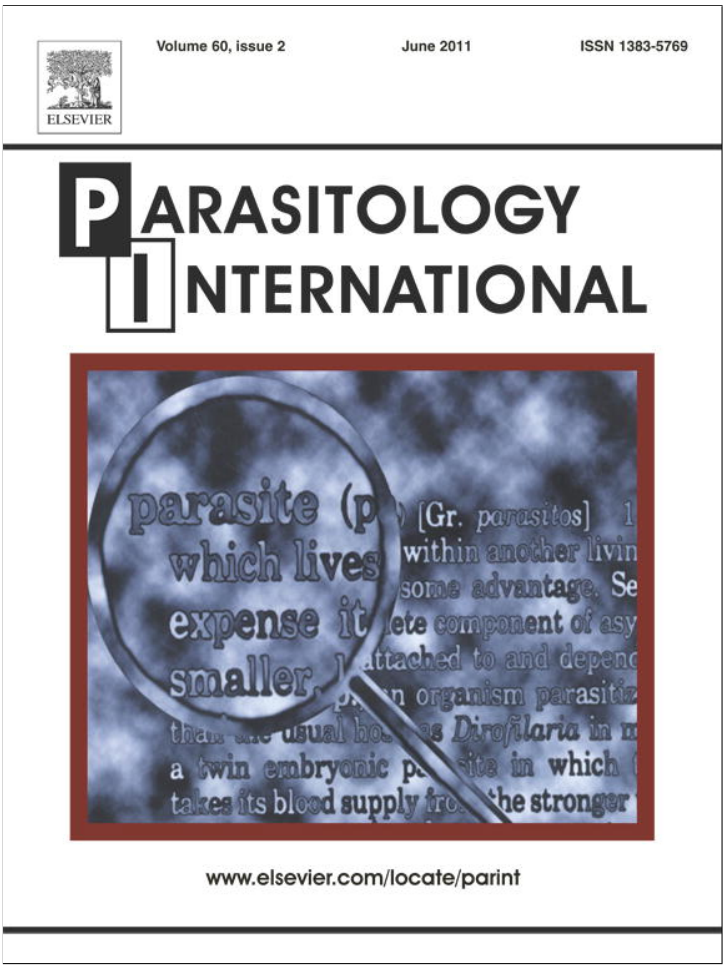

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Short communication

Genetically different clonal isolates of *Trichomonas gallinae*, obtained from the same bird, can vary in their drug susceptibility, an *in vitro* evidence

Elvira Zimre-Grabensteiner [1,2], Najma Arshad [2,3], Aziza Amin, Michael Hess *

Department for Farm Animals and Veterinary Public Health, Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna, Veterinärplatz 1, A – 1210 Vienna, Austria

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ABSTRACT

Trichomonas gallinae is a flagellated protozoon which parasitizes in the upper digestive tract of different birds, especially columbiformes (doves and pigeons) and falconiformes. The parasite is also a common inhabitant of the crop of psittacine birds and is frequently detected in budgerigars. The lesions associated with *T. gallinae* infection of the upper digestive tract range from mild inflammation of the mucosa to large caseous lesions that block the lumen of the oesophagus. Nitroimidazoles are considered to be the drugs of choice for the treatment of trichomonosis. However, only a few studies report the existence of resistant strains of *T. gallinae* to these drugs. Thus, in the present investigation cloned cultures of *T. gallinae* obtained from budgerigars and pigeons were analysed for the first time for their *in vitro* susceptibilities against four 5´-nitroimidazole derivates, including metronidazole, dimetridazole, ronidazole and ornidazole. Significantly different minimal lethal concentrations (MLCs) were observed for them against all four drugs. The lowest MLCs revealed the *Trichomonas* isolates obtained from two budgerigars, ranging from 2.0 ± 0.3 to 3.0 ± 0.7 μg/ml for metronidazole and dimetridazole, and from 2.0 ± 0.6 to 6.7 ± 1.7 μg/ml for ornidazole and ronidazole. Contrary to this, the highest MLCs were recorded for one *Trichomonas* isolate obtained from a pigeon, ranging from 83.3 ± 6.7 (for dimetridazole and ronidazole) to 103.3 ± 3.3 μg/ml (for metronidazole and ornidazole). The data obtained for the resistance testing were further compared with already available genetic data of the small subunit rRNA gene sequences and ITS-1, 5.8S rRNA and ITS-2 sequences, indicating a certain correlation between *in vitro* results and strain relationships.

