1. TITLE PAGE

Endemic infection of the common mynah *Acridotheres tristis* with *Trichomonas gallinae* the agent of avian trichomonosis.

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(Short informative running title.)

Trichomonas gallinae infection of mynah.

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2. SUMMARY

Avian trichomonosis is an architypal disease of wild columbids and those birds that predate them. Increasingly though, it has been reported in passerines; a recent and ongoing epidemic in the chaffinches and greenfinches of Europe and outbreaks amongst house finches, American goldfinches and purple finches in North America. The parasite, *Trichomonas gallinae*, causes lesions in the upper respiratory tract which can cause mortality associated with dehydration and emaciation. This paper reports for the first time, the widespread, endemic and often asymptomatic infection of common mynah (*Acridotheres tristis*) around the Faisalabad District, Pakistan. Parasite typing was used to investigate the potential for transmission among the frequent sympatric species. Type C parasites were found in mynah, and while this is analagous to the pandemic finch strain which is Type A, it is the first known example of passerine infections of this parasite genotype. Subtype analysis showed the strain to be C4 a subtype which has a widespread distribution in columbids.

Key words:

Trichomonosis, Trichomoniasis, *Trichomonas gallinae*, subtyping, passerine, sturnidae, mynah, columbid, ITS1/5.8S/ITS2, Fe-hydrogenase

KEY FINDINGS

- The common mynah is susceptible to Trichomonas gallinae which is normally a disease of pigeons and doves, but which has caused dramatic species declines in European finches in recent years.

- Common mynah infection is widespread and frequent in the area surveyed, but appears to cause little pathology suggesting the common mynah as a previously undetected reservoir for this parasite.

- Only one genotype of parasite was found, the is different to the one affecting European finches indicating that the common mynah infections are not linked to the finch epidemic.

3. INTRODUCTION

The flagellated protozoan parasite *Trichomonas gallinae* is an etiological agent of Avian trichomonosis, ordinarily affecting the upper respiratory and gastrointestinal tract of birds. It is frequently reported in columbids and birds of prey and is also known as cankar and frounce. Infected birds generally present with esophageal lesions, lethargy, difficulty in breathing, watery eyes, drooling and wet feathers.

Columbiformes are beleived to be the primary reservoir host of this parasite, with *Columba livia* considered as an efficient transmitters of *T. gallinae* throughout the world (Forrester & Foster, 2008; Stabler, 1954). The disease is a conservation concern for endangered columbids such as the pink pigeon (Bunbury *et al.*, 2007) and migratory columbids such as the Turtle dove have the potential to spread the parasite over long distances (Stockdale *et al.*, 2015). Several migratory passerine species, which are host to

this disease, such as the greenfinch *Chloris Chloris* and chaffinch *Fringilla Coelebs* which have been linked to trichomonosis during their migratory behaviour (Lawson *et al.*, 2011b).

In recent years the genotype, prevalence and host range of *Trichomonas gallinae* has been subject to significant scrutiny in Europe (Chi *et al.*, 2013; Ganas *et al.*, 2014; Lawson *et al.*, 2006; Lawson *et al.*, 2011a; Lawson *et al.*, 2011b; Robinson *et al.*, 2010; Zu Ermgassen *et al.*, 2016) and North America (Anderson *et al.*, 2009; McBurney *et al.*, 2015), however, in many other parts of the world the distribution of genotypes of *Trichomonas gallinae* remains largely uninvestigated with no major studies reported from Asia and the Indian subcontinent. This is a region where columbid populations such as the blue rock pigeon (*Columba livia*) occur in sympatric flocks and share roost with other avian species. Notably, the common mynah, *Acridotheres tristis*, is a passerine species described as the most invasive of all avian species. It occurs in mixed roosts with *Columba livia* and this close contact suggests that the species may share pathogens. Indeed it has been suggested that the invasive nature of *Acridotheres tristis* and its ability to adapt to new niches has meant that it is susceptible to a wide variety of parasite infections (Clark *et al.*, 2015) and we considered whether it might be susceptible to infection with *Trichomonas gallinae* and so might possess the potential to act a shuttling vector of avian trichomonas between the main *Columba livia* reservoir and other ecological niches to which it is able to adapt with increased exposure of infected birds to avian livestock being of particular concern.

