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A reassessment of the role of oxygen scavenging enzymes in the emergence of metronidazole resistance in trichomonads

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ABSTRACT

Trichomonads are an order of parasitic protists which infect a wide range of hosts. The human parasite *Trichomonas vaginalis* and the bovine parasite *Tritrichomonas foetus* which also infects cats and swine are of considerable medical and veterinary importance, respectively. Since trichomonads are microaerophiles/anaerobes they are susceptible to 5-nitroimidazoles such as metronidazole. 5-nitroimidazoles are exclusively toxic to microaerophilic/anaerobic organisms because reduction, i.e. activation, of the drug can only occur in a highly reductive environment. 5-nitroimidazoles have remained a reliable treatment option throughout the last decades but drug resistance can be a problem.

Clinical resistance to 5-nitroimidazoles has been studied in more detail in *T. vaginalis* and has been ascribed to defective oxygen scavenging mechanisms which lead to higher intracellular oxygen concentrations and, consequently, to less drug being reduced. Two enzymes, flavin reductase (FR) and NADH oxidase have been suggested to be the major oxygen scavenging enzymes in *T. vaginalis*. The loss, or at least an impairment of FR which reduces oxygen to hydrogen peroxide, has been proposed as the central mechanism that enables the emergence of 5-nitroimidazole resistance. In this study we explored if *T. foetus* also encodes a homolog of FR and if it is, likewise, involved in resistance.

T. foetus was indeed found to express a FR but it was only weakly active as compared to the *T. vaginalis* homolog. Further, activity of FR in *T. foetus* was unchanged in metronidazole-resistant cell lines, ruling out that it has a role in metronidazole resistance. Finally, we measured oxygen scavenging rates in metronidazole-sensitive and –resistant cell lines and found that NADH oxidase and FR are not the major oxygen scavenging enzymes in trichomonads and that oxygen scavenging is possibly a consequence, rather than a cause of metronidazole resistance.

1. Introduction

Trichomonads are an order of parasitic protists (Trichomonadida) that infect a wide range of animal species. Two species are particularly of great importance as they pose a threat to either human health or to live stock and domestic animals: *Trichomonas vaginalis* and *Tritrichomonas foetus*. The former, *T. vaginalis*, is one of the most prevalent sexually transmitted pathogens worldwide and causes trichomoniasis, a urogenital infection which mainly affects women (Bouchemal et al., 2017). The latter, *Tritrichomonas foetus*, is a highly versatile veterinary parasite that infects the urogenital tract of cattle and the digestive tract of swine and cats, respectively (Dąbrowska et al., 2019). Whereas *T. foetus* seems to be a harmless commensal in swine, it can cause abortions in cows and severe diarrhea in cats, mainly in kittens. The prevalence of the parasite is very high in catteries and cattle herds all around the world (Dąbrowska et al., 2019).

Trichomonads have an anaerobic metabolism rendering them susceptible to 5-nitroimidazole drugs such as metronidazole or ronidazole (Leitsch, 2019). Nitroimidazoles are prodrugs which have to be reduced at their nitro groups before they can exert their toxic effect. Due to the very low redox potentials of these compounds, however, reduction only takes place in microaerophiles and anaerobes which have a strongly reductive cellular environment. After reduction of the nitro group, pleiotropic effects ensue, involving damage to DNA and proteins and the depletion of non-protein thiol pools (Leitsch, 2019). This dependence on an anaerobic metabolism for activity renders 5-nitroimidazoles

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comparably safe in humans and animals, although their potential carcinogenicity and teratogenicity are still a matter of debate. Due to this, 5-nitroimidazoles have been banned from use in food-producing animals, including cattle. However, ronidazole is currently the drug of choice to treat tritrichomoniasis in cats (Gookin et al., 2017).

Metronidazole and other 5-nitroimidazoles have proven highly reliable but resistance does occur in trichomonads. The first cases of nitroimidazole-resistant *T. vaginalis* were reported soon after the introduction of the drug (Leitsch, 2019). Likewise, metronidazole resistance in *T. foetus*-infected bulls was observed from early on (McLoughlin, 1967) and also ronidazole-resistant *T. foetus* has been isolated from cats (Gookin et al., 2010).

Importantly, nitroimidazole resistance in trichomonads can be grouped into so-called aerobic resistance and anaerobic resistance, respectively. The former occurs in clinical isolates and only becomes manifest in the presence of oxygen (Kulda, 1999; Leitsch, 2019), whereas the latter is induced in the laboratory by extended exposure to sublethal concentrations of the drug and also becomes manifest under anaerobiosis. Anaerobic resistance is caused by a strongly impaired uptake of the drug, arguably due to the loss of drug activating pathways (Kulda, 1999; Leitsch 2019). Aerobic resistance occurs in *T. vaginalis* (Meingassner and Thurner, 1979) and *T. foetus* (Meingassner et al., 1978) alike, but has been better studied in the former. In metronidazole-resistant clinical *T. vaginalis* strains oxygen scavenging is impaired (Yarlett et al., 1986), leading to higher intracellular oxygen levels and supposedly less nitroimidazole drug being reduced (Müller and Lindmark, 1976). Impaired oxygen scavenging has been linked to decreased levels of flavin reducatse FR1 (Leitsch et al., 2014), an enzyme that reduces oxygen to hydrogen peroxide. Indeed, flavin reductase (FR) activity and the degree of metronidazole resistance are negatively correlated (Leitsch et al., 2012), the most resistant *T. vaginalis* isolates having no flavin reductase activity at all. Importantly, flavin reductase activity is also absent from *T. vaginalis* with anaerobic metronidazole resistance (Leitsch et al., 2009). It has remained unknown if a homolog of FR1 also exists in *T. foetus* but both parasites have an NADH oxidase (Linstead and Bradley, 1988; Cerkasovová and Cerkasov, 1974; Lamien-Meda and Leitsch, 2020) which reduces oxygen to water. Interestingly, however, this enzyme activity could not be linked to 5-nitroimidazole resistance so far.

