Original Research Article

IN VITRO ANTIPARASITIC ACTIVITY OF CAMEL MILK AGAINST *BLASTOCYSTIS SP.*

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ABSTRACT

Aim: the aim of the current study was to investigate *in vitro* anti-protozoal activity of camel, cow, and goat raw milks against *Blastocystis sp.* strains isolated from symptomatic patients.

Place and Duration of Study: the study was carried out in two major health care centres of Makkah city, Saudi Arabia between 01 January and 30 March 2017.

Methodology: Stool specimens collected from patients and healthy individuals, were examined by microscopy and *in vitro* cultured using Dulbecco's modified Eagle medium. Cultures were examined after 24, 48, and 72 hrs. *Blastocystis sp.* subtyping was performed on genomic DNA extracts of positive cultures by polymerase chain reaction using sequence-tagged-site primers. *Blastocystis sp.* parasites susceptibility assays were performed in 2 ml final volumes seeded with $2x10^5$ parasites and incubated for 48 h at 37°C. Concentrations of 250 µl/ml, 125 µl/ml, 62.5 µl/ml, 31.2 µl/ml, and 15.6 µl/ml of bovine, goat and camel raw milk were tested for their anti-parasitic activity against two *Blastocystis sp.* isolates identified as ST1 and ST3 subtypes. Metronidazole at (0.1 mg/ml) was used as positive antiparasitic control in all assays.

Results: Out of seven positive cultures, two isolates were identified as ST1 subtype and five isolates as ST3 subtype. A significant *in vitro* killing effect was obtained with camel raw milk at minimal concentration of 31.2 µl/ml compared to cow raw milk ($P<0.05$) and goat raw milk ($P<0.05$), on both subtypes. Both, cow and goat raw milk did not show a noticeable *in vitro* killing effect at the highest dose of 250 µl/ml.

Conclusion: Raw camel milk revealed a substantial dose-dependent *in vitro* antiparasitic activity against *Blastocystis sp.* ST1 and ST3 subtypes, opening a promising perspective for its use in the control of this wide spread gastrointestinal parasite both in humans and livestock. In contrast, cow and goat raw milks did not show noticeable anti-*Blastocystis sp.* activity against both subtypes.

Keywords: *Blastocystis sp.*, SSU rDNA STS sub-typing, camel raw milk, *in vitro* antiparasitic activity.

1. INTRODUCTION

Blastocystis can be described as a unicellular anaerobic parasite that inhabits the lower gastrointestinal tract of humans in addition to many animals [1]. This emerging parasite has a worldwide distribution. In the past few years, a remarkable increase in prevalence studies had exhibited its epidemiological importance, with variable documented prevalence is high as 60% in some tropical, subtropical and developing nations [2]. *Blastocystis sp.* parasites display varied morphological forms; they may appear as vacuolar, granular, ameboid, cystic, avacuolar or multivacuolar [3]. The pathogenic potential of *Blastocystis sp.* is debatable;

Comment [RGK1]: as

several reports discussed the controversy of its capability to cause disease [4-8]. *Blastocystis* parasites have been identified in patients with various gastrointestinal or even allergic skin symptoms, but also in healthy people. It has been suggested that diverse genotypes or subtypes may have different pathogenic potentials [9]. Different molecular approaches such as PCR by small subunit ribosomal DNA (SSU rDNA) Sequence-tagged-site primers are used to study genetic variation among *Blastocystis sp.* isolates [10-13].

Antiparasitic activity of milk from humans and different animals has been investigated by many authors. Bovine, goat and camel milks were the most investigated ones [14 15]. Milk includes numerous compounds such as lacto-peroxidase, lactoferrin, immunoglobulin G, secretory immunoglobulin A, and Lysozymes [16]. The protective effect of these proteins had been screened against several bacterial strains like *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Lactococcus lactis* and rotavirus [17]. Camel milk lacto-peroxidase has been identified as bacteriostatic and bactericidal against Gram-positive and Gram-negative strains, respectively. Its high content in anti-viral antibodies is protective against rotavirus [17].

