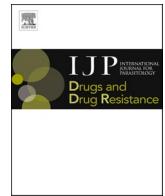




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Evaluation of the inhibitory efficacy of quaternary ammonium compounds on *in vitro* growth of *Theileria equi* parasite in MASP culture

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ABSTRACT

Equine piroplasmiosis has become a global problem of the equine husbandry sector. Haemoprotozoans evolved very quickly and developed resistance against most of the current available drugs. Phospholipid membrane synthesis by choline kinase enzyme is vital for propagation of intra-erythrocytic protozoa parasites. This pathway was targeted in the present study. Quaternary ammonium salts (QAS) and their analogues act against choline and hamper the biosynthesis process for phosphatidylcholine. We analysed anti-*T. equi* activity of three QAS - decamethonium bromide (DMB), decyl trimethyl ammonium bromide (DTAB) and dodecyl trimethyl ammonium bromide (DDTAB). *Theileria equi* parasites *in vitro* treated with different concentrations of DMB, DDTAB and DTAB. Drug treated *T. equi* failed to multiply further in the viability test. The IC₅₀ value of DMB, DDTAB and DTAB for growth inhibition of *T. equi* was 14.0 μM, 469.51 nM and 558.40 nM, respectively. DMB, DDTAB and DTAB treated *T. equi* parasites were observed to be devoid of internal structures, showing pyknotic and degenerative appearances. Various concentration of DMB, DDTAB and DTAB were analysed for their cytotoxicity and haemolytic activity on horse's PBMCs and RBCs. DMB was less than 10% cytotoxic to PBMCs, while DDTAB and DTAB were 40%–50% cytotoxic at 1000 μM concentrations. The respective CC₅₀ values were 7202.96 μM, 1026.26 μM and 1263.95 μM. DMB and DTAB showed least haemolytic activity (<3%); whereas DDTAB was more haemolytic to RBCs at highest concentration of 2000 μM. The respective CC₅₀ values of these drugs were 224495.3 μM, and 39101.35 μM; 713.54 μM. Specific selective index for DMB, DDTAB and DTAB values with respect to host's PBMC and RBC cells, were 514.50, 2185.81, 2263.52 and 16035.38, 1519.75, 70023.91, respectively. These data indicated its non-toxicity to host's cells and selective potential of anti-*T. equi in vitro* activity.

1. Introduction

Global climate change has affected the distribution pattern of vector-borne diseases and disease conditions by apicomplexan parasites have emerged. Theileriosis is the utmost prevailing haemoprotozoan disease of animals (Rashid et al., 2018) and is responsible for huge economic losses to livestock owners. Theileriosis in equines is caused by *Theileria equi* an intra-erythrocytic protozoa and movement of the infected horses has been restricted across the international border (Mehlhorn and Schein, 1998). Development of drug resistance in theileriosis

necessitates the search for new target-based drug molecules (Kumar et al., 2003; Vial and Gorenflot, 2006).

During an asexual erythrocytic development of *Theileria equi*, numerous vital components are synthesized viz phospholipid, DNA, heme etc, which are essential in maintaining its life cycle in the natural host. Erythrocyte needs an immense amount of the phospholipids for its membrane synthesis (Holz and Bull, 1977; Vial and Ancelin, 1992, 1998). A majority of phospholipid metabolism (up to 85%) are governed by two key components - Phosphatidyl-choline and phosphatidyl-ethanolamine (Vial and Ancelin, 1992). Phosphatidyl-choline is the most abundant phospholipid synthesized by

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Abbreviations

QAC	Quaternary ammonium compounds
QAS	Quaternary ammonium salts
DMB	Decamethonium bromide
DTAB	Decyl trimethyl ammonium bromide
DDTAB	Dodecyl trimethyl ammonium bromide
h	Hours
PBMCs	Peripheral blood mononuclear cells
RBCs	Red blood cells
IC ₅₀	50% Inhibitory concentration
CC ₅₀	50% Cytotoxic concentration
SSI	Specific selective index
MASP	Micro-aerophilous stationary phase
VYM	Vega Y Martinez phosphate buffered saline
NPPs	New permeation pathways

