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METHODS AND RESOURCES ARTICLE

Successful storage of *Trichomonas gallinae* on Whatman FTA cards following culture

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Abstract

Logistical constraints concerning parasite sample storage can hinder progress with the discovery of genetic variation on a global scale. New storage methods are being developed to address this, but require testing in order to understand how widely applicable these methods are. Whatman FTA cards have been tested previously under laboratory conditions for storing low-concentration *Trichomonas gallinae* isolates with the conclusion that they are not suitable, but have not been tested under field conditions. Here, we conducted a field-test, comparing FTA cards with storage in ethanol for *T. gallinae* samples collected and cultured from wild Columbiformes in Africa using standard field methods, before transportation to the UK. After 6 months storage, both methods resulted in an overall prevalence of 100% following PCR amplification (n = 59), suggesting that FTA cards are suitable for estimation of *T. gallinae* prevalence. However, samples stored in ethanol produced more, and longer, sequences than those stored on FTA cards. These data suggest storage in ethanol is preferable for the acquisition of high quality genetic strain data, but that FTA cards can be used successfully to ascertain infection prevalence and identify parasite strains under field conditions.

Keywords *Trichomonas gallinae* · Whatman FTA cards · Ethanol · Storage · Genetic variation

Introduction

International research efforts screening wild birds for *Trichomonas gallinae* and identifying genetic strain composition is contributing greatly to the characterization of intra-specific variation and can be used to understand transmission dynamics during epidemics (Chi et al. 2013; Ermgassen et al. 2016). Continuing these efforts on a global scale could pave the way for *T. gallinae* to be developed as a model system for understanding host-parasite relationships. To facilitate this goal, refinements to sample storage immediately after collection are required to render the process easier in areas that lack the facilities to carry out the currently adopted protocol

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for *T. gallinae* storage. This problem is currently limiting sample collection on a global scale.

Trichomonas gallinae is a microaerophilic protozoan parasite that resides in the upper digestive tract of birds (Stabler 1954). The clinical presentation of infection, trichomonosis, has been responsible for epidemics in wild finch populations across Europe (Neimanis et al. 2010; Peters and Ludwichowski 2010; Robinson et al. 2010; Lawson et al. 2011b; Ganas et al. 2014; Zadravec et al. 2017). Mortality from trichomonosis has led to a change in conservation status to endangered for the UK population of greenfinch *Cardualis chloris*, (Stanbury et al. 2017). A clonal strain of *T. gallinae* (A1) has been identified by morphological and subsequent molecular analysis as a causative agent of trichomonosis in European birds (Robinson et al. 2010; Lawson et al. 2011a). Globally, however, it is not the sole etiological agent of trichomonosis outbreaks, as a different variant (A2) was isolated from Band-tailed Pigeons *Patagioenas fasciata* during epidemics of the disease in California (Girard et al. 2014b). A new species of *Trichomonas* (*T. stableri* n.sp.) was also detected during these epidemics confirming a non-clonal etiology of avian trichomonosis in Band-tailed Pigeons (Girard et al. 2014a). Multiple additional—apparently non-pathogenic—strains of *T. gallinae* also circulate in wild bird populations (Thomas 2017). None of these discoveries would have been possible without identifying the parasite strains using molecular techniques. It is becoming standard practice to include this type of analysis for the accurate and reliable identification of etiological agents of infection, but expansion of geographical and temporal monitoring is imperative for progress within this field. In this context, *T. gallinae* has features that lend it to being a model system, such as being widespread, abundant and easily assayed. However, further refinement of the assay protocol is required to remove barriers currently experienced by research groups attempting to sample the parasite without access to a laboratory.