The common mynah belongs to the order Passeriformes and like the starlings belong to the family sturnidae. It is considered omnivorous, and constructs often large but mostly temporary roosts close to human habitation; inhabiting rural and urban gardens, cultivations among the university and college campuses and the road side light vegetation. The mynah has a relatively short home range of about three to five kilometers

during its daily diurnal visitations. It intensively feeds on figs and seeds, unripe fruits, insects, small size frogs and snakes but may also take small, juvenile and dead birds on occasion.

Our study was based in the Indian subcontinent where *Acridotheres tristis* is native, from 20km radius of Faisalabad. Faisalabad is the third most populous city in Pakistan, the second largest in the eastern province of Punjab and its latitude and longitude coordinates are 31.42°, 73.09°. The surrounding countryside, irrigated by the lower Chenab River, produces cotton, wheat, sugarcane, vegetables fruits and tree plantations dominated by *Salmalia malabarica*, *Terminalia arjuna*, *Cedrella toona*, *Dalbergia sissoo*, *Ficus bengalensis*, Eucalyptus species, *Mangifera dactylifera*. Faisalabad has been classified as a hot desert climate (BWh) by the Köppen-Geiger climate classification system. In this region eight different sites were identified and selected with sympatric flocks of mynah and blue pigeons, i.e., University of Agriculture Faisalabad (UAF), Gatwala Forest plantation (GAT), Postgraduate agricultural research station (PARS), Amipur Canal Rest House (AMI), Satina Canal Rest House (SAT), Burala Canal Rest House (BRU), Tarkhani Canal Rest House (TAR) and Moongi Canal Rest House (MON). These study sites are divided into three categories, i.e., urban (UAF, GAT), semi urban (PARS, AMI, SAT) and rural (BRU, TAR, MON) (Figure 1).

4. MATERIALS AND METHODS

Ethical Approval

This work was approved by the Directorate of Graduate Studies and Research Board as permissable by the University of Agriculture, Faisalabad ethics committee. The approval number was 37195-98, dated 31.12.13.

Screening of mynah for *Trichomonas gallinae*

The mynahs were captured (N=167), from different locations of Central Punjab with the mist nets. The nets were erected straight into the respective fields, close to the trees viz, *Salmalia malabarica, Terminalia arjuna, Cedrella toona, Dalbergia sissoo, Ficus bengalensis, Eucalyptus* species, which served as a roost to the majority of birds. The nets were carefully watched and mostly the mynahs were captured during the late evening hours; the time they return to roost. The captured population contained 75 males and 92 females from different locations of Faisalabad district were maintained in an aviary in the department (Zoology, Wildlife and Fisheries). Upon capture, birds were evaluated clinically on the basis of their body weight (g), wing length (mm), tarsus length (mm), tail length (mm), beak length (mm) and head circumference (mm). Birds were then screened for *T. gallinae* as follows: A sterile calcium-alginate cotton swab tip was inserted gently within the oral cavity of the common mynah to obtain the saliva coated swabs. The swabs were used to inoculate InPouchTM TV culture packs (BioMed Diagnostics, USA) according to the manufacturer's instructions (BioMed Diagnostics, Santa Clara, California, USA). Cultures were incubated (37°C) to access the growth performance of the parasite (*T. gallinae*) for a period of 7-10 days. Trichomonosis cases confirmed microscopically from culture were subjected to the DNA extraction and subsequently genotyped.

Genotyping of Mynah isolates

Trichomonas gallinae DNA was obtained from the parasite culture using QIAGEN mini kit according to the manufurers instructions (QIAGEN, Valencia, California). We have previously proposed a simple binomial sequence based genotyping system for *Trichomonas gallinae* based on the ITS region and Iron dehydrogenase (*FeHyd*) gene

sequences which is now widely adopted and undertaken routinely. Genotyping was undertaken essentially as previously described (Chi *et al.*, 2013). Briefly, DNA from *T. gallinae* cultures were subjected to ITS region amplification using TFR1 (TGCTTCAGTTCAGCGGGTCTTCC) and TFR2 (CGGTAGGTGAACCTGCCGTTGG) primers. A fragment of the Fe-hydrogenase gene was also amplified from positive samples TrichhydFOR (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCGAAT) primers. Negative (water) and positive (purified *T. gallinae* DNA from an infected greenfinch) controls were included in each PCR run. All amplicons were directly sequenced using a commercial service (Source BioScience, Nottingham, UK).

