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Research paper

Flow cytometry evaluation of *in vitro* susceptibility of bovine isolates of *Tritrichomonas foetus* to metronidazole

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ABSTRACT

Bovine Trichomonosis, an endemic sexually transmitted disease in countries with extensive livestock and natural service, represents one of the most common causes of reproductive failure. 5-nitroimidazoles and their derivatives are used for its treatment, mainly metronidazole (Mz). The emergence of resistance mechanisms adopted by the parasites against the drug and failure of the treatments suggest the need to investigate susceptibility and obtain current values. The available information of *in vitro* susceptibility of these drugs comes from the use of a diversity of methodologies and criteria, especially observation of the mobility of the parasite under the optical microscope to evaluate its viability. These techniques are arduous and time consuming and lead to a subjective assessment dependent on the operator, the methodology used, and the morphology adopted by the protozoan. In this sense, flow cytometry has proven to be a fast and efficient method to evaluate viability in other protozoa. The aim of this study was to evaluate the *in vitro* susceptibility of six bovine isolates of *Tritrichomonas foetus* to Mz in aerobic (AC) and anaerobic (ANC) conditions by means of the calculation of the 50% inhibitory concentration (IC_{50}), by flow cytometry, and also to analyze minimum lethal concentration (MLC) by means of recovery tests post-treatment *in vitro*. IC_{50} values ranged from 1.06 to 1.25 µM in ANC and from 1.44 to 3.03 µM in AC, these being the only ones reported at 48 h for these protozoa. With respect to MLC at 48 h, the results were from 3.67 to 7.35 µM in ANC, and from 7.35 to 14.7 µM for AC, where two isolates (Tf0 and Tf2) for AC and one (Tf2) for ANC showed higher values than those described in the literature. Flow cytometry has proven to be an effective, rapid and objective methodology and very useful in susceptibility tests. The data obtained through these tests allow us to describe the susceptibility exhibited by these protozoa, this being valuable information when establishing dosages in Mz treatments.

1. Introduction

Bovine Trichomonosis (BT), a sexually transmitted disease with worldwide distribution, is endemic in most countries that have extensive livestock farming with natural breeding as a reproductive method (Filho et al., 2018; Mardones et al., 2008; Rae et al., 2004). It represents one of the most common causes of reproductive failure due to embryonic and fetal loss resulting in a marked decrease in the pregnancy rate of a bovine herd and important economic losses. The etiological agent of BT is the flagellated protozoan *Tritrichomonas foetus* (Wenrich and Emmerson, 1933). Is also naturally present as a commensal in pigs (Doi et al., 2013) and in domestic cats *T. foetus* causes feline enteric trichomonosis (Levy et al., 2003; Yaeger and Gookin, 2005). However, several studies suggest a revision because they could be different species (Slapeta et al., 2012; Walden et al., 2013). For years, the drugs available for the treatment of BT have been 5-nitroimidazoles and their derivatives, mainly metronidazole (Mz) (Bader et al., 2016; Kather et al., 2007; Kulda et al., 1984; Meingassner et al., 1978). Its antimicrobial action is based on the metabolic reduction of the nitro group to a toxic intermediate in hydrogenosomes, which results in the release of reactive free radicals that cause cell deterioration, fundamentally DNA degradation, leading to cell death (Kulda, 1999; Vázquez et al., 2001). In recent years, the appearance of two types of resistance mechanisms has been reported, an aerobic one and another