The current storage protocol for *T. gallinae* requires the parasite to be isolated from culture media using centrifugation and subsequently re-suspended in phosphate buffered saline (PBS) where it is frozen at -20 °C until DNA extraction (Riley et al. 1992; Lennon et al. 2013), which requires any field site to have both a centrifuge and freezer. Whatman FTA cards (GE Healthcare Life Sciences, UK; hereafter referred to as FTA cards) are available in a range of technology formats (FTA classic card, mini card, micro card and Elute card). All formats capture nucleic acids and preserve them at room temperature making this system ideal for collection of samples in the field and preservation during storage and transportation. Storing *T. gallinae* isolates on FTA Elute cards has previously been tested in laboratory, but not field, conditions. Two different concentrations (10 and 100 trichomonads/40 µl) of two *T. gallinae* isolates were inoculated onto FTA Elute cards, in triplicate, with DNA extractions performed 48 h, two and three weeks later (Holt et al. 2015). Only three samples (one at the weaker concentration and two from the stronger concentration) from one isolate extracted after 48 h were detected as positive from PCR (Holt et al. 2015). This suggested that FTA Elute cards are not a suitable storage solution for *T. gallinae,* at least not in the long-term or for samples of low concentration (Holt et al. 2015). However, FTA cards have not been tested under field conditions, where the requirement for culture of parasites prior to isolation (Dunn et al. 2016) may lead to higher concentrations of parasites present in media, and thus an increase in subsequent detection rates.

Here, we trialled two different methods of storing *T. gallinae* samples collected from African Columbidae and cultured before transport from Senegal to the UK for genetic analysis. After incubation in culture media, each sample was split between FTA Classic cards and ethanol. After 6 months of storage, we extracted DNA and compared PCR amplification and DNA sequencing success between the two methods.

Methods

Sample collection

Five dove species were caught in an enclosed area of acacia scrubland at a site near Sandiara, west Senegal (14° 25′ 53.9″ N, 16° 47′ 35.0″ W) during January and February 2015. This site is an enclosed area that has been protected from grazing, allowing semi-mature woodland to form, consisting mostly of acacia bushes used by roosting doves at high densities. European Turtle doves *Streptopelia turtur*, Laughing doves *Spilopelia senegalensis*, Namaqua doves *Oena capensis*, Vinaceous doves *Streptopelia vinacea* and Black-billed Wood doves *Turtur abyssinicus* were caught using whoosh nets and mist nets (Redfern and Clark 2001) and placed into cloth bags prior to processing. All birds were ringed using individually numbered colour rings, and aged and sexed where possible (Redfern and Clark 2001).

All birds were swabbed for *T. gallinae* using a moistened sterile viscose swab that was inserted into the oral cavity and used to swab the mouth cavity, oesophagus and crop. The swab was inoculated into an individual InPouch TF culture kit (Biomed Diagnostics, Oregon), sealed and incubated at room temperature (ranging from 25 to 30 °C) for 7–11 days in order to culture any parasites present. After the incubation period, 0.5 ml of the culture media was transferred to a FTA Classic card using a separate disposable pipette for each sample, and allowed to air dry before being placed into an envelope with a desiccant sachet, ready for transport and subsequent storage. The remaining 2 ml of culture media was transferred to three 1.5 ml eppendorf tubes, leaving enough room for an equal volume of absolute ethanol to be

subsequently added to each tube. FTA cards and eppendorf tubes were stored at room temperature for 6–27 days before transport to the UK. On arrival in the UK, FTA cards were stored at room temperature, and eppendorfs were stored at − 20 °C until further processing.

DNA extraction

Samples in ethanol were centrifuged at 13,000 rpm for 10 min and the supernatant removed to leave a parasite pellet. One eighth of the circle (approx. 2 mm × 2 mm) that held the parasite sample on the FTA card was cut out using a sterile pair of scissors and placed into an eppendorf tube. The scissors were disinfected between each sample with 10% bleach and thoroughly dried. DNA was extracted from all samples using a modified ammonium acetate method (Nicholls et al. 2000). Briefly, the parasite pellet was digested overnight in digestion buffer (20 mM EDTA, 50 mM Tris, 120 mM NaCl, 1% SDS, pH 8.0) with 50 µg of Proteinase K (Sigma-Aldrich, UK). Ammonium acetate (4 M) was then used to precipitate out the proteins and ethanol precipitated out the DNA. The resulting DNA pellet was dissolved in 20–50 µl low TE buffer, depending on the size of the pellet, in a water bath at 65 °C. The extracted DNA was stored at − 20 °C.