Trichomonas gallinae, a flagellated protozoon and etiological agent of avian trichomonosis, inhabits the upper digestive tract of different bird species including columbiformes, galliformes, psittaciformes and passeriformes [1]. It can also affect other organs depending on the virulence of the strain. Hence, the disease presents a broad spectrum of clinical patterns ranging from asymptomatic to lethal infections with columbiformes as the main host and reservoir of *T. gallinae* [2]. The emergence of trichomonosis in different garden birds was reported recently in different countries, resulting in a severe decline of certain bird species [3].

The first drugs, used to treat trichomonosis in birds, include copper sulphate [4] and Enheptin, a 2-amino-5-nitrothiazole [5]. To date standard treatment for avian as well as human trichomonosis, the latter caused by *T. vaginalis*, are metronidazole and other 5´-nitroimidazoles [6]. Clinical resistance to these drugs though, has been reported since 1962 for *T. vaginalis* [7]. It was not until the 1990s that the first therapeutic failures have been described for treatment of avian trichomonosis and that the existence of resistant *T. gallinae* strains has been reported [8–11]. However, the interpretation obtained through these investigations is hampered by the absence of well-defined mono-eukaryotic cultures in the test systems, which is crucial as genetically different strains may occur within the same host [12]. Moreover, limited information is available on the prevalence of resistance to nitroimidazoles among clinical isolates of *T. gallinae*. Therefore, the aim of the present study was to test the *in vitro* efficacy of four different nitroimidazoles against six different clonal cultures of *T. gallinae* obtained from 5 naturally infected birds belonging to two different species, applying well established *in vitro* assays [13].

Six clonal cultures of *T. gallinae*, named *T. gallinae*/Budgerigar/Austria/5895-C1/06, *T. gallinae*/Budgerigar/Austria/15935-C3/06, *T. gallinae*/Racing Pigeon/Austria/231-C1/07, *T. gallinae*/Racing Pigeon/Austria/231-C3/07, *T. gallinae*/Racing Pigeon/Austria/7895-C2/06 and *T. gallinae*/Racing Pigeon/Austria/8855-C6/06, respectively were used in the present investigation. The assignment reflects the species of bird/country of origin/diagnostic number-clone number/year of isolation. Establishment of the *in vitro* cultures and molecular characterization together with

* Corresponding author. Tel.: +43 25077 5151; fax: +43 25077 5192.
E-mail address: Michael.Hess@vetmeduni.ac.at (M. Hess).
[1] Present address: Department for Safety and Efficacy Assessment of Human Medicinal Products, Institute for Marketing Authorisation of Medicinal Products & Life Cycle-Management, AGES PharmMed – Medicinal Products and Medical Devices, Austrian Agency for Health and Food Safety, Schnirchgasse 9, A – 1030 Vienna, Austria.
[2] These authors contributed equally to this paper.
[3] Present address: Department of Zoology, University of the Punjab, Lahore, Pakistan.

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differentiation of strains were described recently [12]. The protozoal strains were isolated during the years 2006 and 2007 out of the oropharynx from two budgerigars (*Melopsittacus undulatus*) and three racing pigeons (*Columbia livia* forma domestica) displaying natural infections of trichomonosis. All of these birds were bred and kept in captivity in Austria. Clinical examination of the birds revealed common signs including dilated crop with a thickened crop mucosa, drowsiness or apathy, reduced appetite or anorexia and weight loss. The budgerigars were further vomiting and one pigeon (no. 8855) displayed bilateral conjunctivitis (tested negative for Chlamydiosis). All birds (except pigeon no. 8855) died shortly before or after their presentation at the clinic before any treatment could be started. Post mortems of all birds revealed lesions characteristic for trichomonosis. Viable trichomonads were demonstrated in all oropharyngeal swabs of the birds under light microscopy.