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related to the anaerobic metabolism, *in vivo* and *in vitro*, respectively (Kulda, 1999). At present there is no effective therapy against BT (Bader et al., 2016; Carvalho and Gadelha, 2007). Some imidazoles not have been approved for use in livestock in many countries (Bader et al., 2016; Cobo and Campero, 2002; Corbeil, 1994; Love et al., 2017). That is why the usual practice is not to treat *T. foetus* positive bulls and sent to slaughter. Data concerning the *in vitro* susceptibility of these drugs comes from the use of a great variety of methodologies carried out with different criteria to elucidate the values of parameters such as minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC), where mobility prevails as a form of feasibility assessment (Bader et al., 2016; Kather et al., 2007; Meingassner et al., 1978). This makes it difficult to compare and interpret results. Susceptibility studies are often difficult, tedious and slow; they involve the use of an optical microscope to perform differential counts of mobile and non-mobile organisms (Humphreys et al., 1994) and entail a subjective evaluation dependent on the operator, the technique used in the test and the morphology adopted by the protozoon. Flow cytometry has proven to be an efficient method to evaluate cell viability in other protozoa (Barbosa et al., 2014; Humphreys et al., 1994; Uzlikova and Nohynkova, 2014) and provides a fast and accurate method that uses fluorochrome dyes as cell viability markers that have already been described and are very useful for this type of assay (Barbosa et al., 2014). Fluorescein diacetate (FDA) has been widely used in inclusion trials to determine cell viability (Humphreys et al., 1994; Morin-Adeline et al., 2015). Metabolically active cells are able to retain and hydrolyze the dye and emit fluorescence (Humphreys et al., 1994; Morin-Adeline et al., 2015). The 50% inhibitory concentration (IC_{50}), is a parameter widely used to measure the potency of a drug *in vitro*. In *T. foetus,* IC_{50} has only been evaluated in a few studies and with other methodologies (Bader et al., 2016; Liu et al., 2016; Nisha Kumar et al., 2014). MLC is another parameter often chosen in susceptibility tests and is the most often used in *T. foetus*. The aim of this study was to investigate the *in vitro* susceptibility to Mz of *T. foetus* bovine isolates in aerobic (AC) and anaerobic (ANC) conditions through the calculation of IC_{50} and also to analyze MLC by subcultures into complete drug-free medium after treatment.

2. Materials and methods

2.1. Isolation of protozoa

Six isolates of *T. foetus* (Tf0, Tf1, Tf2, Tf3, Tf4 and Tf5) of bovine origin were obtained from samples of preputial smegma from three provinces in Argentina (Salta, Santa Fe and Buenos Aires). The samples were kept at 37 °C in Diamond's trypticase yeast extract-maltose medium (TYM) (Diamond, 1957) supplemented with inactivated equine serum (Natocor), with the addition of 100 mg/L of streptomycin (Sigma), 5 µg/mL of ciprofloxacin (Sigma) and 100 mg/L of chloramphenicol (Genbiotech). The presence of *T. foetus* DNA was detected by DNA extraction and PCR with specific primers for sequences in the 5.8S ribosomal RNA gene, as described by Felleisen et al. (1998) (Fig. 1S).

2.2. In vitro susceptibility test to metronidazole

2.2.1. Determination of the initial inoculum

In order to select the optimal number of cells in exponential phase capable of surviving for 48 h (experimental time) *in vitro*, the initial inoculum was determined. For this, different inoculums of Tf2 were cultured (5×10^4, 1×10^4, 5×10^3, 1×10^3 and 5×10^2 parasites/mL) in TYM medium supplemented with equine serum in 1.5 mL tubes (Deltalab, Spain) in triplicate. They were incubated for 7 days at 37 °C. Live cells were counted every 24 h using flow cytometry and the growth curves were determined.

2.2.2. Susceptibility assays

In vitro susceptibility testing to Mz was performed under both aerobic and anaerobic conditions. *T. foetus* isolates (n = 6) were cultured in TYM medium without the addition of antibiotics until the exponential growth phase (48 h). Then they were adjusted to the concentration required for the inoculum by counts made with flow cytometry. The tests were carried out in 1.5 mL plastic tubes (Deltalab, Spain). An inoculum of 5×10^3 parasites/mL of each isolate was placed in each tube in the presence of serial dilutions of Mz in triplicate. A stock solution (100 mM) of Mz (Sigma) was prepared using DMSO (99.7%, Sigma) as a solvent. Then, the stock solution was diluted in TYM medium to the required concentration. The final concentrations of Mz analyzed were: 58.8, 29.4, 14.7, 7.35, 3.67, 1.82, 0.91 µM. In ANC, two lower concentrations were added for analysis: 0.46 and 0.23 µM. Two controls were carried-out in triplicate for each isolate, one without any additions (parasites in TYM media only) and one containing parasites, TYM media, and DMSO at a final concentration of 0.058%. This amount of DMSO added was equivalent to the highest volume of DMSO used in the largest aliquot of stock solution. The tubes were incubated at 37 °C for 48 h in bags (AnaeroGen Compact, Oxoid TM). The ascorbic acid and activated carbon in the kit react in contact with the air and finally the oxygen is absorbed quickly and produces carbon dioxide generating ANC. After that time, the cells were counted by flow cytometry to estimate IC_{50} of each isolate. In order to evaluate MLC, subcultures after treatment were performed in 8 mL glass tubes (Deltalab, Spain) with Mz-free TYM medium at 37 °C for 7 days. The tubes were observed every 24 h in an inverted optical microscope to demonstrate the presence of motile *T. foetus* trophozoites; in this way, MLC was defined as the lowest concentration of the drug where no growth of parasites was observed in an antibiotic-free medium.