PCR amplification of the ITS1/ 5.8S/ ITS2 ribosomal region and sequencing

The primers TFR1 [f] (5′-TGCTTCAGTTCAGCGGGT CTTCC-3′) and TFR2 [r] (5′-CGGTAGGTGAACCTGCCG TTGG-3′) (Gaspar da Silva et al. 2007) were used to target a 400 bp length of the ITS1/5.8S/ITS2 ribosomal region of the genome (hereafter referred to as the ITS region). The PCR recipe consisted of 0.8 × Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.5 µM each of forward and reverse primer (Sigma-Aldrich, UK), and 1 µl of template DNA in a total reaction volume of 10 µl. A positive and negative control were included in each PCR run to confirm successful amplification and lack of contamination respectively. A touchdown PCR thermal cycling program was performed as follows: 15 min at 95 °C followed by 11 cycles of 1 min at 94 °C, 30 s at 66 °C (decreasing by 1 °C every cycle until 56 °C), 1 min at 72 °C; then 24 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C with a final elongation step of 10 min at 72 °C. PCR reactions were run on either a Gene Amp 9700 PCR system (Applied Biosystems, Foster City, CA) or a DNA Engine Tetrad 2 (Bio-Rad Laboratories Inc, CA, USA). PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide and visualised by UV light. The presence of an amplicon at the expected product size indicated the presence of *T. gallinae* infection. If the result was negative, the PCR was repeated to confirm. Samples were purified and sequenced at the NERC Biomolecular Analysis Facility (NBAF Sheffield) on the ABI3730 DNA Analyser (Applied Biosystems, CA, USA).

Sequence analysis

Sequences returned from Sanger sequencing were manually assessed for sequencing errors, trimmed, and the forward and reverse sequences aligned in BioEdit (Hall 2005). The NCBI-BLAST algorithm (Altschul et al. 1997) was used to determine the closest sequence match for each sequence. If the sequence was of poor quality (less than 70% query cover) it was removed from the analysis. A Mann–Whitney U test was used to test for differences in sequence length between the two storage methods.

Results

Fifty-nine samples collected from European Turtle doves (n = 44), Laughing doves (n = 4), Namaqua doves (n = 9), Vinaceous doves (n = 1) and Black-billed Wood doves (n = 1) were stored on both Whatman FTA card and in ethanol. The efficacy of storage methods for subsequent genetic analysis was compared based on PCR and Sanger sequencing results (Table 1). *T. gallinae* prevalence was 100% for both methods.

Comparing results from the same sample, the ethanol storage method obtained sequences from 15 samples that the FTA card storage method did not. The FTA card method obtained one sequence from a sample for which the ethanol storage method failed. Where sequence reads were obtained for samples from both storage techniques (n = 30), there were no conflicts on strain identification, with six strains of *Trichomonas* sp. identified; consistent with additional studies from the same site (Thomas 2017; Young et al. unpubl. data) and reported in more detail elsewhere (Thomas et al. 2022). Overall, the samples stored in ethanol gave longer sequence reads than the samples stored on FTA cards (Mann Whitney-U, $W = 111$, $p < 0.001$; Fig. 1).

Discussion

The samples stored in ethanol performed better than the samples stored on FTA cards in terms of being able to obtain a DNA sequence and the quality of DNA sequence data. In contrast to the findings of Holt et al. (2015), we were able to amplify *T. gallinae* DNA from FTA cards up to six months after the samples had been collected, although we cannot compare the concentrations of trichomonads that were initially inoculated onto the FTA cards in each case as this was beyond the scope of our study, and more representative of

Table 1 Comparison of samples stored in ethanol and on Whatman FTA card. PCR and DNA sequencing was repeated on a subset of samples

Stage	Ethanol stored samples			FTA card stored samples		
	Good quality (%)	Poor quality (%)	Total N	Good quality (%)	Poor quality (%)	Total N
PCR #1	88	12	59	76	24	59
Sequence #1	73	27	53	47	53	45
PCR #2	100	0	27	97	3	39
Sequence #2	52	48	27	23	77	39