Kennedy or CDP - choline pathway (Vial and Ancelin, 1998; Pessi et al., 2005). Choline kinase is the first key enzyme in the Kennedy pathway and its inhibition arrested *in vitro* and *in vivo* growth of *Theileria* parasites (Choubey et al., 2007; Ancelin et al., 1998, 2003; Ancelin et al., 2003a,b; Roggero et al., 2004). Lehane et al. (2004) investigated the uptake of choline kinase by *Plasmodium* parasitized erythrocytes. They reported that choline influx was inhibited competitively by quinine. Quinine (established anti-malarial drug) inhibited the uptake of choline via the competitive inhibition of the choline transporter, indicating that this pathway is essential for the survival of malarial parasites.

Earlier studies indicated that quaternary ammonium salts and their analogues act against choline (by mimicking its structure), impeding the biosynthesis process for phosphatidylcholine (Ancelin et al., 1991). Mono and bis quaternary ammonium salts were found highly effective against *P. falciparum* at IC₅₀ values of as low as 0.33 nM and 3 pM, respectively (Calas et al., 2000). Bis-quaternary ammonium salts also can selectively accumulate in *Plasmodium*-infected erythrocytes as compared to unparasitized erythrocytes (Staines et al., 2000; Stead et al., 2001; Biagini et al., 2003; Ancelin et al., 2003a,b). Bis-quaternary ammonium compounds compartmentalize themselves in the parasite's food vacuole and prevent formation of free heme (hemozoin) contributing to its antimalarial activity (Biagini et al., 2003). *Theileria* and *Babesia* are malaria-like protozoans infecting host-erythrocytes. These haemo-protozoans lack a central food vacuole but may be able to synthesize phospholipids *de novo* and as such do not produce hemozoin. Existence of these contrasting pathobiology among animals *Theileria*, *Babesia* and *Plasmodium* species protozoa prompted us to investigate anti-theilerial activities of quaternary ammonium compounds. We aimed to explore the growth inhibition potential of decamethonium bromide, decyl trimethyl ammonium bromide and dodecyl trimethyl ammonium bromide against *in vitro* cultured *Theileria equi* parasite.

2. Materials and methods

2.1. *In vitro* MASP culture of *Theileria equi*

Theileria equi was *in vitro* cultured by micro-aerophilous stationary phase (MASP) technique. *Theileria equi* negative and positive horses (reared at ICAR-National Research Centre on Equines animal shed) were identified by performing ELISA (Kumar et al., 2013). Defibrinated whole blood was collected aseptically from a *T. equi* negative horse (as above) and centrifuged at 1500 g for 5 min. The supernatant plasma and the top white cells layer were discarded. Sedimented erythrocytes were washed

with 1:1 volume of Vega Y Martinez phosphate buffered saline (VYM) by centrifuging at 1500 g for 5 min. Erythrocytes washing procedure with VYM was repeated three-times. Final pelleted erythrocytes were suspended in 1:1 volume of VYM buffer and stored at 4 °C for further use in MASP. The culture medium M 199 (Sigma-Aldrich, India) was used for MASP culture of *T. equi* and supplemented with 40% defibrinated equine serum, antibiotic solution (containing 60 IU/ml penicillin and 60 mg/ml streptomycin) and 200 µM hypoxanthine solution. *Theileria equi* MASP cultures were maintained at a temperature of 37 °C with micro-aerophilic atmosphere of 5% CO₂, 3% O₂, and 95% N. Erythrocytes collected from ELISA positive horse (as above) were seeded in the MASP culture for propagation of *T. equi* parasite and used for *in vitro* drug trial studies.