2.2.3. Flow cytometry assay

The cell counts performed in the initial inoculum determination and susceptibility assays were carried out in an Attune® (Applied Biosystems, Thermo Fisher Scientific) acoustic focusing flow cytometer. The cell population was discriminated based on frontal dispersion (size) and lateral dispersion (granularity), and the detection of fluorescence emitted by the viability dye fluorescein diacetate (FDA, Sigma), which allows the determination of the integrity of the membrane and the cellular activity (Morin-Adeline et al., 2015). In order to establish the characteristic location of the trophozoites, the parameters were adjusted (voltage of channel BL1 for FDA) using exponential phase cultures, cultures in the death phase and a mixture of both independently. For each count, cells were washed in PBS (phosphate buffered saline) and incubated with FDA (10 µg/mL, Sigma) at 37 °C for 10 min. The events counted by the cytometer were analyzed with the Attune ™ NxT Software.

2.2.4. Statistical analysis

All the tests were performed in triplicate on two different days. IC_{50} values were determined using GraphPad PRISM (V7) statistical software. Dose-response curves were constructed using a four- parameter logistic regression assuming a variable slope model.

3. Results

3.1. In vitro susceptibility testing to Metronidazole

3.1.1. Determination of the initial inoculum

The growth curves obtained (Fig. 2S) allowed to establish the optimal starting cell density for the study of susceptibility at 48 h between 1×10^3 and 1×10^4 parasites/mL. This range of initial concentrations corresponds to the cells that are in the exponential growth phase, which is necessary to carry out this test. An intermediate concentration value (5×10^3 parasites/mL) was selected to perform the present study.