5. RESULTS

Trichomonas gallinae is a common endemic infection of *Acridotheres tristis*

Based on 33 culture positives from 167 birds screened, we approximate the regional prevalence of *Trichomonas gallinae* to be 20%. Of those 33 birds that did culture positive just 11 exhibited markers of poor health/conditions and in just 4 cases were lesions in the upper digestive tract observable with most infected birds appearing to carry the infection asymptomatically (Table 1). We found infected mynah in each of the study locations (Fig. 2) indicating that the infection is widespread in this region. Interestingly, although at least some mynah were infected in each of the study sites, most of the infections detected were of male birds and the number of cases at BRU and AMI where contact between common mynah and blue pigeon is the most limited also showed the lowest infection rates.

Trichomonas gallinae isolates from *Acridotheres tristis* are all of the C4 subtype

Genotypic analysis indicated that all the common mynah isolates among the Faisalabad samples were shared the same genotype. Of the 33 sequences procured from mynah, all were identified to be *T. gallinae* (ribo)Type C by the finding that their sequences were identical to the Type C reference sequence EU215362. Similarly all of the sequences obtained from the *FeHyd* amplicons were identical to the reference sequence KC529662 An illustrative phylogenetic tree is provided to show the evolutionary relationship of these isolates and other avian isolates which have previously been characterised (Fig. 4). The C4 genotype has previously been identified in UK and USA columbids (AA and KMT, unpublished) but the first description of this genotype from asia/indian-subcontinent or in a passerine species.

6. DISCUSSION

A new genotype of *Trichomonas gallinae* in passerine birds

Our study reports the highest prevalence for *T. gallinae* carriage recorded in any non columbid species and is the first example of a host species other than columbids in which the parasite is widely carried without apparent pathology. It is striking because all genotyped isolates associated with outbreaks of passerine disease have previously been caused by the A1 genotype which is associated with considerable pathogenicity and mortality, particularly in passerine hosts. In this study C4, a genotype that is less associated with such virulence characteristics than A1, is prevalent in live, asymptomatic birds suggesting that mynah has the potential to be a vector for this parasite between the main columbid reservoir and to niches which ordinarily would be isolated from it. It is also interesting that in the UK where an epidemic caused by the A1 genotype finch strain of *T. gallinae* has been taking place since 2006, starlings (a very common species in the same family as the mynah, the Sturnidae) have not been one of at least 12 passerine

187 species diagnosed with the disease by the Garden Wildlife Health project, a national wild
188 bird disease surveillance programme (B. Lawson, personal communication).
189
190 **Mynah - as a new reservoir or a dead end host?**
191 On the basis of this study, we conclude that the common mynah has the potential
192 to serve as a reservoir for avian trichomonosis throughout the study sites of Faisalabad, Pakistan
193 and perhaps throughout its entire (tropical and subtropical) range. This finding is particularly
194 concerning because of the recognized ability of mynah to adapt to new niches and particularly
195 ones from which columbids may normally be excluded extending the potential for exposure of
196 susceptible domestic and wild avifauna to infection. Transmission of influenza from free living
197 mynah to farmed poultry has previously been demonstrated (Body *et al.*, 2015) and similarly our
198 observation of mynah infection by a known pathogen of avian livestock will be of particular
199 concern to poultry producers where mynah may gain access to flocks. Nevertheless, the
200 observations in this study do not in any way address transmission to and from the mynah as a
201 host. We cannot yet speculate on whether the infections observed are spillover from sympatric
202 columbids or passed within the species by other mynah. Certainly, mynah feed in sympatric
203 flocks with blue rock pigeons throughout this region and so may be infected from them. It is also
204 not clear whether mynahs pass the infections to partners and young or even whether the
205 infection is normally pathogenic, fatal or resolves. It is also not clear why there is only one subtype
206 discernable, whether susceptibility is limited to this subtype, whether it represents a mynah
207 adapted strain or whether the pathogenicity of other subtypes means they are quickly eliminated
208 from mynah populations when transmissions do occur. These intriguing questions are though
209 tractable and answering them will be important in gauging the risk posed by mynahs to other
210 susceptible avian populations.
211