2.2. *In vitro* growth inhibitory assay

Theileria equi parasitized RBCs were collected from the MASP culture and adjusted to 1% parasitaemia by diluting with un-infected RBCs (ELISA *T. equi* negative horse, as above). *Theileria equi in vitro* growth inhibitory assay was performed in 48 well culture plates. Decamethonium bromide (DMB), Decyl trimethyl ammonium bromide (DTAB) and dodecyl trimethyl ammonium bromide (DDTAB) were obtained commercially (Sigma-Aldrich, India). Stock solutions (1000 µM) of these drugs were prepared in deionized distilled water. Working concentrations of DMB (1, 5, 10, 20, 50, 100 and 200 µM) and DTAB and DDTAB (125, 250, 500, 750, 1000 nM) were obtained by diluting their respective stock concentration with *T. equi* complete culture medium. Fifty microliter of *T. equi* parasitized RBCs (at 1% parasitaemia) were dispensed per well (in triplicate) together with 500 µl of the culture complete medium containing the indicated drug concentrations (as above). These *in vitro* cultures with or without drug molecules concentrations were incubated at 37 °C in an atmosphere of 5% CO₂, 3% O₂, and 95% N, for a period of 96 h. The overlaid culture medium was replaced with fresh medium containing indicated drug molecule concentration after every 24 h. IC₅₀ value was calculated by standard curve fitting technique (Bork et al., 2004).

2.3. Viability test

After 96 h of *in vitro* treatment with different drugs (as per individual concentration), 20 µL of drug-treated/un-treated parasitized RBCs were collected and transferred to a fresh 48 well culture plate containing 30 µL of parasite-free normal horse RBCs in 500 µl of *T. equi* complete growth medium (without any drug molecule). The overlaid growth medium was replaced after every 24 h for the next 72 h, and *T. equi* parasite recrudescence/viability was determined by examining its Giemsa-stained blood smears (Bork et al., 2004).

2.4. *In vitro* cytotoxicity assay

In vitro cytotoxicity of different concentrations of drug was assessed on PBMCs by resazurin-based cell viability assay (Gopalakrishnan et al., 2016). PBMCs were separated on histopaque-1077 (Sigma-Aldrich, India) from the whole blood collected from a healthy horse. These PBMCs were resuspended in 1 ml complete growth medium consisting of RPMI-1640 supplemented with 2 mM L-glutamine, 60 µg/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum (Sigma Aldrich, India). Final cells concentration was adjusted to 3 × 10⁵ cells/100 µL and 100 µL volume was distributed to each well of the 96 well culture plate. Simultaneously, 50 µL phytohaemagglutinin (PHA @ 10 µg/ml) was also added to each of these wells. The culture plate was incubated at 37 °C having 5% CO₂ in air for 48 h. Further, these PBMCs were treated

with 100 µl volume of the different respective concentration of drugs (as above). Negative (without drug complete culture medium) and positive (complete culture medium with 1% triton-X100 solution) control were also maintained during cytotoxicity drug trial. Culture plate was further incubated for 24 h at 37 °C in an incubator (5% CO₂ in air). A 25 µL volume of resazurin dye (150 µg/ml) was added to each well and culture plate was again incubated for next 4 h. The change of dye colour was monitored by measuring optical density (OD) at 570 nm and 650 nm. The effective OD value for each well was calculated by deducting OD₅₇₀ value from its respective OD₆₅₀ value. Effect of different drug molecules on PBMCs in terms of per cent viable cell population was determined as below:

$$\text{PBMCs viability (\%)} = \frac{\text{OD of test sample} - \text{OD of positive control}}{\text{OD of negative control} - \text{OD of positive control}} \times 100$$

$$\% \text{ Cytotoxicity} = \frac{\text{OD of negative control} - \text{OD of test sample}}{\text{OD of negative control}} \times 100$$

The IC₅₀ of each drug molecule on PBMCs was also calculated from a regression equation.

$$\text{Percentage hemolysis} = \frac{\text{OD of different drug concentration} - \text{OD of negative control}}{\text{OD of positive control} - \text{OD of negative control}}$$

2.5. In vitro haemolytic assay

Haemolytic activity of each drug was assessed on horse RBCs as per standard haemolytic assay (Raghava et al., 1994). Fresh RBCs were separated from the whole blood collected from a healthy horse by centrifuging at 1200 g for 10 min. RBCs pellet was washed three times with PBS (phosphate buffer saline) by centrifugation. Different concentrations of each drug molecules (DMB, DTAB and DDTAB) were prepared in solubilising buffer (10% dimethylformamide in PBS). Twenty microliters of RBCs suspension were added to each well of 96 well culture plate containing 180 µL of different concentrations of drug molecules. Positive (RBCs suspended in distilled water) and negative (RBCs suspended in PBS) control were also maintained in the assay. The 96 well plate was incubated further at 37 °C for 90 min. The contents of each well after incubation was transferred into 2 ml micro-centrifuge tube, followed by centrifugation at 3000g for 5 min. Supernatants from each micro-centrifuge tubes were transferred to new 96 well plates and the OD was measured at 543 nm in UV spectrophotometer. Percentage of haemolysis was determined as below:

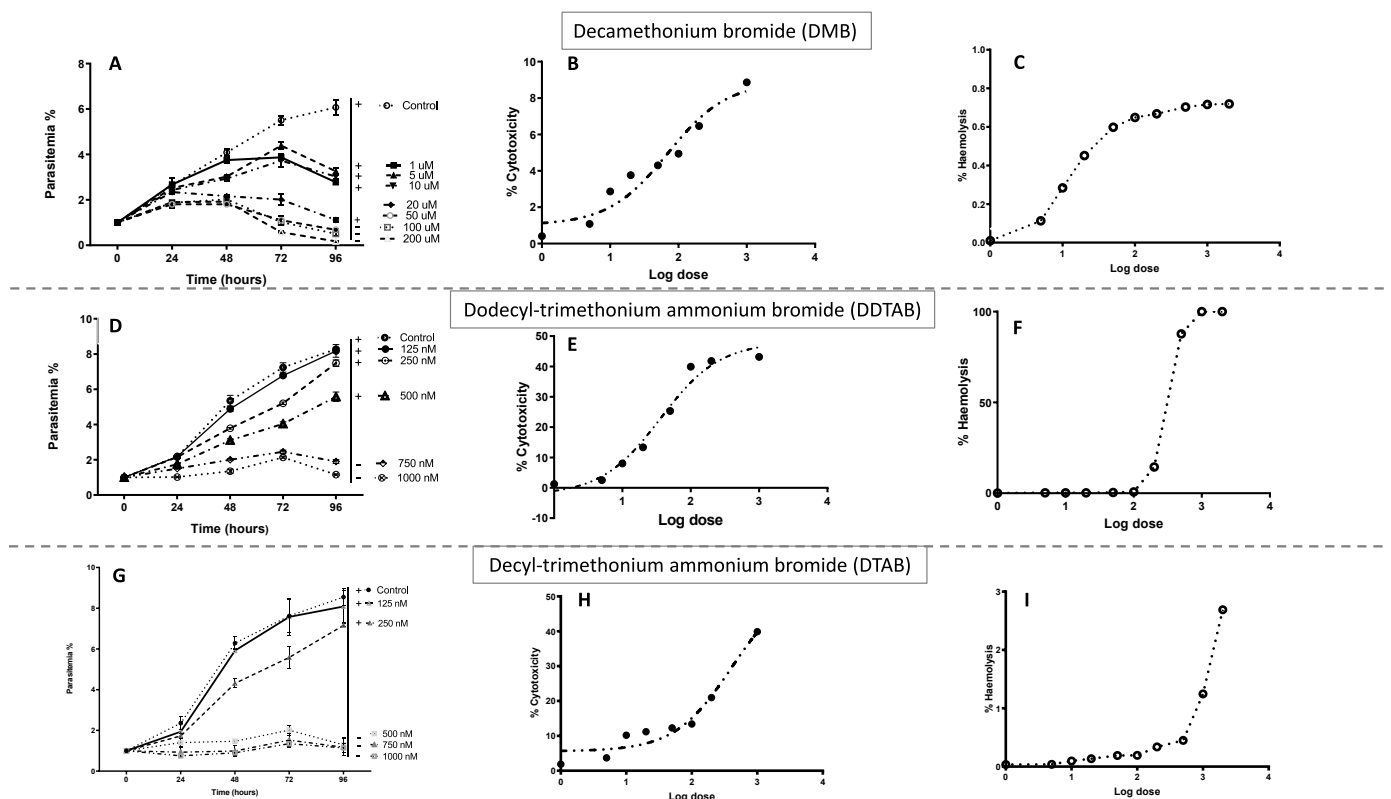


Fig. 1. Liner *in vitro* growth inhibition curves of *Theileria equi* (A, D, G) and cytotoxicity assay on horse’s peripheral blood mononuclear cells (PBMCs; B, E, H) and red blood cells (RBCs; C, F, I) after treatment with different concentrations of decamethonium bromide (DMB), dodecyl trimethyl ammonium bromide (DDTAB) and decyl trimethyl ammonium bromide (DTAB). Each value in the individual graph (A, D, G) represents percent parasitaemia (Mean ± SEM) observed at different time interval. ‘+’, ‘-’ numeral on right hand side of the graph represented viability status of the *T. equi* parasite after 96 h of viability test.

