomonad infection in clinically and sub-clinically infected columbid birds

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TECHNICAL NOTE

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Compliance with Ethical Standards: Faecal samples were collected from birds captured and handled under licence from the British Trust for Ornithology (to JCD and RCT). Oral swabs were taken under licence from the Home Office.

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Abstract

Trichomonosis, caused by the protozoan *Trichomonas gallinae*, is an emerging infectious disease in finches, and is more commonly found in columbids and raptors. Infections can be sub-clinical or cause morbidity and mortality, but the parasite is currently only detectable by incubation of an oral swab. Here, we test whether *T. gallinae* parasites can be detected by PCR from faecal or non-cultured samples from the oral cavity and gastrointestinal tract of infected Turtle Doves (*Streptopelia turtur*). PCR did not detect *T. gallinae* parasites in any faecal samples screened, and in only 1 of 11 oral / gastrointestinal samples (from the mouth of a nestling suspected to have died from trichomonosis). We conclude that both oral swabs and parasite culture are still necessary to detect the sub-clinical presence of *T. gallinae* infection in birds.

Main article

Trichomonosis is an emerging infectious disease in finches (Aves: Fringillidae) within the UK and across Europe (Robinson et al. 2010; Lawson et al. 2011). The protozoan agent of trichomonosis, *T. gallinae*, is globally distributed and more commonly found in columbids and raptors where it can have sub-clinical or chronic impacts (Bunbury et al. 2008a) as well as causing both adult and nestling mortality (Krone et al. 2005; Bunbury et al. 2008b; Amin et al. 2014; Stockdale et al. 2015).

We recently highlighted the importance of monitoring sub-clinical infection in vulnerable populations, rather than just monitoring mortality (Stockdale et al. 2015). However screening techniques for *T. gallinae* infection are invasive and wild birds can be difficult to sample in the field, requiring the location and capture of individuals, followed by swabbing of the mouth, oesophageal tract, and crop and subsequent incubation of the swab (reviewed by Amin et al. 2014). *Trichomonas gallinae* is a parasite of the oesophageal tract; however, there are occasional morphological reports of *T. gallinae* from faecal samples (e.g. Ponce Gordo et al. 2002; Badparva et al. 2014). Here, we test whether screening non-cultured faecal and seed samples from birds with known *T. gallinae* infection using sensitive PCR techniques may provide an alternative, less invasive, method to screen live birds for the presence of sub-clinical *T. gallinae* infection.

We obtained faecal samples from Turtle Doves (*Streptopelia turtur*) handled as part of a wider autecological study of Turtle Dove ecology in south-east England (UK) (e.g. Dunn et al. 2015; Stockdale et al. 2015). Samples were collected either directly from birds during handling, or from the inside of clean bird bags. All samples (n=78) were frozen as soon as possible after collection (1 – 8 h) until subsequent analysis. We also obtained seed samples from the mouth (n=2), crop (n=4), proventriculus (n=2) and gizzard (n=3) of five recently dead nestlings (recovered dead either in the nest after chilling/abandonment, underneath the nest, or nearby following depredation). Trichomonosis was suspected in only one nestling due to an empty crop. Seed samples were frozen 1-8 h after collection.

DNA was extracted from seed samples using a standard 'salting out' procedure, and from each faecal sample using a QIAamp DNA Stool Mini Kit (Qiagen, Manchester, UK) following a modified protocol. To maximise DNA yield, we extended the inhibitor binding step to 5 min, extended the digestion step to 30 min, extended the drying step to 3 min centrifugation and finally reduced the elution volume to 100μl following a 5 min incubation. DNA extraction was confirmed in all cases by amplification of a 280-355 bp amplicon within the ITS-2 region using primers designed to target dietary components (Dunn et al. unpubl.). We obtained two positive controls of DNA from *T. gallinae* parasites collected using standard crop swabs and culture procedures (e.g. Bunbury et al. 2005; Lennon et al. 2013; Thomas et al. unpubl.). All PCRs for *T. gallinae* detection were run in a 50ul reaction volume with 1 X PCR Buffer, 2mM $MgCl_2$, 0.2mM each dNTP, 0.5 μM each primer (TFR1 and TFR2; Gaspar da Silva et al. 2007) 1.25 U GoTaq Flexi (Promega, Madison, WI) and 1μl template DNA. The PCR protocol consisted of an initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 45 sec, 63°C for 30 sec and 72°C for 45 sec, and a final extension at 72°C for 5 min and was carried out on a Gene Amp ® PCR System 9700.