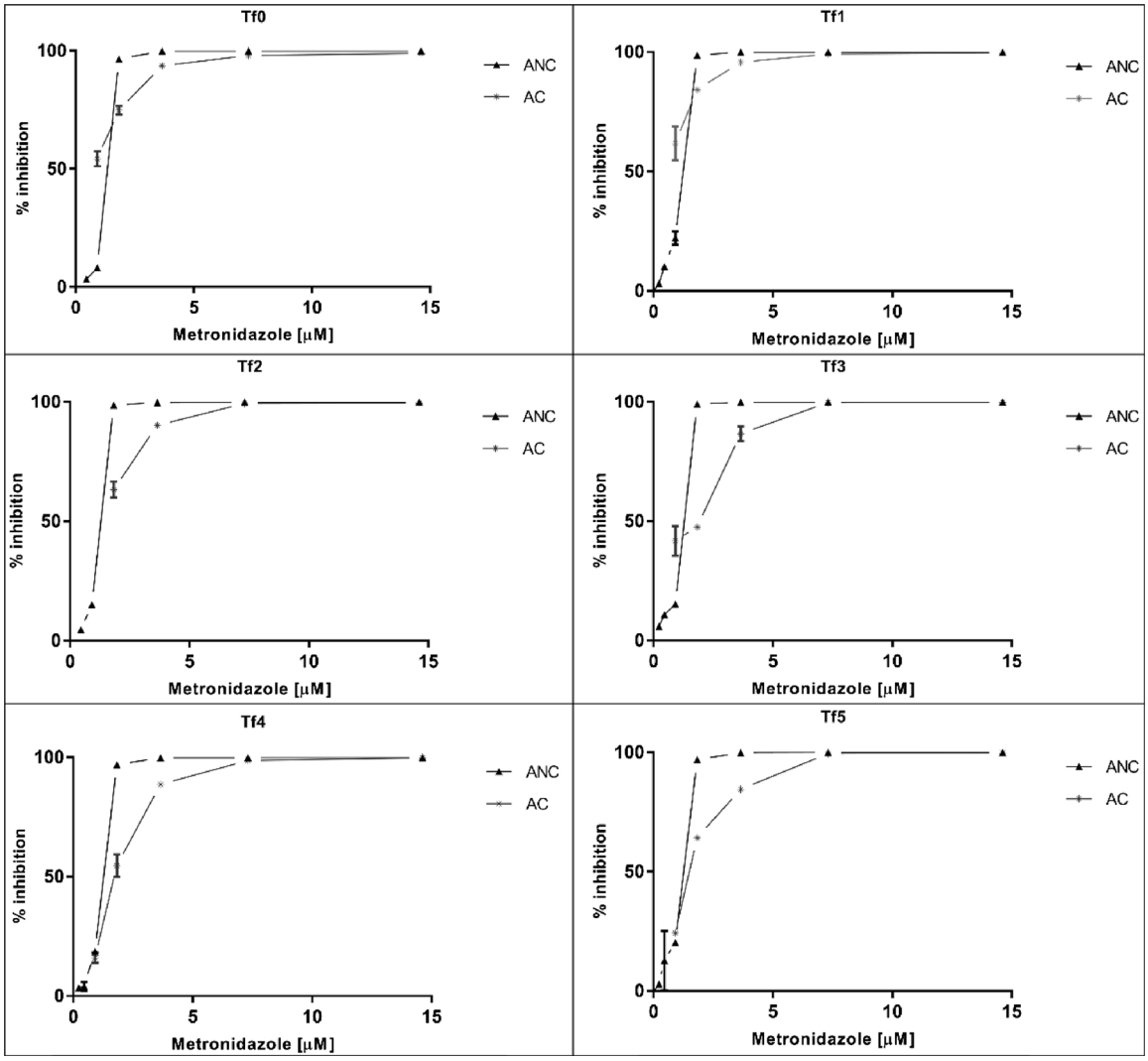

Fig. 1. Dose-response curves generated by flow cytometry. Standard curves were constructed and used to calculate the growth inhibition percentages of *Tritrichomonas foetus* trophozoites (Tf0, Tf1, Tf2, Tf3, Tf4 and Tf5) after treatment with metronidazole. For each graph, all the values were expressed as Mean ± SEM. ANC: anaerobic condition. AC: aerobic condition.

3.1.2. Susceptibility assays

Table 1 summarizes the IC_{50} and MLC values obtained at 48 h in anaerobic and aerobic conditions. All isolates showed an inhibited growth *in vitro* after an incubation period of 48 h with 1.06 to 1.25 μM of Mz in ANC and from 1.44 to 3.03 μM in AC. This is evidenced in the dose-response curves generated by flow cytometry that are represented in Fig. 1.

MLC values obtained by subculture of the parasites in drug-free medium showed variation between 3.67 to 7.35 μM for ANC and from 7.35 to 14.7 μM for AC (Table 1). Live cells (FDA positive) of Tf2 isolate after treatment with Mz under aerobic and anaerobic conditions are shown in Figs. 2 and 3. The dose-response curves generated by flow cytometry of the other isolates are represented in Figs. 3S and 4S.

4. Discussion

Mz has been used for years to treat diseases caused by anaerobic bacteria and protozoa in both animals and humans (Muller and Lindmark, 1976; Samuelson, 1999; Uzlikova and Nohynkova, 2014). Although in some countries its use has not been approved (Bader et al., 2016; Love et al., 2017), treatment with Mz is an alternative to the slaughter *T. foetus* positive bulls. Two distinct types of resistance have been recognized and named aerobic and anaerobic (Cerkasovová et al., 1984; Schwebke and Burgess, 2004) and the emergence of resistant strains (Kulda et al., 1984; McLoughlin, 1967) has led to ineffective treatments, so there is currently no effective therapy for BT (Bader et al., 2016; Carvalho and Gadelha, 2007).