2.6. Specific selectivity index (SSI)

The extent of selectivity of different drug molecules against *T. equi* in comparison to horse PBMCs or RBCs at respective IC₅₀ concentration was calculated using the below mentioned standard formula:

$$\text{Specific Selectivity Index (SI)} = \frac{\text{IC}_{50} \text{ of drug molecule on horse PBMCs}}{\text{IC}_{50} \text{ of drug molecule on protozoan parasite}}$$

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 software (San Diego California, USA). Two-way ANOVA followed by Bonferroni post-hoc test ($p < 0.05$) was computed to know the anti-*T. equi* activity of these drug molecules. The 'p' values < 0.05 were considered statistically significant differences between the treated groups and control cultures. The correlation between drug molecule concentrations, cytotoxicity and haemolytic activity was also evaluated.

3. Result

3.1. In vitro growth inhibitory assay

Theileria equi in vitro growth inhibition observation at 24 h exhibited significant difference from its respective control well ($p < 0.05$) at higher concentrations of DMB (100 μM and 200 μM ; Fig. 1A); DDTAB (750 and 1000 nM; Fig. 1D); DTAB (500, 750 and 1000 nM; Fig. 1G). While, at 48 h, 72 h and 96 h of in vitro drug treatment with different concentration of DMB (5 μM –200 μM), DDTAB (250 nM–1000 nM), DTAB (250 nM–1000 nM) displayed significant ($p < 0.05$) growth inhibition from respective control well. The IC₅₀ value of DMB, DDTAB and DTAB for growth inhibition of *T. equi* on 96 h of culture was 14.0 μM , 469.51 nM and 558.40 nM, respectively.

Theileria equi parasites treated in vitro (for 96 h) with different concentrations of DMB (50 μM –200 μM), DDTAB (500 nM–1000 nM) and DTAB (750 nM and 1000 nM) failed to multiply further in the viability test (Fig. 1A, D, 1G). While *T. equi* parasites were live and showed recrudescence after 96 h of in vitro treatment with remaining concentrations of these drugs.

3.2. Morphological changes observed in parasites

Decamethonium bromide, DDTAB and DTAB treated *T. equi* parasites were observed. They were devoid of internal structures, showing pyknotic and degenerative appearances (Fig. 2). Moreover, the drug treated

parasites were showing no demarcation between cytoplasm and nucleus (Fig. 2). Changes in the morphology of treated parasite were indicating the efficacy of these drug molecules.

3.3. In vitro cytotoxicity and haemolytic assay

Various concentration of DMB, DDTAB and DTAB were analysed for their cytotoxicity (1 μM –1000 μM) on PBMCs cell lines and haemolytic activity (10 μM –2000 μM) on horse's RBCs. Decamethonium bromide was less than 10% cytotoxic to horse PBMCs, while DDTAB and DTAB were 40%–50% cytotoxic to horse PBMCs at highest concentrations (1000 μM ; Fig. 1B, E, 1H). The respective cytotoxic concentration (CC₅₀) as deduced by regression analysis of these drugs were 7202.96 μM , 1026.26 μM and 1263.95 μM .