Pigeon no. 8855 was one out of 40 pigeons from a flock located at the Clinic for Avian, Reptile and Fish Medicine. A high prevalence of *T. gallinae* had been recorded in the birds and several nitroimidazoles, including dimetridazol, carnidazole, metronidazole, ronidazole and ornidazole, were used to combat the disease. However, all different treatment regimes failed and an infestation of the pigeons with imidazole-resistant *T. gallinae* was suspected.

Water-soluble powder preparations of four 5´-nitroimidazole derivatives (Sigma-Aldrich, Austria), including dimetridazole, metronidazole, ornidazole and ronidazole, were tested in the present study. Stock solutions (1.0 mg/ml) of the drugs were prepared in the test media described below and stored in the dark at 4 °C. Unfortunately, the powder form of carnidazole was not available in Austria and also impossible to import. Tablets of carnidazole (Brieftauben-Spartrix®, Janssen-Cilag, Germany) were available but they were found to be insoluble in water and the test medium. Therefore, carnidazole had to be excluded from the trial.

Susceptibility testing was performed in 2.0 ml Eppendorf tubes with closed lids each of them containing a total volume of 1.0 ml test medium incorporated with different concentrations of the drugs and 10^5 protozoa [13]. The cells were exposed to the nitroimidazoles for a total of 72 h. Counting of viable cells was done after 24, 48 and 72 h. The MLC was determined as the concentration after 24 h of incubation at which no live or motile protozoa were detectable. The experiments were first performed with xenic cultures. To find out if the bacteria in the cultures had an effect on the substances, the tests were also done with axenic cultures. In brief, trichomonads were grown xenically in Medium 199 + Earle´s salts + L-glutamine + 25 mM HEPES-L-amino acids [Gibco™] containing 15% fetal bovine serum (FBS) and rice starch (1 mg/ml; Sigma Aldrich) [13] and axenic in Hollander fluid (HF) medium without agar [14] for 48 h at 37 °C. For the axenization process a mixture of meropenem (6 μg/ml), streptomycin (200 μg/ml) and penicillin (200 IU/ml) was added to the cultures containing bacteria for only three passages to get axenic cultures as published recently [14]. The final concentrations of the nitroimidazoles ranged from 0.1 to 200 μg/ml in the test system. Resistances of the clonal cultures to the imidazole drugs were defined as aerobic MLCs of greater than or equal to 50 μg/ml [15].

For each test, negative and positive controls (with regard to growth of protozoa) were also included. All experiments were performed in duplicate and repeated three times.

Data were analyzed by ANOVA followed by Tukey's test. The means and standard errors were recorded. *P* values of ≤ 0.05 were considered significant. All of the data analysis was performed with SPSS software (SPSS for Windows, 11.0.0. ed., 2001, SPSS, Chicago IL).

The anti-protozoal activities of the four 5´-nitroimidazole derivatives could be demonstrated, and the MLCs for all substances could be determined after the testing of the different clonal cultures (Table 1). The effects were independent of the bacteria as no differences of the MLCs of the isolates could be noticed testing them under xenic or axenic conditions (data not shown). However, significant differences ($p<0.05$) in the *in vitro* susceptibilities of the *Trichomonas* strains to the four nitroimidazole drugs were observed (Table 1). The most sensitive isolates proved to be the ones obtained from the two budgerigars exhibiting the lowest MLCs for the substances, ranging from 2.0 ± 0.3 up to 6.7 ± 1.7 μg/ml. Contrary to this, the most resistant isolate was shown to be *T. gallinae*/Racing Pigeon/Austria/8855-C6/06 with MLCs ranging from 83.3 ± 6.7 (for dimetridazole and ronidazole) and 103.3 ± 3.3 μg/ml (for metronidazole and ornidazole). Hence, the isolate was considered to be resistant to the four nitroimidazole drugs (MLCs ≥ 50 μg/ml). Interestingly, the two *Trichomonas* isolates (clones 1 and 3, *T. gallinae*/Racing Pigeon/Austria/231-C1/07, *T. gallinae*/Racing Pigeon/Austria/231-C3/07, respectively) which were obtained from racing pigeon no. 231 differed significantly ($p<0.05$) in their *in vitro* responses to all four substances (Table 1). Whereas, clone *T. gallinae*/Racing Pigeon/Austria/231-C1/07 proved to be rather sensitive to the four chemotherapeutics, clone *T. gallinae*/Racing Pigeon/Austria/231-C3/07 revealed much higher MLCs to the four drugs and was even found to be resistant to two of them, ornidazole and ronidazole, respectively. Moreover, clone *T. gallinae*/Racing Pigeon/Austria/231-C3/07 displayed similar MLCs as clone *T. gallinae*/Racing Pigeon/Austria/7895-C2/06.