The aim of numerous studies has been to determine the *in vitro* effect of the drug against protozoa of different species. With respect to studies with trichomonads, most have been carried out in *Trichomonas vaginalis* given its relevance in human health (Elwakil et al., 2017; Upcroft and Upcroft, 2001; Vanácová et al., 2001; Vázquez et al., 2001), while only few studies were performed in *T. foetus* (Bader et al., 2016; Bouma et al., 1998; Gookin et al., 2006; Kather et al., 2007; Kulda et al., 1984; Love et al., 2017; Meingassner et al., 1978). The susceptibility values available come mostly from the analysis of standard cell line that have been maintained in culture for years (Bader et al., 2016; Meingassner et al., 1978). The methodology used to determine MIC and MLC of various drugs in trichomonads consists in the post treatment viability evaluation based on the mobility of the parasites by direct observation under an optical microscope (Kather et al., 2007; Love et al., 2017). IC_{50} is a useful quantitative measure to evaluate the potency of a given antagonistic drug in pharmacological research. The IC_{50} values obtained in this study fluctuated between 1.06 and 1.25 μM for ANC and between 1.44 and 3.03 μM for AC. Some of these values are higher than those observed by other authors at 24 h for example 1.7 μM (Bader

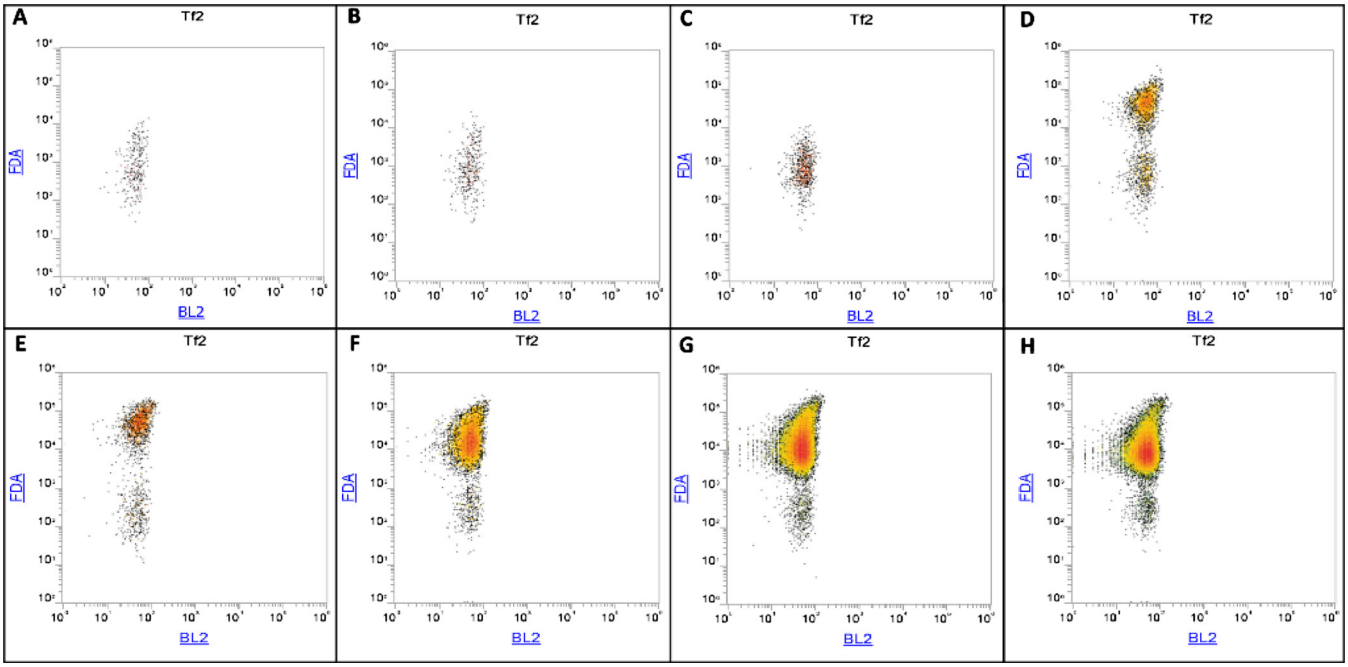

Fig. 2. Results of the flow cytometry. In the Y axis, is shown the live cells of Tf2 (FDA positive) treated with different concentrations of metronidazole under aerobic conditions. A: 58.8 μM Mz, B: 29.4 μM Mz, C: 14.7 μM Mz, D: 7.35 μM Mz, E: 3.67 μM Mz, F: 1.82 μM Mz, G: 0.91 μM Mz and H: control.

