Parasitic diseases can negatively influence domestic animal fitness (1) and increase the extinction risk of wild and captive natural populations by reducing their survival rate and reproductive fitness (2,3). Trichomonosis is a global infectious disease caused by *Trichomonas gallinae*, a single-celled flagellate protozoan (4), which usually affects Columbiformes as primary hosts (5,6,7,8). Trichomonas gallinae was first described by Rivolta in 1878 when it was found in an oral swab from a pigeon (*Columba livia*), common mynah *Acridotheres tristis*, chicken *Gallus gallus domesticus*, turkey *Meleagris gallopavo*, and ducks *Anatidae* were collected and tested for *T. gallinae* infection with InPouch™ TV culture kits. The results showed that the overall prevalence of *T. gallinae* in these samples was 26.4% (*n* = 72). The PCRs were used to detect the internal transcribed spacer (ITS) region of *T. gallinae*, and the results of the sequence analysis indicated genetic variation. Among 48 sequences, we found 15 different ribotypes, of which 12 were novel. Three had been previously described as ribotypes A, C, and II. To our knowledge, this study demonstrated the presence of *T. gallinae* strain diversity in Saudi Arabian birds for the first time and revealed that ribotypes A and C are predominant among Riyadh birds.

**Key words:** ITS region, ribotype, *Trichomonas gallinae*, trichomonosis, trichomoniasis, trichomonads, columbid, genotypic diversity, protozoan parasite, prevalence

**Abbreviations:** ITS = internal transcribed spacer; MEGA = molecular evolutionary genetics analysis; NCBI = National Center for Biotechnology Information; rRNA = ribosomal RNA; TFRI = transferrin receptor 1; TFR2 = transferrin receptor 2

The infection can be transmitted directly via feeding of squabs or when adult males and females exchange food as breeding behavior or indirectly via sharing of food or water (8,17). Trichomonosis can be directly diagnosed through symptoms, such as lesions in the digestive and upper respiratory tracts and swelling in the throat, nostrils, and eyes (18,19). However, infected adult birds might not show signs of the disease because they can develop tolerance to the infection (17). Death occurs from breathing difficulties, starvation, or both (20,21,22).

Trichomonosis has threatened several rare wild bird species around the world. For example, it was introduced to Mauritius with exotic bird species and transmitted to the endemic bird species on the island, including the echo parakeet (*Melopsittacus undulatus*) and pink pigeon (*Columba mayeri*) (8,17). As a result, the populations of these two avian species have drastically declined in number in Mauritius (8,17). In addition to wildlife threats, there are increasing concerns that this disease could negatively affect the economic sectors and recreational values of certain countries (23).

Several *T. gallinae* strains with differing virulence have been identified. For example, Sansano-Maestre *et al.* (13) used the 5.8S ribosomal RNA (rRNA) region and restriction fragment length
swabs were then immediately inoculated individually into InPouch TV culture kits (BioMed Diagnostics, White City, OR), according to the manufacturer’s instructions. The InPouch TV cultures containing T. gallinae were transferred to 1000-µl Eppendorf tubes and then centrifuged at 7245 × g for 3 min. The supernatant was removed, and the cell pellet was mixed with 500 µl DNAzol with gentle pipetting to lyse the cells. Subsequently, the cell lysates were centrifuged at 2795 × g for 4 min at 4 C, and the resulting supernatant was transferred to a new Eppendorf tube. To precipitate DNA, 500 µl of 100% ethanol was added to each tube, followed by mixing through inversion and 3 min of centrifugation at 1789 × g at 4 C. The supernatant was removed, and the DNA pellet was washed in 75% ethanol. The DNA pellets were left to air dry for 4 min before adding 100 µl of nuclease-free water to resuspend the DNA. The extracted DNA was stored at −20 C for subsequent work. The quantity of DNA for each isolate was estimated by using spectrophotometry based on an absorbance reading at 260 nm. The extracted DNA was confirmed visually on a 1% agarose gel stained with ethidium bromide under an ultraviolet transilluminator.

A PCR was conducted to amplify the ITS region with the forward primer (TFR1; 5′-TGCTTCAAGTTGCAGGGCTTTC-3′) and the reverse primer (TFR2; 5′-CCGTTACGTAACCTGGCGTACG-3′) (24,27) according to the method described by Robinson et al. (28). The PCR amplification was carried out in a 25-µl reaction volume containing 15 µl of Green Master Mix (2×; Thermo Fisher Scientific, Waltham, MA), 3 µl each of forward (TFR1) and reverse (TFR2) primers (Eurofin Genomics, Anzingen, Germany), 3 µl of doubly distilled water, and 1 µl of template DNA. Each PCR was run with a negative control containing no template DNA and a positive control containing the T. gallinae DNA.