Camel milk lactoferrin showed anti-cancer effect by reducing colorectal cancer cells proliferation *in vitro* [18]. Camel milk have proven to be anti-schistosomal against *Schistosoma mansoni* in infected mice [19]. The present study is the first report on antiparasitic activity of bovine, goat and camel raw milk against *Blastocystis sp.* isolates from symptomatic patients.

2. MATERIAL AND METHODS

2.1 Samples collection and parasites identification:

A total of 1136 stool samples were collected from two major health care centres in Makkah city, Saudi Arabia between 01 January and 30 March 2017 from patients and healthy individuals, after their consent. *Blastocystis sp.* parasites positive fecal specimens were diagnosed by microscopy carried out as explained before [20]: briefly, two direct wet mount preparations of 2 mg of feces emulsified in one drop of physiologic saline and one drop of Lugol's iodine were examined under both, low power (×10) and high power (×40) objectives. *Blastocystis sp*. parasites can be recognized by morphological features as vacuolar, granular, ameboid, or cystic with very variable sizes [2].

Comment [RGK2]: the authors still did not describe the forms they took as *Blastocystis* in the stools.

2.2 *Blastocystis sp. in vitro* culture:

About 0.5 g of each *Blastocystis sp.* microscopically positive stool samples were immediately cultured in 11×100-mm sterile screw-capped tubes containing 2 ml of media and incubated I at 37 °C in anaerobic gas pack (BD gas pack-Becton, Dickinson, USA). The culture medium consisted in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 12 mg/ml ampicillin and 4 mg/ml streptomycin supplemented with 20 % inactivated horse serum (Gibco) sterilized by filtration as described by [21]. A drop of culture was examined after 24, 48, and 72 hours by direct microscopy. After three passages, parasites from positive subcultures of each isolate were pooled, counted in haemocytometer chambers (Improved Neubauer, Hausser Scientific), and cryo-preserved separately as $1x10^6$ parasites/ml of DMSO freezing medium in liquid nitrogen.

2.3 Molecular subtyping of *Blastocystis sp.* isolates:

Genomic DNA was extracted from positive subcultures by using QIAmp DNA extraction kit (QIAmp, QIAGEN Inc, Germany) according to manufacturer's protocol. Quantity and quality of isolated DNA were determined by measuring the 260 and 280 nm absorbance in a spectrophotometer (SpectraDrop, SpectroMax, life technology, USA). *Blastocystis sp.* subtyping was performed by PCR using sequence-tagged-site primers according to [22] **(table 1**). DNA extracts (2 µl) were amplified in PCR reactions of 25 µl with AmpliTaq Gold

UNDER PEER REVIEW

360 master mix (Applied biosystems, USA) under the following conditions: one cycle of initial denaturing at 94°C for 5 min, 40 cycles including denaturation at 94°C for 30 s, annealing at different temperatures as indicated in table 1 for 30 s, and extension at 72°C for 1 min, and a final elongation cycle for 5 min at 72°C. PCR amplifications were carried out in duplicate for each sample and each primer pair.

Table 1: Primer Pairs for *Blastocystis sp.* STs SSU rDNA identification by PCR.