Table 1
IC_{50} and minimum lethal concentration (MLC) values obtained in the different isolates of *Tritrichomonas foetus* under anaerobic (ANC) and aerobic (AC) conditions at 48 h.

	ANC		AC	
	IC50 [μM]	MLC [μM]	IC50 [μM]	MLC [μM]
Tf0	1,25	3.67	1,44	14.7
Tf1	1,06	3.67	1,48	7.35
Tf2	1,15	7.35	2,74	14.7
Tf3	1,16	3.67	3,03	7.35
Tf4	1,13	3.67	1,87	7.35
Tf5	1,11	3.67	1,64	7.35

et al., 2016), 0.72 μM (Nisha Kumar et al., 2014) and 0.55 μM (Liu et al., 2016). The data obtained in this study provides information about IC_{50} obtained at 48 h for *T. foetus* not descript before. In AC the IC_{50} values are higher than for ANC values as reported in other studies. The inhibitory concentration of Mz is two to three orders of magnitude higher for aerobic cells than for anaerobic cells consistent with oxygen interfering with the antimicrobial effect of Mz descript before (Kulda, 1999; Land and Johnson, 1997; Muller et al., 1988).

The MLC obtained in ANC (3.67–7.35 μM) and in AC (7.35–14.7 μM) for some isolates were higher than those described in other studies at 48 h (Love et al., 2017; Meingassner et al., 1978) and even higher than those evaluated at 24 h (Kather et al., 2007). It is important to note that in the studies cited the methodology used in the susceptibility tests was the direct observation with an optical microscope, evaluating motility of the parasites after treatment with the drug.