The PCR was performed with the following temperature cycle: initial denaturation at 94 C for 15 min, followed by 35 cycles of denaturation at 94 C for 1 min, annealing at 65 C for 30 sec, and extension at 72 C for 1 min, and a final extension at 72 C for 5 min.

We confirmed PCR amplification under ultraviolet light with a 1% agarose gel stained with ethidium bromide. A band of approximately 350 bp in size was confirmed with a Ready-Load™ 100-bp DNA ladder (Promega, Madison, WI). The positive PCR products were submitted for sequencing to Macrogen Inc. (Seoul, Republic of Korea).

The molecular and phylogenetic relationship of the sequences of trichomonad parasites were determined by software programs molecular evolutionary genetics analysis (MEGA) version 7 (29) and CLUSTAL X version 2.1 (30). All sequence data were aligned with the forward and reverse complement of the reverse primer by the MEGA software. The T. gallinae sequences available in the National Center for Biotechnology Information (NCBI) GenBank database were used to compare the ITS regions. The phylogenetic trees of the datasets obtained from the ITS1/5.8S and rRNA/ITS2 regions were constructed separately by the

### Table 1. Prevalence of *Trichomonas gallinae* infection of five bird species sampled in Riyadh, Saudi Arabia.

| Species                  | Origin     | No. | Lesion existence (%) | Positive culture | Sample prevalence (%) |
|--------------------------|------------|-----|----------------------|------------------|----------------------|
| Feral pigeon (*Columba livia*) | Domestic   | 87  | 43.46                | 55               | 63                   |
| Feral pigeon (*Columba livia*) | Wild       | 17  | 0                    | 3                | 63                   |
| Common mynah (*Acridotheres tristis*) | Wild       | 73  | 0                    | 4                | 9                    |
| Chicken (*Gallus gallus domesticus*) | Domestic   | 58  | 33.33                | 9                | 16                   |
| Turkey (*Meleagris gallopavo*)     | Domestic   | 25  | 0                    | 4                | 16                   |
| Duck (*Anatidae*)            | Domestic   | 13  | 0                    | 0                | 0                    |
| Overall total               |            | 273 | 72                   | 26               |                      |

The percentage of lesion existence of *T. gallinae*-infected samples (positive culture).
neighbor-joining and the Tamura-Nei models and were used to analyze the relationships between taxa by nucleotide sequence analysis (29,30). We used Felsenstein’s bootstrap testing to calculate the associated taxa clustered in the bootstrap values (1000 times) (31). The final dataset from the ITS ribotype included a total of 239 positions.

RESULTS

Of the 273 birds examined in this study, 72 (26.4%) tested positive for T. gallinae infection, including 55 out of 87 (63.3%) with 43.64% lesion existence in domestic pigeon, 3 out of 17 (17.7%) with 0% lesion existence in wild pigeon, 1 out of 73 (0.14%) with 0% lesion existence in wild common mynah (Acridotheres tristis), 9 out of 58 (15.6%) with 33.33% lesion existence in chickens, 4 out of 25 (16%) in turkeys (Meleagris gallopavo), and 0 of 13 (0%) in ducks (Table 1).

In the 72 T. gallinae–positive samples, the ITS region was successfully amplified through PCR by ITS region–specific primers, resulting in a fragment of approximately 300 bp (Table 1). Of these positive PCR samples, 48 were sequenced (Table S1). The phylogenetic tree for the ITS region contained 12 novel lineages, in addition to three previously described ribotypes, A (29 samples), C (six samples), and II (one sample) (Fig. 1). Most samples clustered in lineage A (32) (GenBank GQ150752), which was found in 17 samples, including columbids (feral pigeon), passerines (common mynah), and galliforms (chicken, turkey). Eighteen samples of columbids (feral pigeon) were predominantly infected by lineage C (33) (GenBank EU215362). One isolate of T. gallinae obtained from feral pigeons clustered in the same clade as the sequence of the T. gallinae ITS region lineage II obtained from a racing pigeon (34) (GenBank FN433474). Interestingly, a comparison of the complete ITS region of all novel sequences determined in this study revealed 12 different sequence lineages (KSA1–KSA12), and all obtained sequences were uploaded to GenBank (accession MK771125–MK771135 and MK765029). These lineages demonstrated a degree of sequence divergence between each other, and each formed a separate branch. All of these occurred in columbids (feral pigeon). To our knowledge, these sequences types (ITS; KSA1–KSA12) are novel and reported for the first time in the present investigation in Riyadh.