Sequence analyses of the 18S rRNA gene and the complete genomic region spanning the two ribosomal RNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene done in a separate study [12] showed that the six clonal isolates belong to four different ITS- and four different 18S-sequence types (Table 1). Nucleotide analyses showed that the isolates of the two budgerigars were 100% identical with each other, indicating a possible correlation between genetic relationship and drug sensitivity. The same phenomenon could be noticed for strains *T. gallinae*/Racing Pigeon/Austria/231-C3/07 and *T. gallinae*/Racing Pigeon/Austria/7895-C2/06 which belong to the same ITS- or 18S-sequence type. Comparing ITS- and 18S-nucleotide sequences a complete different set of sequence type was noticed for the *T. gallinae*/Racing Pigeon/Austria/8855-C6/06, displaying complete resistance against the 4 tested nitroimidazoles.

The 5´-nitroimidazoles are the most commonly used drugs to treat protozoal infections such as *Trichomonas* infections in humans and birds [9,11,16]. However, over the years the nitroimidazole drugs seem partially to have lost their efficacy as there has been an increase in the recognition of nitroimidazole-resistant strains in humans [17]) and birds [8–11].

Franssen and Lumeij [9] were the first who carried out an *in vitro* sensitivity testing with eight different isolates of *T. gallinae* obtained from naturally infected pigeons noticing differences in the susceptibilities of the strains to certain nitroimidazole drugs. Some years later, Munoz et al. [11] conducted *in vivo* and *in vitro* experiments in pigeons demonstrating a high proportion of therapeutic failures *in vivo,* such confirming the *in vivo* and *in vitro* resistance of one pigeon isolate to several nitroimidazole drugs. However, in none of the investigations performed so far, mono-eukaryotic cultures of *T. gallinae* were used, making it impossible to distinguish whether the investigated *Trichomonas* isolate represented a culture of genetically identical trichomonads or mixture of different *Trichomonas* species. Thus, the results offer a certain lack of standardization.

Hence, in the present investigation, clonal and molecular characterized *Trichomonas* isolates were used for the first time for *in vitro* sensitivity testing. Different significant ($p<0.05$) susceptibilities of the *Trichomonas* isolates to the investigated chemotherapeutics could be demonstrated, underlying again the benefit of clonal cultures as reported recently for *H. meleagridis* [13].

Genetic variations may be one possible cause for the different sensitivity patterns of the protozoa to the compounds. It was not until recently, that the existence of different genotypes of *T. gallinae* has been reported [12,18,19] demonstrating that this species is rather a species-complex than one species with various genetic types based on 18S RNA gene and ITS-region sequence analysis. This approach enabled for the first time the inclusion of two genetically different *Trichomonas* strains obtained from a single sick bird (racing pigeon no.

Table 1
Minimal lethal concentrations (MLC, in μg/ml) of metronidazole, dimetridazole, ornidazole and ronidazole for the six different cloned xenic trichomonas cultures investigated in the present study. Values are given as Mean ± SEM of three independent experiments performed in duplicate. Data were analysed using one way ANOVA followed by Tukey's test. Values (in columns) having no common superscript are significantly different at $p<0.05$, values in bold indicate resistance (= MLC ≥ 50 μg/ml) of the isolate to the respective drug. The results of sequence and phylogenetic analyses of the trichomonas isolates are also displayed in the table.