It was the aim of this study to identify and characterize a homolog of *T. vaginalis* FR1 in *T. foetus* and to assess if it is involved in metronidazole resistance in this parasite. Due to the recent publication of the *T. foetus* genome (Benchimol et al., 2017) directed data base searches were enabled, allowing the identification and testing of candidate enzymes. Flavin reductase was indeed identified in *T. foetus* but it is only weakly active as compared to *T. vaginalis* FR1 and is not correlated with metronidazole resistance. In addition, oxygen scavenging measurements with an oxygen-sensitive electrode suggest that neither FR1 nor NADH oxidase are the major oxygen scavenging enzymes in both trichomonads. These results complement but also question the proposed model of metronidazole resistance.

2. Materials and methods

2.1. Cell culture

The *T. foetus* strains KV1 (ATCC 30924), 09D2458/2009 (further referred to as 09D2458) which had been previously isolated from a diseased cat (Reinmann et al., 2012), and 1/N (ATCC 30167, "*Tritrichomonas suis*") were grown in sterile-filtered TYI-S-33 medium (Diamond et al., 1978) using Nunc culture tubes (Thermo Fisher Scientific). Metronidazole resistance was induced by culturing cells in ever increasing concentrations of metronidazole, starting with 2 μM.

T. vaginalis C1 (ATCC 30001), BRIS/92/STDL/B7268 (further referred to as B7268), and CDC085 (ATCC 50143) were grown as described (Leitsch et al., 2012). Metronidazole resistance in C1 had been induced before (Leitsch et al., 2009), and a stabilate displaying early-stage metronidazole resistance (i.e. growth in presence of up to 50 μM of metronidazole) which had been stored since at −80 °C was used for this study.

2.2. Recombinant expression of candidate T. foetus flavin reductases

Genes were amplified from cDNA using primers (Supplementary Table 1) bearing appropriate restriction sites and C-terminal 6 × histidine tags to enable purification in Ni-NTA agarose columns after expression of the recombinant proteins in *E. coli*. Amplified genes were cloned into the pET17b vector and introduced into *E. coli* expression strain BL21-AI (Thermos Fisher Scientific) in which expression of recombinant proteins was started upon addition of L-arabinose (0.1% in LB medium). After expression at 37 °C for 3 h, proteins were isolated using Ni-NTA agar columns (Qiagen) according to the manufacturer's instructions.

2.3. Enzymatic assays and determination of enzymatic parameters

Flavin reductase and NADH oxidase activites of purified recombinant flavin reductases were measured similarly as described before (Leitsch et al., 2014; Lamien-Meda and Leitsch 2020), but in 100 mM potassium phosphate pH 6.8 instead of pH 5.5. FMN, FAD, riboflavin, NADPH, and NADH were all purchased from Sigma Aldrich. Flavin reductase and NADH oxidase activities in *T. foetus* and *T. vaginalis* cell extracts were measured as described before (Leitsch et al., 2012; Lamien-Meda and Leitsch, 2020) but a larger amount of cell extract (an equivalent of 50 μg of protein instead of 20 μg) was used. Enzymatic parameters were calculated using GraphPad Prism 8 software. Statistical tests were performed as described in the legend to Fig. 2.

2.4. In-gel nitroblue tetrazolium (NBT) staining

Flavin reductase and NADH oxidase activities in KV1 cell extracts were visualized through enzymatic reduction of NBT (0.2%) in SDS-free PAA gels (10%) after gel electrophoresis according to an earlier published protocol (Leitsch et al., 2014; Lamien-Meda and Leitsch, 2020). Either NADPH (0.5 mM) or NADH (0.5 mM) was used, depending on the enzyme to be visualized. After staining, gels were rinsed in water and bands were excised and submitted for mass spectrometric analysis.

2.5. Mass spectrometry

The submitted gel bands were processed as described (Jiménez et al., 2001). In-gel digestion of proteins was performed with trypsin (20 ng μl^{-1}, Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI) for 8 h at 37 °C (Shevchenko et al., 1996) in an appropriate trypsin buffer (50 mM $NH4^{+}$ HCO_3^{-}, 5 mM $CaCl_2$). Afterwards, peptides were extracted and dried. Peptides were re-solubilised in 0.1% TFA and LC-MS/MS analysis was performed on a nano-HPLC Ultimate 3000 RSLC system (Dionex) in a 25 cm Acclaim PepMap C18 column with direct coupling to a high resolution Q Exactive HF Orbitrap mass spectrometer. Mass spectrometric full scans were performed in the ultrahigh-field Orbitrap mass analyzer and peptide masses were matched to Swiss Prot and NCBI databases.

2.6. Oxygen scavenging measurements

1.5×10^6 cells were harvested from exponential cell cultures and suspended in 14 ml of inoculation medium (TYI–SS–33 medium without ascorbic acid, serum, cysteine and iron) and filled into a 15 ml glass tube. Previously, the medium had been rigorously shaken in a bottle with ample headspace filled with air in order to ensure saturation with oxygen. Afterwards the oxygen microsensor (OX-500; Unisense, Denmark) of an oximeter (OXY-Meter; Unisense, Denmark) was inserted

and the tube carefully sealed with Parafilm M to prevent any inflow of air. Oxygen concentrations in the inoculation medium were measured automatically every 30 s for a total duration of 60 min. Background oxygen removal by medium components were measured by measuring oxygen levels in the same setup in the absence of cells. Statistical tests were performed as described in the legend to Fig. 3.

3. Results

3.1. Identification and characterization of T. foetus flavin reductases

We wanted to identify homologs of *T. vaginalis* flavin reductase 1 (Tv FR1) in *T. foetus* and searched the NCBI database with the BLAST search engine using the amino acid sequence of Tv FR1 (XP_001296413, TVAG_517010) as a query. In total, eight proteins with considerable homology to Tv FR1 (Table 1) were found. These proteins were designated Tf FR1-8 according to the degree of homology to Tv FR1, Tf FR1 being the closest homolog of Tv FR1 in *T. foetus* Tf FR8 the most distant one. When trying to amplify all FR genes from KV1 cDNA, only Tf FR1, FR2, FR3, FR5 and FR8 could be amplified. Tf FR6 could be amplified from KV genomic DNA but not from cDNA, indicating that it is not expressed. Tf FR4 and 7 could be amplified from cDNA from another *T. foetus* isolate, 1/N, which was originally designated as *Tritrichomonas suis*. After several decades of discussion it is now clear that *T. foetus* and *T. suis* are synonyms (Tachezy et al., 2002; Šlapeta et al., 2010; Dąbrowska et al., 2020). In this context it is interesting to note KV1 was isolated from cattle (Kulda and Honigberg, 1969), as was genome strain K (Benchimol et al., 2017) based on whose genome sequence all primers in this study were designed.