DISCUSSION

Trichomonosis is an avian disease caused by the protozoan T. gallinae and characterized by a great variation in strain virulence and pathogenicity, affecting different bird species worldwide. In the last few years, interest in the investigation of T. gallinae strains has increased and spurred the introduction of new molecular techniques. In this paper, we studied the prevalence and genotypes of T. gallinae...
strains in avian species in Riyadh, including birds found at the poultry market and those that were caught in the wild. We demonstrated, to our knowledge for the first time, strains of *T. gallinae* by comparing the ITS ribotype isolated from different bird species in Riyadh.

We found *T. gallinae* to be present in four of the five bird species examined, confirming the cases in Saudi Arabia, with an overall prevalence rate of 26.4%. The prevalence of *T. gallinae* strains found in the poultry market in Riyadh was much higher in domestic pigeons than in other bird species. This result was predictable because feral pigeons have been considered the primary host of *T. gallinae* strains (18,35). However, this parasite can also affect chickens (34). This study is among the few that have reported infection with *T. gallinae* in the common mynah (36) and turkey (37). Although all screened samples of duck in this study were negative for *T. gallinae*, infection with this parasite in the Anatidae family has been confirmed in a few studies (38). The prevalence rates were found to be higher in birds from the poultry market, especially in pigeons, compared with those caught in the wild, which might implicate the pigeons from the poultry market as a mode of transmission of *T. gallinae* among and within bird species. Although signs of infection have been found to be low in adult birds (39), we found a relatively high observation of lesions in both pigeons and chickens (43.46% and 33.33%) of positive screened birds, respectively. Interestingly, 9 of 11 (81.82%) of the new strains of *T. gallinae* reported here were isolated from the infected birds with lesions. These birds might not have yet developed any form of tolerance or resistance to these strains of the parasite.

*Trichomonas gallinae* genotypes were divergent between different bird species, although our sample size was too small to be conclusive. Results revealed the existence of several *T. gallinae* strains circulating in Saudi Arabian avifauna. In this study, a phylogenetic analysis was used to identify 15 unique sequences, which were clearly divided into different branches depending on the ITS ribotype (Fig. 1). Of these 15 sequences, 12 were novel, and three have been previously described. In this study, we further presented data on the genetic diversity of *T. gallinae* found in Saudi Arabian birds, with different nomenclatures to differentiate between them. Of the 15 genetic lineages found in this study, three genotypes have been described in previous studies: genotypes A (32), C (33), and II (34). Regarding the previously described lineages and NCBI, KSA1–KSA12 might be the newly detected lineages because they have not been described in previous studies. Furthermore, these lineages appear to be distinct from lineages A/B and C/D/E (32,33); thus, they might not be as common or widespread as lineages A, B, and C (33,32,40) because they were found only in pigeons in Saudi Arabia. However, judging by the overall occurrence of these three genotypes, our findings propose a widespread distribution of these genotypes among different bird species (32,36,40,41,42,43).

In this study, we found that most birds were infected by *T. gallinae* ribotypes A and C, which is consistent with previous studies that found that the pathogenicity of genotype A was strong compared with genotype C, as demonstrated by the dramatic decline in the U.K. finch population in 2007 (28,41). The C genotype isolated in our study was identified in pigeons and chickens with lesions and was an apparently pathogenic lineage. We also found this symptom in birds infected with a novel genotype, as described in this study. However, most of the birds infected with genotype A appeared to be healthy. Several pigeons and chickens with ulcers in the oral cavity were also observed to be infected with genotype C or a novel genotype. In 2009, Sansano-Maestre *et al.* (13) observed in Spanish samples that genotype C tended to be less virulent than genotype A, commonly associated with macroscopic lesions. Since then, a virulent clonal isolate of genotype A has been associated with both pigeon and passerine pathology and mortality (41). However, because virulence is likely a rapidly evolved trait (44), different strains within distinct genetic lineages will vary markedly in virulence. In support of this postulation, our study seems to suggest that it is genotype C rather than a genotype A with which the pathology of *T. gallinae* infection is primarily associated. Further research on this avian parasitic species, its genetic diversity, and its association with pathogenicity on a global scale will elucidate to what extent, if any, virulent traits can be ascribed to genetic lineages of this parasite. Additionally, further analyses using more isolates and a multilocus sequencing approach are required.

Supplemental data associated with this article can be found at https://doi.org/10.1637/aviandiseases-D-9-00162.s1.