To test the sensitivity of our analysis, we carried out a sixfold 1:10 dilution series on our positive samples of cultured *Trichomonas* parasites. We treated seed samples as non-cultured controls to test the necessity of culturing oral swabs following collection. All individuals from which faecal and seed samples were collected tested positive for *T. gallinae* infection using standard crop swab and culture techniques (Lennon et al. 2013; Stockdale et al. 2015; Thomas et al. unpubl.). Only one faecal sample, collected from a nestling (nestling 23 in Stockdale et al. 2015), was from a clinically affected bird (which had matted feathering around the beak, and yellow caseous lesions within the oesophageal tract which were found upon gross necropsy; Stockdale et al. 2015).

We successfully amplified *T. gallinae* DNA from our positive cultured controls diluted to 1:1,000 (Figure 1). The same PCR protocol failed to amplify DNA from any of our faecal samples, and all but one of our uncultured seed samples (Figure 1). A single seed sample, collected from the mouth of the nestling suspected to have died from trichomonosis, tested positive.

Recent work has suggested that faecal diagnostics can be used to detect blood parasites in some primates, although this technique failed when applied to birds (Martinsen et al. 2015). *T. gallinae* is occasionally reported from microscopic analysis of avian faeces (e.g. Ponce Gordo et al. 2002; Badparva et al. 2014) but these identifications are based on morphology and none of these infections thus far have been confirmed by PCR. It is possible these identifications may be of other trichomonads besides *T. gallinae* (e.g. Amin et al. 2014), or that faecal diagnostics may occasionally be effective for *T. gallinae* infections in other species.

We failed to amplify *T. gallinae* DNA from either faecal samples or uncultured seed samples from Turtle Doves testing positive for *T. gallinae* using standard sampling methods, thus confirming that standard oral swab and culture techniques are both necessary for confirmation of sub-clinical *T. gallinae* infection in wild birds.

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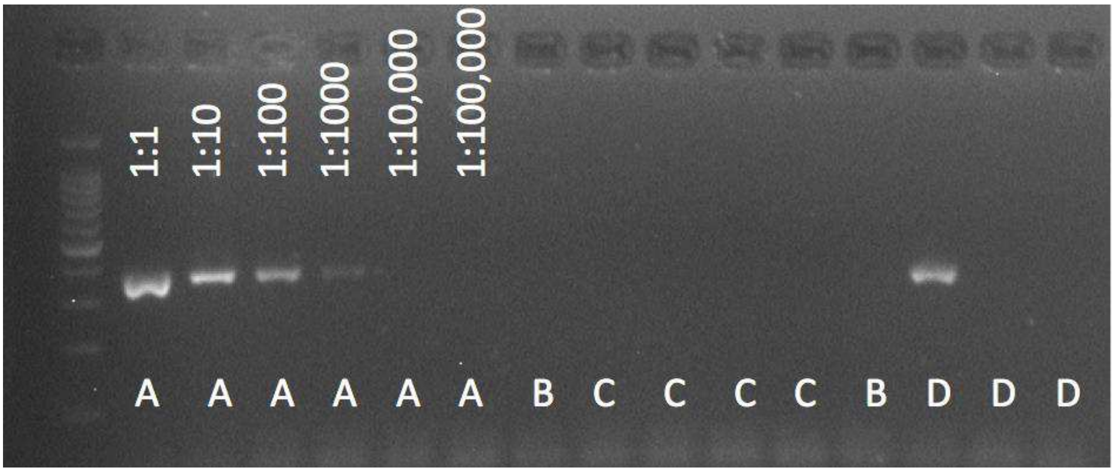
Fig 1 PCR products from a subset of reactions visualised on an agarose gel. Lane 1 contains a 100 bp ladder



A: 1 – 1:100,000 positive control dilution series

B: PCR negatives

C: Faecal samples

D: Seed samples from the gastrointestinal tract