As a next step, all Tf FRs were cloned and expressed in *E. coli* in order to assay their functions. Tf FR3, 5, and 6 could not be expressed in *E. coli*, Tf FR3 and 5 even causing abrupt cessation of growth in *E. coli* BL 21 AI cells after induction of recombinant expression, which indicates considerable toxicity of these proteins. Tf FR4, 7 and 8 did not exhibit any flavin reductase activity when assayed according to the protocol as established before for *T. vaginalis* (Leitsch et al., 2012). In contrast, Tf FR1 and 2 displayed flavin reductase activity (Table 2) with Tf FR1 being clearly faster than FR2 but having a lower affinity for FMN. Under identical assay conditions and with FMN as a substrate, both Tf flavin reductases displayed a Vmax smaller by almost two orders of magnitude than that of *T. vaginalis* FR1 (143.8 ± 18 μmol min^{-1} mg^{-1} at an FMN concentration of 10 μM). Tf FR1 also displayed a much lower affinity for NADPH than Tv FR1 (Km of 200–600 μM NADPH as opposed to about 80 μM).

Table 1
Flavin reductases in *T. foetus* ranked according to their similarity to *T. vaginalis* FR1 (XP_001296413/TVAG_517010) as determined by BLAST search.

Name as used herein	GenBank entry	Name in NCBI database	Size (aa)	Similarity to *T. vaginalis* FR1 (% similarity/identity)
Tf FR1	OHT13998	Flavodoxin-like fold family protein	254	61/45
Tf FR2	OHT05585	Flavodoxin-like fold family protein	243	59/45
Tf FR3	OHS95900	Flavodoxin-like fold family protein	248	58/42
Tf FR4	OHS95389	quinone reductase	253	51/36
Tf FR5	OHS99549	Flavodoxin-like fold family protein	229	54/35
Tf FR6	OHT08335	Flavodoxin-like fold family protein	265	54/33
Tf FR7	OHT08325	Flavodoxin-like fold family protein	254	51/31
Tf FR8	OHT11973	Flavodoxin-like fold family protein	309	47/28

Table 2
Kinetic parameters of Tf FR1 and 2.

Enzyme	Vmax [μmol min^{-1} mg^{-1}]	Km FMN (μM)	Km NADPH (μM)
Tf FR1	4.2	13	220
Tf FR2	1.4	1.4	630

In order to obtain more evidence that FR activity in *T. foetus* is indeed exerted by Tf FR1 and/or Tf FR2, it was attempted to isolate the enzyme responsible for flavin reductase (FR) activity from KV1 cell extracts. KV1 cell extract was separated in a native PAA gel followed by staining with NBT as substrate and with NADPH as coenzyme (Fig. 1A). In accordance with previous work on *T. vaginalis* FR1 (Leitsch et al., 2014) and taking into account the relatively small size of the flavin reductases, the lowest band was excised and submitted for identification by mass spectrometry. Indeed, the band was found to contain Tf FR1 but no other FR (Supplementary Table 2). The same procedure was repeated with NADH as coenzyme to isolate and identify NADH oxidase in *T. foetus* (Fig. 1B). As expected, the "pyridine nucleotide-disulfide oxidoreductase family protein" OHT07162 which is the only homolog of *T. vaginalis* NADH oxidase (Lamien-Meda and Leitsch, 2020) was found amongst the proteins inside the excised gel snippet (Supplementary Table 3). This renders OHT07162 a highly probable NADH oxidase candidate. However, it was not attempted to confirm NADH oxidase activity of OHT07162 by assaying recombinant enzyme preparations.

3.2. Flavin reductase and NADH oxidase activites in T. foetus and T. vaginalis cell extracts

In order to gain more information on the physiological importance of FR in *T. foetus*, FR activity was measured in KV1 cell extracts and was

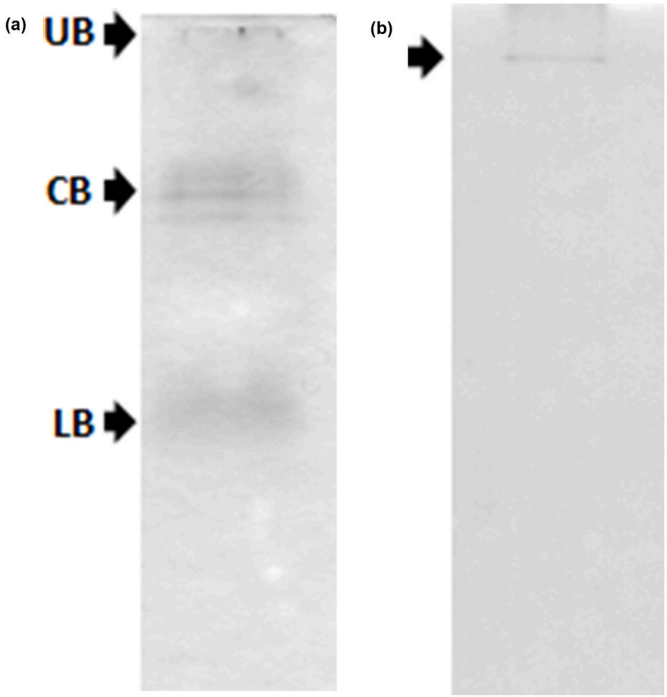
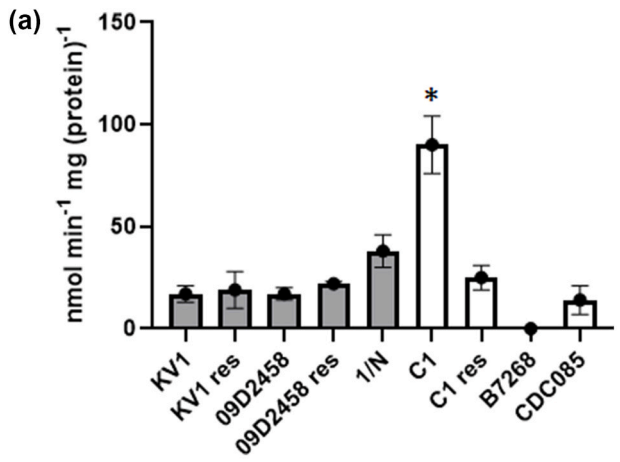
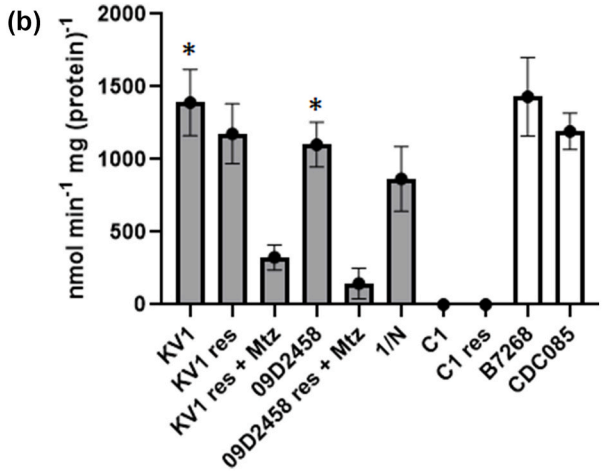