REFERENCES

1. Cox-Witton K, Reiss A, Woods R, Grillo V, Baker RT, Blyde DJ, Boardman W, Cutter S, Lacasse C, McCracken H, *et al.* Emerging infectious diseases in free-ranging wildlife—Australian zoo based wildlife hospitals contribute to national surveillance. *PLOS ONE* 9:e95127; 2014.

2. Lachish S, Jones M, McCallum H. The impact of disease on the survival and population growth rate of the Tasmanian devil. *J Anim Ecol* 76:926–936; 2007.

3. Höner OP, Wachter B, Goller KV, Hofer H, Runyoro V, Thierer D, Fyumagwa RD, Müller T, East ML. The impact of a pathogenic bacterium on a social carnivore population. *J Anim Ecol* 81:36–46; 2012.

4. Stabler RM. *Trichomonas gallinae*, pathogenic trichomonad of birds. *J Parasitol* 33:207–213; 1947.

5. Bunbury N. Trichomonad infection in endemic and introduced columbids in the Seychelles. *J Wildl Dis* 47:730–733; 2011.

6. Stockdale JE, Dunn JC, Goodman SJ, Morris AJ, Sheehan DK, Groic PV, Hamer KC. The protozoan parasite *Trichomonas gallinae* causes adult and nestling mortality in a declining population of European turtle doves, Streptopelia turtur. *Parasitology* 142:490–498; 2015.

7. Abouhosseini Tabari M, Youssefi MR. In vitro and in vivo evaluations of Pelargonium roseum essential oil activity against *Trichomonas gallinae*. *Avicenna J PhytoMed* 8:136–142; 2018.

8. Swinnerton KJ, Greenwood AG, Chapman RE, Jones CG. The incidence of the parasitic disease trichomoniasis and its treatment in reintroduced and wild pink pigeons Columba mayeri. *Ibis* 147:772–782; 2005.

9. McKeon T, Dunsmore J, Raidal SR. *Trichomonas gallinae* in budgerigars and columbids in birds in Perth, Western Australia. *Aust Vet J* 75:652–655; 1997.

10. Mantini C, Dalia-Cornette J, Noda S, Van Der Heijden HM, Capron M, Dei-Cas E, Landman WJ, Ohkuma M, Viscogliosi E. Molecular identification and phylogenetic relationships of trichomonad isolates of galliform birds infected from nuclear small subunit rRNA gene sequences. *Parasitol Res* 106:163–170; 2009.

11. Rogers KH, Girard YA, Woods L, Johnson CK. Avian trichomonosis in spotted owls (Strix occidentalis): indication of opportunistic spillover from prey. *Int J Parasitol Parasites Wildl* 5:305–311; 2016.

12. Cousquer G. First aid and emergency care for the avian casualty. *In Prac* 27:190–203; 2005.

13. Sansano-Maestre J, Garijo-Toledo MM, Gomez-Munoz MT. Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey. *Avian Pathol* 38:201–207; 2009.

14. Burton DL, Doblar KA. Morbidity and mortality of urban wildlife in the midwestern United States. In: Shaw WW, Harris KL, Van Druff L, editors. *Proceedings of the 4th International Urban Wildlife Symposium*. 2004 May 1–5; Tucson (AZ): University of Arizona. p. 171–181; 2004.
15. Lehikoinen A, Lehikoinen E, Valkama J, Väisänen RA, Isomursu M. Impacts of trichomonosiosis epidemics on Greenfinch Chloris chloris and Chaffinch Fringilla coelebs populations in Finland. Ibis 155:357–366; 2013.

16. Brand Phillips R, Snell HL, Vargas H. Feral rock doves in the Galápagos Islands: biological and economic threats. Not Galapagos. 62:6–11; 2003.

17. Bunbury N, Jones CG, Greenwood AG, Bell DJ. Trichomomas gallinae in Mauritian columbids: implications for an endangered endemic. J Wildl Dis. 43:399–407; 2007.

18. Stabler RM. Trichomonas gallinae: a review. Exp Parasitol 3:368–402; 1954.

19. Narcisi EM, Sevoian M, Honigberg BM. Pathologic changes in pigeons infected with a virulent Trichomonas gallinae strain (Eiberg). Avian Dis. 35:55–61; 1992.

20. Stabler RM. Infection with a less virulent strain of Trichomomas gallinae as a protection against a more virulent one. J Parasitol 33:8; 1947.

21. Stabler RM. The effect of furazolidone on pigeon trichomoniasis due to Trichomonas gallinae. J Parasitol 43:280–282; 1957.

22. Tasca T, De Carli GA. Scanning electron microscopy study of Trichomonas gallinae. Vet Parasitol. 118:37–42; 2003.

23. Götze H, Ferroglio E, Höfe U, Fröhlich K, Vicente J. Diseases shared between wildlife and livestock: a European perspective. Eur J Wildl Res. 53:241; 2007.