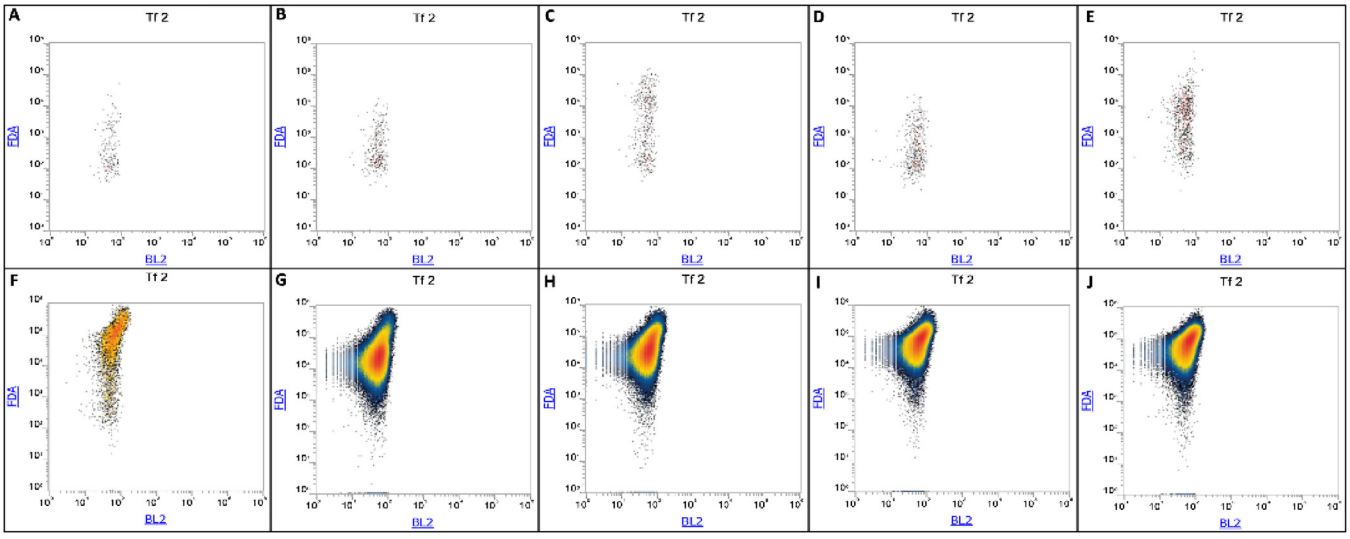

Fig. 3. Results of the flow cytometry. In the Y axis, is shown the live cells of Tf2 (FDA positive) treated with different concentrations of metronidazole under anaerobic conditions. A: 58.8 μM Mz, B: 29.4 μM Mz, C: 14.7 μM Mz, D: 7.35 μM Mz, E: 3.67 μM Mz, F: 1.82 μM Mz, G: 0.91 μM Mz, H: 0.46 μM Mz, I: 0.23 μM Mz and *J*: control.

In this sense, we consider relevant the unification of criteria to establish the MLC.

In our study, in addition to observing survival 48 h after treatment, we performed the subcultures of the trophozoites in Mz-free medium for 7 days in order to determine MLC. The values obtained indicate that all the bovine isolates studied are susceptible to Mz since resistance values were not observed in any of the conditions tested (extremely high concentrations) (Kulda et al., 1984; Vázquez et al., 2001).

There are several methods to perform *in vitro* susceptibility tests that allow the rapid detection of new compounds with activity anti-*T. foetus* (Bader et al., 2016). Once the drug is identified, flow cytometry offers advantages in terms of simplicity, speed and precision that make it an adequate technique for dose-response studies and study of mechanisms of action. In recent years, flow cytometers and software for analysis have improved considerably, allowing faster and less complicated tests (Karl et al., 2009). In a single test, flow cytometry allows rapid and accurate multiple marker evaluation simultaneously. In addition, it is possible to evaluate the action of the compounds in different cellular parameters, such as cell morphology, cell viability, apoptosis, the potential of the mitochondrial membrane, DNA damage and/or proliferation, among others.

Considering the diversity of the methodologies used for drug susceptibility studies, we consider that flow cytometry has numerous technical advantages. In this sense, fluorescent dyes such as FDA that allow the population of live trophozoites to be discriminated from the dead ones are very useful since the analysis does not depend on protozoan motility or the subjectivity of the optical microscope operator. At the same time, flow cytometry allows us to evaluate a large number of events and therefore perform the total count of trophozoites of each sample in a faster and less arduous way than using a hemocytometer. Furthermore, unlike the other methodologies used, flow cytometry allows the detection of round non-motile structures, with no external flagella visible to the naked eye called pseudocysts that could to underestimate of MIC or MLC values. (Mariante et al., 2004; Pereira-neves et al., 2003; Pereira-Neves et al., 2011; Pereira-Neves and Benchimol, 2009).

5. Conclusions

Flow cytometry represents a valuable alternative for the quantitative evaluation of *in vitro* drug susceptibility. The technique allows independence of the subjectivity of the operator and also detects rounded non-motile structures that tend to establish erroneous susceptibility values. In addition, it proved to be an effective technique, fast, precise and less laborious. We believe that the unification of criteria for the evaluation of drug susceptibility to characterize the isolates of the region where the treatment is to be performed is of fundamental importance. The *in vitro* susceptibility values against Mz obtained, allowed to determine an important phenotypic trait for these *T. foetus* isolates that constitutes a useful tool to establish Mz dosages in treatments.

Given the individual isolates exhibited biological variations it is important to have susceptibility information from the place where it will be used in order to establish adequate dosage. This would avoid the selection of resistant parasites due to the use of the drug in sublethal concentrations, since the effective plasma concentration needed may vary between countries and even between regions of the same country. Although the *in vitro* action of other drugs has been investigated, further studies are needed to find an effective BT drug that represents a safe and cost-effective alternative.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.02.004.

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