Decamethonium bromide and DTAB showed least haemolytic activity ($< 3\%$; Fig. 1C and I); whereas DDTAB was more (Fig. 1F) haemolytic to horse RBCs at highest concentration of 2000 μM . The

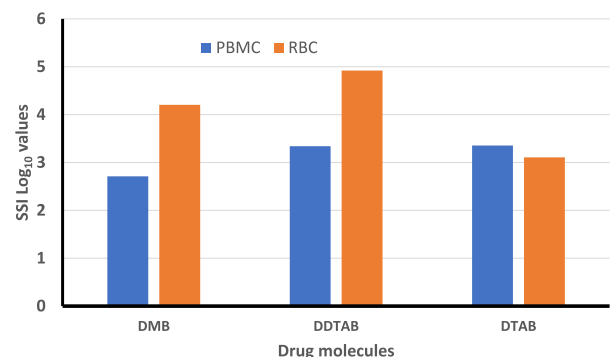


Fig. 3. Specific selectivity index (SSI) of decamethonium bromide (DMB), dodecyl trimethyl ammonium bromide (DDTAB) and decyl trimethyl ammonium bromide (DTAB) with respect to horse PBMCs and RBCs.

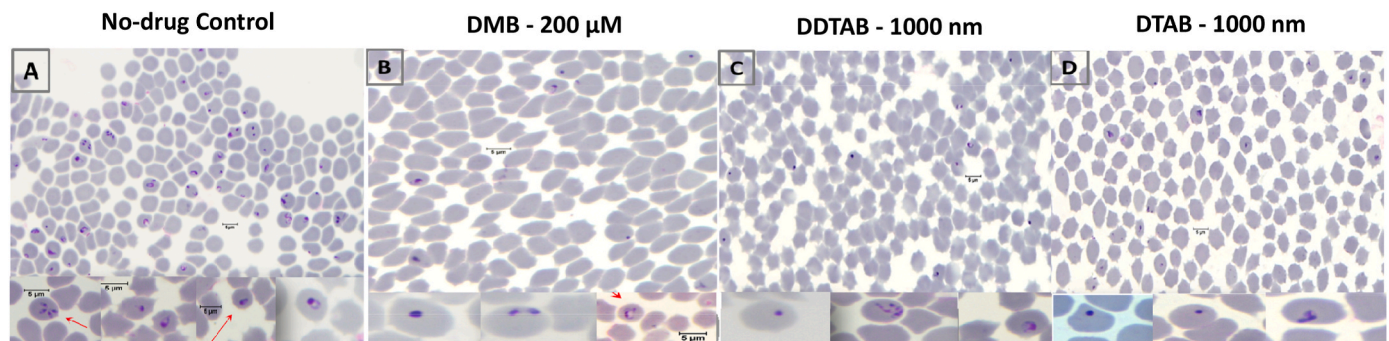


Fig. 2. Microphotographs depicting morphological changes observed in *T. equi* parasites after in vitro treatment (at 96 h) with decamethonium bromide (DMB; B), dodecyl trimethyl ammonium bromide (DDTAB; C) and decyl trimethyl ammonium bromide (DTAB; D). Control (no-drug, at 96 h; A) culture showed pyriform shaped *T. equi* merozoites, whereas the drug treated parasites were degenerated or with condensed nucleus and appeared pyknotic. Giemsa X 1000. Bars 5 μm .

respective CC_{50} as deduced by regression analysis of these drugs were 224495.3 μ M, 39101.35 μ M and 713.54 μ M.

Specific selective index (SSI) for DMB, DDTAB and DTAB values with respect to host's PBMC and RBC cells, were 514.50, 2185.81, 2263.52 (respective Log_{10} values: 2.71, 3.33, 3.35) and 16035.38, 1519.75, 70023.91 (respective Log_{10} values: 4.20, 4.92, 3.10), respectively (Fig. 3).

4. Discussion

The plasma membrane of the host erythrocytes has abundance of membrane transport proteins which are responsible for the flow of nutrients into and out of the cells. *Plasmodium* parasites, require some essential nutrients (after some hour of invasion) which are not sufficiently available within the infected erythrocytes. These are accomplished through the creation of new permeation pathways (NPPs) that facilitate the transport of a broad range of substrates across the erythrocyte membrane (Ginsburg et al., 1985; Desai et al., 1993). The mature host-erythrocyte lacks the pathways for phospholipid synthesis; however, the intra-erythrocytic parasite synthesises a range of phospholipids de novo – phosphatidylcholine from choline. Parasitized erythrocytes from *Plasmodium vinckei* – infected erythrocytes exhibited a significant influx of choline via NPP (Staines et al., 2000). Bis-quaternary ammonium compounds had been shown to enter the *P. falciparum*-infected erythrocyte via the NPP and inhibit *in vitro* growth of the parasite (Biagini et al., 2003). In this perspective, compounds which mimic choline structure have been developed to target phospholipid metabolism for possible use as novel class of antimalarials or apicomplexan parasites.