24. da Silva DG, Barton E, Bunbury N, Lunnnes P, Bell DJ, Tyler KM. Molecular identity and heterogeneity of Trichomonas parasites in a closed avian population. Infect Genet Evol. 7:433–440; 2007.

25. Samour JH, Naldo JL. Diagnosis and therapeutic management of trichomomasiosis in falcons in Saudi Arabia. J Avian Med Surg. 17:136–144; 2003.

26. Naldo JL, Samour JH. Radiographic findings in captive falcons in Saudi Arabia. J Avian Med Surg. 18:242–257; 2004.

27. Felleisen RS. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology. 115 Part 2:111–119; 1997.

28. Robinson RA, Lawson B, Toms MP, Peck KM, Kirkwood JK, Chantrey J, Clatworthy IR, Evans AD, Hughes LA, Hutchinson OC, et al. Emerging infectious disease leads to rapid population declines of common British birds. PLOS ONE. 5:e12215; 2010.

29. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 33:1870–1874; 2016.

30. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends Biochem Sci. 23:403–405; 1998.

31. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39:783–791; 1985.

32. Lawson B, Cunningham AA, Chantrey J, Hughes LA, John SK, Bunbury N, Bell DJ, Tyler KM. A clonal strain of Trichomonas gallinae is the aetiologic agent of an emerging avian epidemic disease. Infect Genet Evol. 11:1638–1645; 2011.

33. Gerhold RW, Yabsley MJ, Smith AJ, Ostergaard E, Mannan W, Cann JD, Fischer JR. Molecular characterization of the Trichomonas gallinae morphologic complex in the United States. J Parasitol 94:1335–1341; 2008.

34. Grabensteiner E, Bilic I, Kolbe T, Hess M. Molecular analysis of clonal trichomonal isolates indicate the existence of heterogenic species present in different birds and within the same host. Vet Parasitol. 172:53–64; 2010.

35. Stabler RM. Effect of Trichomonas gallinae from diseased mourning doves on clean domestic pigeons. J Parasitol 37:473–478; 1951.

36. Farooq HA, Khan HA, Alrefaei AF, Tyler KM. Endemic infection of the common mynah Acridotheres tristis with Trichomonas gallinae the agent of avian trichomonosis. Parasitology. 145:1548–1552; 2018.

37. Mirzaei M, Ghashghaei O, Khedri J. First report of an outbreak trichomoniasis in turkey In Sistan, Iran. J Parasit Dis. 40:61–64; 2016.

38. Shemshadi B, Ranjar-bahadori S, Delfan-abazari M. Prevalence and intensity of parasitic infection in domestic ducks (Anas platyrhynchas) in Gilan Province, Northern Iran. Comp Clin Pathol. 26:165–167; 2017.

39. Bunbury N, Jones CG, Greenwood AG, Bell DJ. Epidemiology and conservation implications of Trichomonas gallinae infection in the endangered Mauritian pink pigeon. Biol Conserv. 141:153–161; 2008.

40. Zimra-Grabensteiner E, Arshad N, Armin A, Hess M. Genetically different clonal isolates of Trichomonas gallinae, obtained from the same bird, can vary in their drug susceptibility, an in vitro evidence. Parasitol Int. 60:213–215; 2011.

41. Chi JF, Lawson B, Durrant C, Beckmann K, John S, Alrefaei AF, Kirkbride K, Bell DJ, Cunningham AA, Tyler KM. The finch epidemic strain of Trichomonas gallinae is predominant in British non-passerines. Parasitology. 140:1234–1245; 2013.

42. Alrefaei AF, Gerhold RW, Nader JL, Bell DJ, Tyler KM. Improved subtyping affords better discrimination of Trichomonas gallinae strains and suggests hybrid lineages. Infect Genet Evol. 73:234–241; 2019.

43. Alrefaei AF, Low R, Hall N, Jardim R, Dávila A, Gerhold R, John S, Steinbiss S, Cunningham AA, Lawson B, et al. Multi-locus analysis resolves the epidemic finch strain of Trichomonas gallinae and suggests introgression from divergent trichomonads. Genome Biol Evol. 11:2391–2402; 2019.

44. Henrik H. Does pathogen plasticity facilitate host shifts? PLOS Pathog. 14:e1006961; 2018.

ACKNOWLEDGMENTS

We extend our appreciation to the Deanship of Scientific Research at King Saud University for funding this work through Research Project NFG-7-18-03-06, and we thank the Researchers Support Services Unit at King Saud University for their technical support.