A 'Good' quality result from PCR is a strong band visualised on gel, 'Poor' quality is either a faint band or non-distinct band; overall prevalence was 100%. A 'Good' quality result from sequencing is both directions of sequencing run being assembled and DNA matched to *Trichomonas* sp., 'Poor' quality is sequence failure or poor quality sequence (small/non-existent peaks or double peaks on chromatogram)

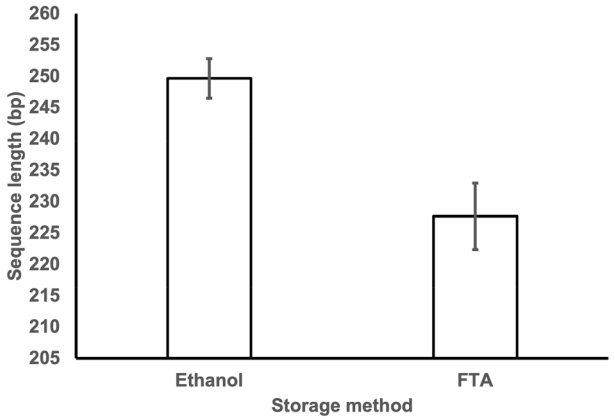

Fig. 1 Mean ± SE length (bp) of *Trichomonas* sp. sequences produced following analysis of samples stored in ethanol or on FTA cards

field conditions. It is possible that much higher concentrations than 10 or 100 trichomonads per 40 µl were inoculated onto our FTA cards, and elsewhere it has been demonstrated that parasite culture is an essential step to identify sub-clinical *T. gallinae* infection in wild birds (Dunn et al. 2016). Holt et al. (2015) suggested the use of FTA cards would not be sensitive enough to detect sub-clinical infections but none of the African species of Columbidae in our study had clinical signs of trichomonosis, showing that it is possible to diagnose sub-clinical infection using FTA cards. Holt et al. (2015) used a different protocol to initially process the FTA cards storing *T. gallinae.* They used FTA Elute cards, which allow the direct elution of DNA through a series of pulse centrifugation steps and washes whereas the FTA Classic cards require a DNA extraction protocol such as was used here. However, a previous study comparing efficiency of DNA extractions from multiple types of preservation paper found no significant differences between storage on FTA Classic or FTA Elute cards, when the same DNA extraction method was used (Love Stowell et al. 2018) The FTA card technology is designed to lyse cells, denature proteins and protect DNA from degradation. Our method of DNA extraction from the FTA card may have been more efficient at recovering the DNA from the matrix than simple DNA elution. Despite the improved performance of FTA cards in preserving *T. gallinae* DNA under field conditions, we recommend that cultured *T. gallinae* media should be mixed with an equal amount of ethanol for storage and transportation from a field site to a laboratory to maximise the acquisition of genetic strain information. However, if the acquisition and/or transport of ethanol is logistically difficult, storage on FTA cards following culture is a viable alternative for strain identification, and for estimation of infection prevalence, although we recommend a repeat PCR on any negative samples. The storage of *T. gallinae* in this form has not been trialled beyond 6 months so it is advisable to perform DNA extraction within this time frame.

This solution has simplified the process for *T. gallinae* storage after growth in culture media. Collection of *T. gallinae* samples from wild bird populations is no longer restricted to sites with access to laboratory facilities or field workers with the necessary laboratory skills. Research into the prevalence of *T. gallinae* infection in wild bird populations and the strains circulating in these populations can hence be pursued on a global scale. Furthermore, this protocol greatly eases *T. gallinae* sample storage post-collection which, in addition to the use of the InPouch culture kits, results in a very simple sample collection and storage system—an important feature for any model host-parasite system.

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Author contributions RCT: Conceptualization, Methodology, Investigation, Formal analysis, Writing; JCD: Conceptualization, Formal analysis, Supervision, Writing; COO: Investigation, Writing—Review & Editing; AJM: Funding acquisition, HH: Methodology, Writing – Review & Editing; Writing—Review & Editing; PVG: Funding acquisition, Writing—Review & Editing; KCH: Supervision,

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Data availability Data will be deposited in an Open Access Repository upon manuscript acceptance.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Ethical approval for this work was granted by the RSPB Ethics committee. All birds were released back to the wild following sample collection.

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