Fig. 1. Isolation of flavin reductase (A) and NADH oxidase (B) from cell extracts of *T. foetus* KV1 via native gel electrophoresis and ensuing NBT staining. In both experiments cell extract was loaded in equivalents of 50 μg protein. UB, upper band; CB, central band; LB, lower band.





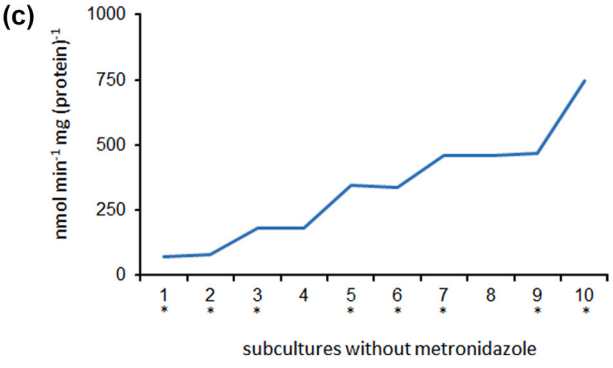


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Fig. 2. **A**, activities of FR in cell extracts of *T. foetus* KV1 (wildtype and resistant), *T. foetus* 09D2458 (wildtype and resistant), *T. foetus* 1/N, *T. vaginalis* C1 (wildtype and resistant), *T. vaginalis* B7268, and *T. vaginalis* CDC085. *Multiple comparisons with an initial Kruskal-Wallis test on ranks ($p < 0.001$), followed by the two-stage step-up method of Benjamini, Krieger, and Yekuteli as a post-hoc test to reveal C1 > KV1 ($q < 0.001$), C1 > KV1 res ($q < 0.01$), C1 > 09D2458 ($q < 0.05$), C1 > 09D2458 res ($q < 0.05$), C1 > C1 res ($q < 0.05$), C1 > B7268 ($q < 0.001$), and C1 > CDC085 ($q < 0.01$). Grey bars: *T. foetus*; white bars: *T. vaginalis*. Error bars indicate SD. **B**, NADH oxidase activity in *T. foetus* KV1 (wildtype, resistant, and resistant grown with metronidazole), *T. foetus* 09D2458 (wildtype and resistant), *T. foetus* 1/N, *T. vaginalis* C1 (wildtype and resistant), *T. vaginalis* B7268, and *T. vaginalis* CDC085. * Multiple comparisons with an initial Kruskal-Wallis test on ranks ($p < 0.001$), followed by a post-hoc Dunn's test: KV1 > KV1 res + Mtz ($p < 0.001$); 09D2458 > 09D2458 res + Mtz ($p < 0.05$). Grey bars: *T. foetus*; white bars: *T. vaginalis*. Error bars indicate SD. **C**, gradual increase of NADH oxidase activity in metronidazole-resistant 09D2458 (09D2458 res) after culture without metronidazole. Asterisks indicate that a measurement was performed. The numbers on the x-axis indicate the number of passages without metronidazole.

found to be very low, i.e. 9- to 10-fold lower than in *T. vaginalis* C1 (Fig. 2A). This low FR activity in KV1 cell was consistent with the low activities of Tf FR1 and FR2 as measured before. In order to rule out that the measured values were strain-specific, FR activity was also determined in the feral isolate 09D2458 and the porcine isolate 1/N. Also in 09D2458 FR activity was very low but in 1/N it was about three times higher amounting to about 40% of the rate as measured in *T. vaginalis* C1 (Fig. 2A).

Next, it was measured if FR activity was decreased in metronidazole-resistant cell lines of the strains tested which would be indicative of a role of Tf FR in metronidazole resistance. Metronidazole resistance could be easily induced in the strains KV1 and 09D2458 by starting at a low concentration of 2 μM metronidazole and subsequently increasing the concentration gradually with every passage, but attempts to do so in 1/N failed repeatedly. Induction of resistance in KV1 and 09D2458 was stopped at 100 μM metronidazole (equalling about 16 μg ml^{-1}) which is higher than serum levels in treated animals but much lower than the maximal level which can be achieved experimentally *in vitro* (Kulda et al., 1984). Interestingly, FR activity was not negatively affected in metronidazole-resistant KV1 and 09D2458 (Fig. 2A) but even slightly elevated (if not to a statistically significant extent). Thus, FR does not have a role in the emergence of metronidazole resistance in these strains. For comparison, FR activity was also measured in a metronidazole-resistant cell line of C1 which had been induced before (Leitsch et al., 2009) and which can grow in the presence of up to 50 μM metronidazole (early stage resistance). In this cell line FR activity was found to be decreased by almost 75% (Fig. 2A), underscoring the importance of FR deficiency already in early stage metronidazole resistance in *T. vaginalis* (Leitsch et al., 2014). As reported before (Leitsch et al., 2012), FR activities were also very low in the metronidazole-resistant *T. vaginalis* isolates B7268 and CDC085 (Fig. 2A).