Subtype	Primers set name	PCR $T_{annealing}$	PCR products size (bp)	Accession N° in GenBank	Sequences
ST 1	SB83	55°C	351	AF166086	F: GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAGGCAGC
ST 2	SB340	57°C	704	AY048752	F: TGTTCTTGTGTCTTCTCAGCTC R:TTCTTTCACACTCCCGTCAT
ST 3	SB227	54°C (multiplex)	526	AF166088	F:TAGGATTTGGTGTTTGGAGA R:TTAGAAGTGAAGGAGATGGAAG
	SB228		473	AF166089	F: GACTCCAGAAACTCGCAGAC R: TCTTGTTTCCCCAGTTATCC
	SB229		631	AF166090	F: CACTGTGTCGTCATTGTTTTG R: AGGGCTGCATAATAGAGTGG
ST4	SB337	57°C	487	AY048750	F: GTCTTTCCCTGTCTATTCTTGCA R:AATTCGGTCTGCTTCTTCTG
ST5	SB336	57°C	317	AY048751	F:GTGGGTAGAGGAAGGAAAACA R:AGAACAAGTCGATGAAGTGAGAT
ST6	SB332	55°C	338	AF166091	F: GCATCCAGACTACTATCAACATT R:CCATTTTCAGACAACCACTTA
ST7	SB155	53°C	650	AF166087	F:ATCAGCCTACAATCTCCTC R: ATCGCCACTTCTCCAAT

2.4 *In vitro* antiparasitic activity assays:

Blastocystis sp. parasites susceptibility assays were performed *in vitro* as described by [23] in 2 ml final volumes seeded with $2x10^5$ parasites and incubated for 48h at 37°C. Concentration of 250 µl/ml, 125 µl/ml, 62.5 µl/ml, 31.2 µl/ml, and 15.6 µl/ml µl of bovine, goat and camel raw milk were tested in duplicate for their antiparasitic activity against two *Blastocystis sp.* isolates identified as ST1 and ST3. Metronidazole was used at a concentration of 0.1 mg/ml as an effective antiparasitic positive control. Two other cultures without additions were used in parallel of each assay as parasites growth controls. After 48 h, 1.5 ml of supernatant media were carefully aspirated out after centrifugation at 800 rpm for 5 min. Sediments were then agitated to distribute evenly the parasites in the remaining media before counting in presence of 0.4% trypan blue (Sigma-Aldrich Corp. USA) as viability indicator [24 25]; only parasites that did not take up trypan blue stain were counted. Counting was performed by two investigators in triplicate for each assay. The entire experiment was repeated three times using different raw milk collections. Raw milks were collected in veterinary college from controlled animals certified as free of known microbial and parasitic infections.

2.5 Statistical analysis:

The data were analysed using the Chi-square test. A P-value < 0.05 was statistically significant. Statistical analysis was performed using SPSS version 21.

UNDER PEER REVIEW

3. RESULTS

During the three months collection period, seven *Blastocystis sp.* positive samples were detected by microscopy among a total of 1136 examined stool samples from symptomatic and healthy individuals. Two isolates were identified as ST1 subtype and five isolates as ST3 subtype by specific sequence-tagged-site (STS) primers **(Figure 1)**.

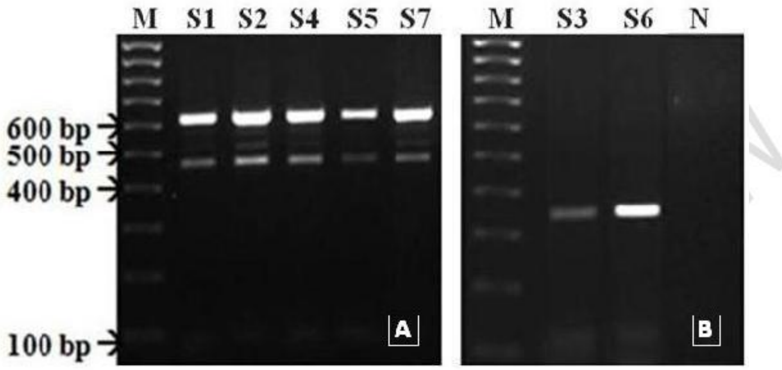

Figure 1: Sequence-tagged Sites (STS) SSU rDNA primer-based PCR analysis of *Blastocystis sp.* subtypes of positive samples from symptomatic patients (S1-S5) and asymptomatic individuals (S6 and S7) using: SB227 (ST3-526bp), SB228 (ST3-473bp), and SB229 (ST3-631bp) combined primer pairs as a multiplex reaction for ST3 subtype (Panel A), and SB83 (ST1-351bp) primer pair for ST1 subtype detection (Panel B). Negative control (lane N) and 100 bp molecular size marker (lane M) separated in parallel.