The DMB, DDTAB and DTAB are quaternary ammonium salt and have shown promising *in vitro* anti-plasmodial activities at lower concentrations. In our *in vitro* studies DMB, DDTAB and DTAB successfully inhibited the growth of *T. equi* with IC_{50} value of 14.0 μ M, 469.51 nM and 558.40 nM, respectively. The *T. equi* parasites were completely dead in *in vitro* culture at $\geq 50 \mu$ M or ≥ 500 nM concentration of these drug molecules. Hexadecyltrimethylammonium bromide (HDTAB – a quaternary ammonium compound) has efficiently inhibited the *in vitro* growth of *T. equi* and *Plasmodium falciparum* at IC_{50} value of 14 μ M (Gopalakrishnan et al., 2016) and 10 μ M (Choubey et al., 2007), respectively. Ancelin and Vial (1986) also reported *in vitro* anti-*P. falciparum* activities of DMB, DDTAB and DTAB at respective IC_{50} value of 1 μ M, 500 nM and 700 nM concentrations.

Quaternary ammonium salts are cationic compounds containing alkyl groups. The alkyl chain with 10–12 methylene groups were found to be more active against *P. falciparum* (Calas et al., 2000). The DDTAB drug molecule has 12 carbon atoms, whereas DTAB has 10 carbon atoms. Quaternary ammonium compounds with longer alkyl chain would exist with a particle size of 60–110 nm at low concentration resulting more affinity with target (Cheng and Ran, 2014). The coexistence of small compacted (size) particles and larger aggregates (large alkyl chain), initiate drug-target aggregation and enhanced efficacy of the drug molecules. We evaluated lowest anti-*T. equi* efficacy of DDTAB in nano-molar concentration. DDTAB has more carbon atoms than DTAB attributing to its target-specific efficacy at lower concentration.

It is imperative to know the selectivity of the tested drug candidate between host cells and target parasite for its future applicability. Respective drug candidates may show promising *in vitro* efficacy trial and simultaneously may be significant cytotoxic to the host cells. In the present experiment we also intended to investigate the cytotoxic and haemolytic activity of tested drug molecules. The results of the present study showed that these three drug molecules (DMB, DDTAB and DTAB) have no significant toxicity against host's PBMC and erythrocytes cell line. However more toxicity of these drug molecules in the natural host biological system needs to be analysed before accepting them as therapeutic drug. Evaluation of SSI value for a drug molecule is very critical for documenting its bioactivity specifically against the pathogen of

interest. Awouafack et al. (2013) recommended an acceptance criterion of $SSI \geq 10$ for a selective drug molecule. In this study, the tested drug molecules were observed to be very safe and non-toxic to the host's cell lines as very high SSI values were recorded and these were - 500 to 2200 (PBMCs cell lines) and 1.2×10^3 to 83.0×10^3 (RBC cell lines). QAC have prominent surface-activity and widely used in bactericidal and fungicidal preparation. Bioavailability of these compounds was observed to be less upon oral and subcutaneous administration in mouse model (Ancelin et al., 2003a,b). This might forbid its anti-plasmodial usage, but it can be improved by modifying these compounds for systemic usage.

5. Conclusions

It can be concluded that DMB, DDTAB and DTAB are the quaternary ammonium salts and potential, selective *T. equi in vitro* growth inhibitors. These drug molecules have very high SSI values and are non-toxic to host's PBMC and RBC cell lines. These drugs molecules may be taken up further for their *in-vivo* anti-*T. equi* potential in horses.

Credit authors' statement

AS and AG performed the whole experiments. CM performance *in vitro* cytotoxicity trials. RKD performed haemolytic trials, SK conceptualized, designed, and supervised the whole study. Also drafted the final version of manuscript. RK performed statistical calculations and prepared the graphs. All authors read and approved the final manuscript.

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Ethics declarations

Prior approval was taken for equine sampling in the present study from the Institutional Animal Ethics Committee of ICAR-NRCE, Hisar.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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