In order to obtain more information on a possible link of metronidazole resistance and oxygen scavenging in *T. foetus*, also NADH activity was measured in cell extracts of the same strains (Fig. 2B). KV1 displayed very distinct NADH oxidase activity which was well comparable to earlier measurements (Müller, 1973). In the metronidazole-resistant KV1 cell line, NADH oxidase activity was found to be decreased by 75% (Fig. 2B). This effect, however, was only observed when cells were grown in the presence of metronidazole (100 μM). When metronidazole-resistant KV1 were grown over-night without metronidazole, NADH oxidase activities were similar to those in wildtype KV1 (Fig. 2B). This, however, did not reflect a reversal to the metronidazole-sensitive state as these cells could be readily grown again in the presence of metronidazole. In fact, metronidazole resistance was found to be stable for at least 15 subcultures without metronidazole. This indicates that the lowered NADH oxidase activity as observed in

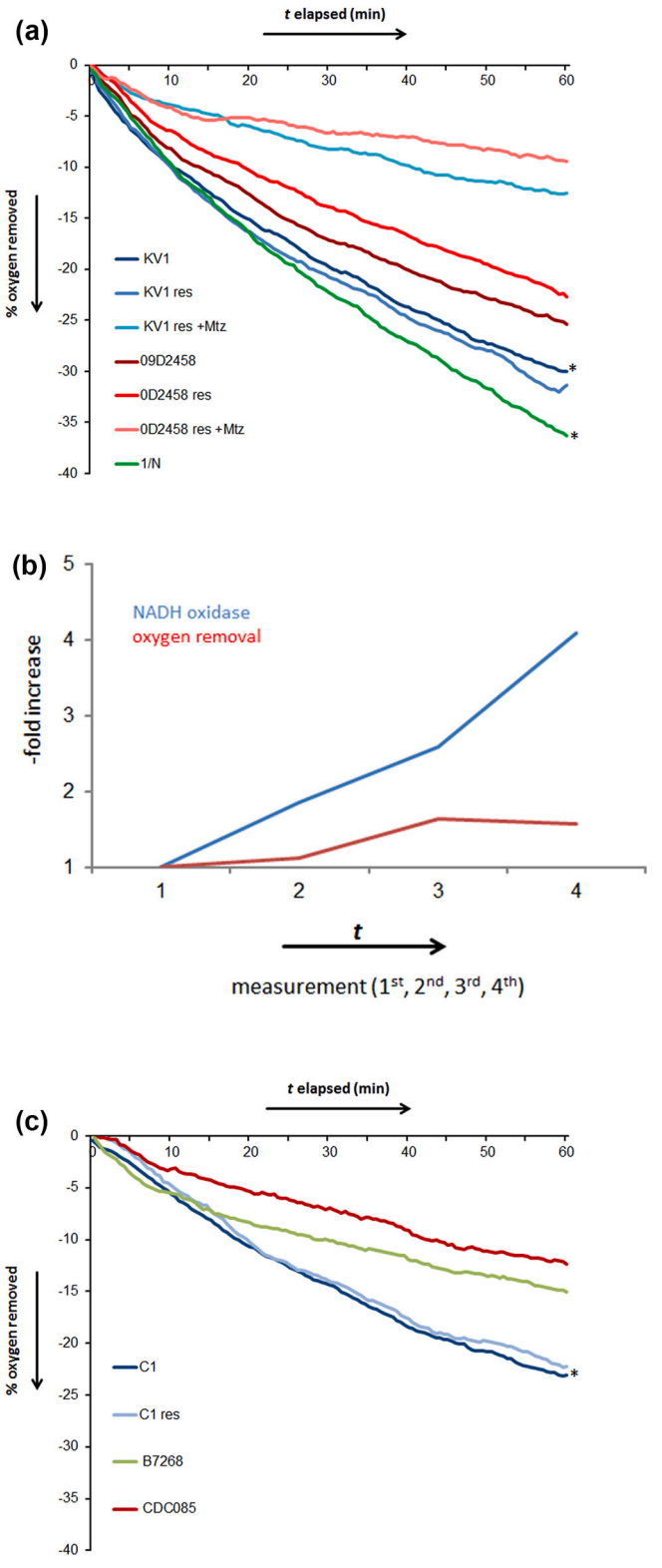

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Fig. 3. Oxygen scavenging measurements with *T. foetus* and *T. vaginalis*. For all measurements cells (15×10^6) were incubated in growth medium without cysteine, serum, iron and ascorbic acid for 60 min and removal of oxygen was measured with an oxygen-sensitive electrode. Curves represent averages of at least four biological replicates. **A**, oxygen scavenging rates of *T. foetus* KV1 (wildtype, resistant, and resistant grown with metronidazole), *T. foetus* 09D2458 (wildtype, resistant, and resistant grown with metronidazole), and *T. foetus* 1/N. *Multiple comparisons with an initial Kruskal-Wallis test on ranks ($p < 0.001$), followed by the two-stage step-up method of Benjamini, Krieger, and Yekuteli as a post-hoc test to reveal KV1 faster than KV1 res + Mtz ($q < 0.05$), KV1 faster than 09D2458 res + Mtz ($q < 0.05$), 1/N faster than KV1 res + Mtz ($q < 0.05$), and 1/N faster than 09D2458 res + Mtz ($q < 0.05$). **B**, unequally pronounced increase of oxygen scavenging rates and NADH oxidase activities in metronidazole-resistant *T. foetus* 09D2458 (09D2458 res) after several passages without metronidazole. Four measurements were performed with the same culture and on the same day each: after 6, 7, 9, and 10 passages without metronidazole. Values of the first measurement were set to 1 with the following three measurements indicating the –fold increments relating to the first measurement. **C**, oxygen scavenging rates of *T. vaginalis* C1 (wildtype and resistant), *T. vaginalis* B7268, and *T. vaginalis* CDC085 cell lines. * Student's t-test showing that C1 faster than CDC085 ($p < 0.01$).

metronidazole-resistant KV1 grown with metronidazole is not a cause of metronidazole resistance but rather a consequence, brought about by the inhibition of the enzyme. A similar observation had been made before in *T. vaginalis* G3 (Leitsch et al., 2014). Strain 09D2458 displayed an NADH oxidase activity well comparable to that of KV1 and, as observed before with resistant KV1, NADH oxidase activity of resistant 09D2458 was strongly decreased when cells had been sub-cultured in the presence of metronidazole prior to enzyme assays (Fig. 2B). In contrast to our observations with the resistant cell line of KV1, however, NADH oxidase activity in resistant 09D2458, did not recover fully after one passage without metronidazole. Rather, NADH oxidase activity increased incrementally with every passage without metronidazole (Fig. 2C) and reached about 70% of the original level after ten passages. The porcine isolate 1/N had a slightly lower NADH oxidase activity than the two other *T. foetus* strains, albeit not to a statistically significant extent (Fig. 2B).