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273 Table 1: Occurrence of different strains (*Trichomonads*) as isolated from the available
274 bird species based on *FeHyd* gene with their GenBank reference.
275

Host species	**Type**	**Origin**	**GenBank**
Greenfinch	A1	UK	JF681136
Sparrow hawk	A1.1	UK	KC529660
Wood pigeon	A1.2	UK	KC962158
Wood pigeon	A1.3	UK	KC529661
Madagascar turtle dove	A2	Seychelles	JF681141
Rock pigeon	C1	North America	AF446077
Wood pigeon	C2	UK	KC529664
Rock pigeon	C3	UK	KC529663
Wood pigeon	C4	UK	KC529662
Feral pigeon	C8	UK	KY569256
Feral pigeon	C9	UK	KY569257
Socorro dove	C10	UK	KY569258
Tawny owl	C11	UK	KY569259

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277

278 Table 2: Characteristic lesions (Trichomonosis) of common mynah consistent with
279 culture using the ITS region and *FeHyd* sequence.

#	Case ID	SEX	Clinical Signs	Lesions	Culture	ITS	*FeHyd*
1	S1A4Y4	M	+	-	-	C	C4
2	S1A8Y8	M	-	-	+	C	C4
3	S1A1B1	F	+	+	+	C	C4
4	S1A6Y6	M	-	-	+	C	C4
5	S1A8Y3	M	-	-	+	C	C4
6	S2A3G3	F	-	-	+	C	C4
7	S2A3O3	M	-	-	+	C	C4
8	S1A1O1	M	-	-	+	C	C4
9	S2A6O6	F	+	-	+	C	C4
10	S2A10O10	M	-	-	+	C	C4
11	S3A4BL4	M	-	-	+	C	C4
12	S3A8W3	F	-	-	+	C	C4
13	S4A9BR9	M	-	-	+	C	C4
14	S4A7BR7	M	-	-	+	C	C4
15	S4A8P8	M	-	-	+	C	C4
16	S5A8Y8	F	-	-	+	C	C4
17	S5A2B2	M	+	-	+	C	C4
18	S5A6Y2	F	-	+	+	C	C4
19	S5A8Y3	F	-	+	+	C	C4
20	S5A9Y9	M	-	-	+	C	C4
21	S6A8G8	F	-	-	+	C	C4
22	S6A3O3	M	-	-	+	C	C4
23	S6A6O6	F	+	-	+	C	C4
24	S6A9G7	F	-	-	+	C	C4
25	S7A7W7	M	+	-	+	C	C4
26	S7A3BL3	F	-	-	+	C	C4
27	S7A5BL5	M	-	-	+	C	C4
28	S8A10BR10	M	-	-	+	C	C4
29	S8A5A5	M	-	-	+	C	C4
30	S7A4A4	M	-	-	+	C	C4
31	S8A9BR4	M	+	+	+	C	C4
32	S8A10BR5	F	+	-	+	C	C4

Figure 1: *T. gallinae* is a frequent infection of common mynah (*Acridotheres tristis*) across the Faisalabad district. The map shows eight different ecological study sites. Prevalence of infection is indicated as the proportion of birds captured and released at each site. Overall prevalence is also noted. University of Agriculture Faisalabad=UAF, Gatwala Forest Plantation=GAT, Postgraduate Agricultural Research Station=PARS, Amipur Canal Rest House=AMI, Satina Canal Rest House =SAT, Burala Canal Rest House =BRU, Trkhani Canal Rest House =TAR and Moongi Canal Rest House = MON.

Figure 2: *T. gallinae* phylogeny using the neighbour-joining method for the *FeHyd* region from the sequences described in Table 2. *Trichomonas vaginalis* is included as an outgroup. The bootstrap consensus tree was constructed using the Tamura–Nei model and 2000 replicates. Tree topology was tested using 719 positions. †Finch Strain ‡Mynah Isolates