Cloned cultures	MLCs of the test compounds in μg/ml				Sequence types of the clonal cultures[a]		Classification after phylogenetic analyses[a]
	Metronidazole	Dimetridazole	Ornidazole	Ronidazole	ITS-sequence type	18S-sequence type	
Trichomonas gallinae/Budgerigar/ Austria/5895-C1/06	2.0 ± 0.3^{c}	2.5 ± 0.3^{c}	6.7 ± 1.7^{c}	6.7 ± 1.7^{c}	ITS-IV	18S-VI	*T. gallinae*-like
Trichomonas gallinae/Budgerigar/ Austria/15935-C3/06	2.7 ± 0.3^{c}	3.0 ± 0.7^{c}	2.0 ± 0.6^{c}	2.7 ± 0.3^{c}	ITS-IV	18S-VI	*T. gallinae*-like
Trichomonas spp./Racing Pigeon/ Austria/ 231-C1/07	8.8 ± 1.3^{c}	5.0 ± 0.0^{c}	16.7 ± 1.7^{c}	5.0 ± 0.0^{c}	ITS-III	18S-VIII	*Trichomonas* sp.
Trichomonas spp./Racing Pigeon/ Austria/ 231-C3/07	28.3 ± 1.7^{b}	33.3 ± 3.3^{b}	**76.7 ± 3.3^{b}**	**65.0 ± 2.9^{b}**	ITS-II	18S-I	*T. tenax*-like
Trichomonas spp./Racing Pigeon/ Austria/ 7895-C2/06	25.0 ± 2.9^{b}	36.7 ± 3.3^{b}	**73.3 ± 6.7^{b}**	**61.7 ± 1.7^{b}**	ITS-II	18S-I	*T. tenax*-like
Trichomonas gallinae/Racing Pigeon/ Austria/ 8855-C6/06	**103.3 ± 3.3^{a}**	**83.3 ± 6.7^{a}**	**103.3 ± 3.3^{a}**	**83.3 ± 6.7^{a}**	ITS-I	18S-II	*T. gallinae*-like

[a] According to the classification published recently [12].

231) in an *in vitro* testing. The two strains – possibly representing two different *Trichomonas* species according to phylogenetic analyses – displayed significantly different drug sensitivities in the present study. One of the strains was further shown to be resistant (MLCs greater than 50 μg/ml) to two of the tested drugs.

Interestingly, this strain showed 100% nucleotide identity with a *Trichomonas* strain isolated out of another diseased pigeon (no. 7895) which also behaved similar in the *in vitro* testing. The most drug sensitive isolates proved to be the genetically close related isolates from the two budgerigars displaying similar ($p>0.05$) MLCs for all four nitroimidazoles. Contrary to this, strain no. 8855 was shown to be the most resistant one of the present study exhibiting MLCs ≥ 83.3 ± 6.7 μg/ml to all four drugs. The genetic profile of this strain was found different to those reported for the other isolates. As it was obtained from a pigeon kept within the clinic's own aviary the treatment regime is well documented. The birds from this aviary often displayed *Trichomonas* infections as the flock was located half outside the building with access to wild birds as a possible source for the infection. It could also well be that some of the pigeons received subtherapeutic dosages of the drugs as these were most of the time administered to the whole flock through the drinking water. Some birds also made efforts to drink rain water instead of the medicated water. All of these factors could have contributed to the selection of such a resistant strain. Treatment failure of avian trichomonosis due to the widespread use of subtherapeutic dosages of nitroimidazoles has been documented elsewhere [8,10]. Unfortunately, a second strain isolated from this pigeon proliferated *in vitro* only moderately and could not be maintained in culture over a long period. Thus, it could not be included in the present study.

Induction of metronidazole resistance due to the exposure of the cells to different dosages of the drug has already been demonstrated *in vitro* for other trichomonads, such as *T. vaginalis* [20] and *Tritrichomonas foetus* [21].

To conclude, in the present investigation four 5´-nitroimidazole drugs were tested for the first time for their *in vitro* efficacy against clonal cultures of *T. gallinae*-like parasites and differences in the sensitivities of the protozoal isolates were found. For the first time genetically different isolates obtained from the same bird were investigated, indicating a certain correlation between *in vitro* results and genetic relationship. A correlation between *in vitro* and *in vivo* resistance of one *Trichomonas* strain could be demonstrated. Future investigations will now focus on the isolation and characterisation of *T. gallinae*-like strains out of different bird species, subsequently using them in controlled infection experiments and performing combined *in vitro* and *in vivo* drug sensitivity testing.

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