In the resistant clinical *T. vaginalis* strains B7268 and CDC085 NADH oxidase levels were comparable to those in the three *T. foetus* strains (Fig. 2B). Importantly, however, strain C1 lacks NADH oxidase activity because its NADH oxidase gene (TVAG_049830) has a frame shift mutation resulting in a prematurely terminating polypeptide (Lamien-Meda and Leitsch, 2020).

3.3. Measurement of oxygen scavenging rates in T. foetus and T. vaginalis

We wanted to test if altered activities of FR and NADH oxidase in the metronidazole-resistant cell lines would lead to impaired oxygen scavenging as reported before for metronidazole-resistant *T. vaginalis* clinical isolates (Yarlett et al., 1986). According to the absent NADH oxidase activity and the strongly decreased FR activity in metronidazole-resistant C1 (Fig. 2), we expected this cell line to display minimal oxygen scavenging rates.

In order to address these hypotheses we measured the oxygen scavenging rate of each cell line in tightly sealed culture tubes with an oximeter (Fig. 3A). The oxygen scavenging rate in metronidazole-resistant *T. foetus* KV1 was more than 50% lower than that in wildtype KV1, but only if cells had been grown with metronidazole prior to the assays. After growth without metronidazole, oxygen scavenging rates of both cell lines were practically identical (Fig. 3A). A similar observation was made with 09D2458 and its resistant cell line (Fig. 3A). The porcine 1/N displayed the fastest oxygen scavenging rate. In order to assess the impact of NADH oxidase on oxygen scavenging more directly we measured NADH oxidase activity and oxygen scavenging in cultures of resistant 09D2458 on the same day (Fig. 3B). NADH oxidase activity and oxygen scavenging both increased with the number of passages without

metronidazole, but the former increased at a far higher pace. The last measurement, performed after ten passages without metronidazole, even revealed a sharp increase of NADH oxidase activity accompanied by a slight decrease in the oxygen scavenging rate (Fig. 3B). This finding suggests that NADH oxidase activity is not directly indicative of the oxygen scavenging rate.

Oxygen scavenging also proved to be totally independent of NADH oxidase activity in *T. vaginalis* (Fig. 3C). The two resistant clinical strains B7268 and CDC085 removed oxygen more slowly than the metronidazole sensitive strain C1, although the former have pronounced NADH oxidase activities whereas the latter has no NADH oxidase activity at all (Fig. 2B). Moreover, the metronidazole-sensitive and -resistant C1 cell lines displayed identical oxygen scavenging rates (Fig. 3C) although FR activity in the resistant cell line is down to about 25% as compared to the sensitive cell line (Fig. 2A). Taken together, these results indicate that FR and NADH oxidase are not determining oxygen scavenging rates and are not the major oxygen scavenging enzymes in *T. foetus* and *T. vaginalis*. Furthermore, metronidazole resistance is not necessarily linked to impaired oxygen scavenging.

4. Discussion

In this study we wanted to explore if metronidazole resistance mechanisms as described for *T. vaginalis* (Leitsch et al., 2014; Leitsch, 2019) do also apply for another trichomonadid parasite, *T. foetus*. One of the postulated key events in the emergence of metronidazole resistance in *T. vaginalis* is the loss of, or at a least sharp decrease in FR activity (Leitsch et al., 2009, 2010, 2014) which is predominantly exerted by Tv FR1 but also by other, less active homologs (Leitsch et al., 2014). Flavin reductase reduces molecular oxygen to hydrogen peroxide via FMN (and, less efficiently, other flavins) and has been suggested to be the major source of hydrogen peroxide in *T. vaginalis* (Chapman et al., 1999). As shown herein, FR also exists in *T. foetus*, again as a set of several homologs, i.e. eight as opposed to seven in *T. vaginalis*. Only with two of these, Tf FR1 and Tf FR2, FR activity could be demonstrated (Table 2), and only one, Tf FR1, was found in NBT-stained bands in native polyacrylamide gels (Fig. 1A). This indicates that Tf FR1 is the major FR in *T. foetus*. Surprisingly, however, it proved to be almost two orders of magnitude slower than its counterpart in *T. vaginalis* with altogether less favourable parameters than the latter (Table 2). FR activity was also much lower in cell extracts of all three *T. foetus* strains assayed than in cell extracts of *T. vaginalis* C1 (Fig. 2A). In case of KV1 and 09D2458 FR activities were 9 to 10-fold lower than in *T. vaginalis* C1 and even lower than in many metronidazole-resistant *T. vaginalis* strains (Leitsch et al., 2012). FR activities were not found to be further decreased in metronidazole-resistant cell lines of KV1 and 09D2458, whereas in metronidazole-resistant *T. vaginalis* C1 FR activity was decreased by about 75%. We conclude that a decrease in FR activity is a hallmark event in the emergence of metronidazole resistance in *T. vaginalis* but not in *T. foetus*, arguably because FR activities are already very low in *T. foetus* anyway. Things could be different in strain 1/N, however. In this strain, FR activity amounts to about 40% of the rate in *T. vaginalis* C1 (Fig. 2A). It is interesting to hypothesize that induction of metronidazole resistance failed in *T. foetus* 1/N because FR activity was too high and could not be downregulated. In *T. vaginalis*, loss of FR activity is achieved through epigenetic regulation and not due to gene mutation (Leitsch et al., 2014), but it is currently unknown if similar epigenetic regulatory mechanisms are in place in *T. foetus* as in *T. vaginalis* (Pachano et al., 2017; Lizarraga et al., 2020).

In contrast to FR, NADH oxidase activity, very likely exerted by enzyme OHT07162 as determined in this study (Fig. 1B), was lower in metronidazole-resistant *T. foetus* KV1 and 09D2458, but only when cells had been grown before with metronidazole (Fig. 2B). This suggests an inhibitory effect of metronidazole on *T. foetus* NADH oxidase, similar to that observed previously in *T. vaginalis* (Leitsch et al., 2014), which argues against a causal relation of NADH oxidase activity and metronidazole resistance.

Unexpectedly, the oxygen scavenging rates of the *T. foetus* and *T. vaginalis* cell lines studied proved to be independent of FR and NADH oxidase activities (Fig. 3). The enzymes which remove the bulk of oxygen in trichomonads remain to be discovered, as are the true physiological roles of FR and NADH oxidase. Furthermore, the notion that metronidazole resistance in trichomonads is necessarily accompanied by impaired oxygen scavenging must be revised. Although the removal of oxygen was found to be slowed down by about 50% in the resistant *T. foetus* KV1 and 09D2458 cell lines, this was only the case if the cells had been grown in the presence of metronidazole prior to the measurements. Since the resistant KV1 cell line retained resistance even after 15 subcultures without metronidazole this suggests that tolerance to metronidazole was not caused by impaired oxygen scavenging. Rather, impaired oxygen scavenging proved to be an effect of the supplementation of the growth medium with metronidazole. It is important to emphasize that the resistant cell lines studied here were in an early stage of resistance. After prolonged exposure to metronidazole in laboratory culture resistant trichomonads might permanently display impaired oxygen scavenging due to epigenetic regulation mechanisms, thereby blurring causal relations. This might indeed also apply to metronidazole-resistant clinical *T. vaginalis* isolates such as B7268 and CDC085 which display lowered oxygen scavenging rates independently of the supplementation of the growth medium with metronidazole (Fig. 3C). However, as metronidazole-resistant clinical isolates vary in many aspects from *in vitro* metronidazole-resistant cell lines (Leitsch, 2019), it would be premature as of now to dismiss impaired oxygen scavenging as a causative factor of metronidazole resistance *in vivo*.

Although the results of this study show that FR and NADH oxidase are not the major oxygen scavenging enzymes in *T. vaginalis* and *T. foetus*, they further corroborate the importance of FR downregulation in the emergence of metronidazole resistance in *T. vaginalis*, both *in vivo* and *in vitro*. The smaller amounts of hydrogen peroxide produced in strains with decreased FR activity might enhance tolerance to metronidazole to a relevant extent. Further research will have to focus on how and why tolerance to metronidazole and hydrogen peroxide correlate. It is interesting, however, to speculate on possible contexts. The inhibition of thioredoxin reductase (TrxR) by metronidazole (Leitsch et al., 2007, 2009, 2016), and thereby indirectly of peroxiredoxins, can be expected to result in delayed removal of hydrogen peroxide. Hydrogen peroxide, however, is detrimental in the strongly reductive intracellular environment of microaerophiles and anaerobes because it reacts with thiols and ferrous iron, leading to the formation of highly reactive hydroxyl radicals. The upregulation of TrxR expression as a compensating response, however, might also prove harmful due to TrxR's capability to reduce non-canonical substrates such as nitro compounds. Indeed, TrxR can reduce and thereby activate metronidazole in *T. vaginalis* (Leitsch et al., 2009), *Entamoeba histolytica* (Leitsch et al., 2007), and *Giardia lamblia* (Leitsch et al., 2011). Accordingly, TrxR was found to be nearly inactive in a highly metronidazole-resistant *T. vaginalis* C1 cell line due to the absence of its FAD cofactor (Leitsch et al., 2009). Thus, down-regulation of FR which is the major source of hydrogen peroxide in *T. vaginalis* (Chapman et al., 1999) might have a dual effect: it leads to a higher tolerance to metronidazole because metronidazole-induced inhibition of TrxR does not lead to an accumulation of hydrogen peroxide, and it facilitates the emergence of high level metronidazole resistance by enabling the loss of TrxR activity which can be only tolerated by the cell if endogenous hydrogen peroxide levels are very low. It is important to emphasize, however, that TrxR activity is not decreased in clinical metronidazole-resistant *T. vaginalis* isolates such as B7268 and CDC085 (Leitsch et al., 2012), arguably because the enzyme is necessary for survival in the host.

As a conclusion, the results of this study suggest that FR and NADH oxidase are not the major oxygen scavenging enzymes in *T. vaginalis* and *T. foetus*. Although the sharp decrease of FR activity in a *T. vaginalis* C1 cell line with early stage metronidazole resistance constitutes further

evidence for an involvement of FR in metronidazole resistance in *T. vaginalis*, similar observations could not be made in *T. foetus*. The physiological roles of NADH oxidase and FR in trichomonads remain to be discovered. Possibly, the near or total loss of FR activity in metronidazole-resistant *T. vaginalis* renders these isolates vulnerable to alternative therapies. Future research will have to focus on this question.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.04.004.

References

Bouchemal, K., Bories, C., Loiseau, P.M., 2017. Strategies for prevention and treatment of *Trichomonas vaginalis* infections. Clin. Microbiol. Rev. 30, 811–825.

Benchimol, M., de Almeida, L.G.P., Vasconcelos, A.T., de Andrade Rosa, I., Reis Bogo, M., Kist, L.W., de Souza, W., Benchimol, M., 2017. Draft genome sequence of Tritrichomonas foetus strain K. Genome Announc. 5, e00195-17.

Cerkasovová, A., Cerkasov, J., 1974. Location of NADH-oxidase activity in fractions of *Tritrichomanas foetus* homogenate. Folia Parasitol. 21, 193–203.

Chapman, A., Linstead, D.J., Lloyd, D., 1999. Hydrogen peroxide is a product of oxygen consumption by *Trichomonas vaginalis*. J. Biosci. 24, 339–344.

Dąbrowska, J., Karamon, J., Kochanowski, M., Sroka, J., Zdybel, J., Cencek, T., 2019. Tritrichomonas foetus as a causative agent of tritrichomonosis in different animal hosts. J. Vet. Res. 63, 533–541.

Dąbrowska, J., Keller, I., Karamon, J., Kochanowski, M., Gottstein, B., Cencek, T., Frey, C.F., Müller, N., 2020. Whole genome sequencing of a feline strain of *Tritrichomonas foetus* reveals massive genetic differences to bovine and porcine isolates. Int. J. Parasitol. 50, 227–233.

Diamond, L.S., Harlow, D.R., Cunnick, C.C., 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. 72, 431–432.

Gookin, J.L., Stauffer, S.H., Dybas, D., Cannon, D.H., 2010. Documentation of in vivo and in vitro aerobic resistance of feline *Tritrichomonas foetus* isolates to ronidazole. J. Vet. Intern. Med. 24, 1003–1007.

Gookin, J.L., Hanrahan, K., Levy, M.G., 2017. The conundrum of feline trichomonosis. J. Feline Med. Surg. 19, 261–274.

Jiménez, C.R., Huang, L., Qiu, Y., Burlingame, A.L., 2001. Enzymatic approaches for obtaining amino acid sequence: on-target ladder sequencing. Curr. Protoc. Protein. Sci. Chapter 16. Unit 16.4.

Kulda, J., Honigberg, B.M., 1969. Behavior and pathogenicity of *Tritrichomonas foetus* in chick liver cell cultures. J. Protozool. 16, 479–495.

Kulda, J., Cerkasov, J., Demes, P., Cerkasovová, A., 1984. *Tritrichomonas foetus*: stable anaerobic resistance to metronidazole *in vitro*. Exp. Parasitol. 57, 93–103.

Kulda, J., 1999. Trichomonads, hydrogenosomes and drug resistance. Int. J. Parasitol. 29, 199–212.

Lamien-Meda, A., Leitsch, D., 2020. Identification of the NADH-oxidase gene in *Trichomonas vaginalis*. Parasitol. Res. 119, 683–686.

Leitsch, D., Kolarich, D., Wilson, I.B.H., Altmann, F., Duchêne, M., 2007. Nitroimidazole action in *Entamoeba histolytica*: a central role for thioredoxin reductase. PLoS Biol. 5, 1820–1834.

Leitsch, D., Kolarich, D., Binder, M., Stadlmann, J., Altmann, F., Duchêne, M., 2009. *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. Mol. Microbiol. 72, 518–536.

Leitsch, D., Kolarich, D., Duchene, M., 2010. The flavin inhibitor diphenyleneiodonium renders *Trichomonas vaginalis* resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways. Mol. Biochem. Parasitol. 171, 17–24.

Leitsch, D., Burgess, A.G., Dunn, L.A., Krauer, K.G., Tan, K., Duchene, M., Upcroft, P., Eckmann, L., Upcroft, J.A., 2011. Pyruvate:ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. J. Antimicrob. Chemother. 66, 1756–1765.

Leitsch, D., Drinić, M., Duchêne, M., 2012. Down-regulation of flavin reductase and alcohol dehydrogenase-1 (ADH-1) in metronidazole-resistant isolates of *Trichomonas vaginalis*. Mol. Biochem. Parasitol. 183, 177–183.

Leitsch, D., Janssen, B.D., Kolarich, D., Johnson, P.J., Duchêne, M., 2014. *Trichomonas vaginalis* flavin reductase 1 and its role in metronidazole resistance. Mol. Microbiol. 91, 198–208.

Leitsch, D., Muller, J., Muller, N., 2016. Evaluation of *Giardia lamblia* thioredoxin reductase as drug activating enzyme and as drug target. Int. J. Parasitol. Drugs Drug Resist. 6, 148–153.

Leitsch, D., 2019. A review on metronidazole: an old warhorse in antimicrobial chemotherapy. Parasitology 146, 1167–1178.

Linstead, D.J., Bradley, S., 1988. The purification and properties of two soluble reduced nicotinamide:acceptor oxidoreductases from *Trichomonas vaginalis*. Mol. Biochem. Parasitol. 27, 125–133.

Lizarraga, A., O'Brown, Z.K., Boulias, K., Roach, L., Greer, E.L., Johnson, P.J., Strobl-Mazzulla, P.H., de Miguel, N., 2020. Adenine DNA methylation, 3D genome organization, and gene expression in the parasite *Trichomonas vaginalis*. Proc. Natl. Acad. Sci. U.S.A. 117, 13033–13043.

Meingassner, J.G., Mieth, H., Czok, R., Lindmark, D.G., Müller, M., 1978. Assay conditions and the demonstration of nitroimidazole resistance in *Tritrichomonas foetus*. Antimicrob. Agents Chemother. 13, 1–3.

Meingassner, J.G., Thurner, J., 1979. Strain of *Trichomonas vaginalis* resistant to metronidazole and other 5-nitroimidazoles. Antimicrob. Agents Chemother. 15, 254–257.

McLoughlin, D.K., 1967. Drug tolerance by *Tritrichomonas foetus*. J. Parasitol. 53, 646–648.

Müller, M., 1973. Biochemical cytology of trichomonad flagellates. I. Subcellular localization of hydrolases, dehydrogenases, and catalase in *Tritrichomonas foetus*. J. Cell Biol. 57, 453–474.

Müller, M., Lindmark, D.G., 1976. Uptake of metronidazole and its effect on viability in trichomonads and *Entamoeba invadens* under anaerobic and aerobic conditions. Antimicrob. Agents Chemother. 9, 696–700.

Pachano, T., Nievas, Y.R., Lizarraga, A., Johnson, P.J., Strobl-Mazzulla, P.H., de Miguel, N., 2017. Epigenetics regulates transcription and pathogenesis in the parasite *Trichomonas vaginalis*. Cell Microbiol. 19, e12716.

Reinmann, K., Müller, N., Kuhnert, P., Campero, C.M., Leitsch, D., Hess, M., Henning, K., Fort, M., Müller, J., Gottstein, B., Frey, C.F., 2012. *Tritrichomonas foetus* isolates from cats and cattle show minor genetic differences in unrelated loci ITS-2 and EF-1α. Vet. Parasitol. 185, 138–144.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., Mann, M., 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. U.S.A. 93, 14440–14445.

Šlapeta, J., Craig, S., McDonell, D., Emery, D., 2010. *Tritrichomonas foetus* from domestic cats and cattle are genetically distinct. Exp. Parasitol. 126, 209–213.

Tachezy, J., Tachezy, R., Hampl, V., Sedinová, M., Vanacová, S., Vrlík, M., Van Ranst, M., Flegr, J., Kulda, J., 2002. Cattle Pathogen *Tritrichomonas Foetus* (Riedmüller, 1928) and Pig Commensal *Tritrichomonas Suis* (Gruby & Delafond, 1843) Belong to the Same Species.

Yarlett, N., Yarlett, N.C., Lloyd, D., 1986. Metronidazole-resistant clinical isolates of *Trichomonas vaginalis* have lowered oxygen affinities. Mol. Biochem. Parasitol. 19, 111–116.