Two isolates, named S1 identified as ST3 subtype and S3 identified as ST1 subtype, from GIT symptomatic patients were used for raw milk susceptibility *in vitro* assays, separately and in duplicate in three different experiments. A significant *in vitro* killing effect was obtained with camel raw milk at minimal concentration of 31.2 µl/ml, compared to bovine raw milk (**P<0.05) and goat raw milk (**P<0.05). **(Table 2)**.

Table 2: Camel raw milk antiparasitic effectiveness against ST1 and ST3 *Blastocystis sp.* subtypes compared to bovine and goat raw milks at different concentrations:

Concentration of raw milk (µl/ml)	Subtype	Parasites' count (mean±SD)x10^5		P-value
		Camel raw milk	Bovine raw milk (B) Goat raw milk (G)	
15.6	ST1	14.67±4.36	(B) 20.33±5.81 (G) 24.00±7.06	0.204 0.311
	ST3	16.67±4.16	(B) 22.33±6.81 (G) 23.00±5.19	0.286 0.175
31.2	ST1	7.33±1.53	(B) 16.00±4.00 (G) 18.33±3.61	0.004 0.003
	ST3	6.00±1.00	(B) 18.00±4.00 (G) 19.33±3.21	0.007 0.002

UNDER PEER REVIEW

62.5	ST1	0.67±0.23	(B) 15.33±6.11 (G) 19.67±5.13	0.003 0.004
	ST3	0.57±0.31	(B) 16.33±4.51 (G) 20.67±5.03	0.004 0.002
125	ST1	1.02±0.15	(B) 15.67±3.21 (G) 15.67±4.04	0.003 0.003
	ST3	0.83±0.15	(B) 17.00±3.61 (G) 17.33±4.16	0.001 0.002
250	ST1	0.91±0.26	(B) 14.00±4.36 (G) 13.33±5.51	0.002 0.005
	ST3	1.07±0.38	(B) 15.67±2.08 (G) 14.67±5.03	0.0001 0.01

Maximum killing effect was noted at a starting concentration of 62.5 µl/ml of camel raw milk **(Figure 2)**.

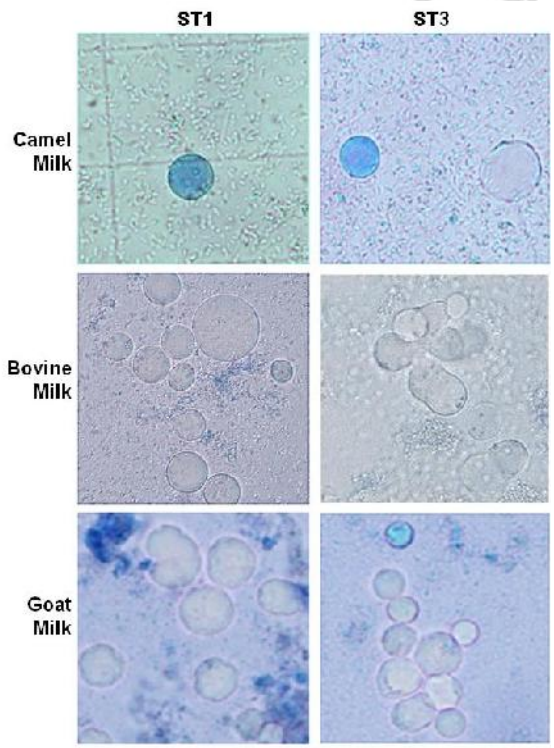

Figure 2: ST1 and ST3 *Blastocystis sp.* parasites counting in haemocytometer (improv Neubauer chamber) after 48h culture incubation in presence of 62.5 µl/ml of cam bovine, and goat raw milks.

At this concentration, camel raw milk showed the highest significant killing effect compared to cow raw milk (**P<0.05) and goat raw milk (**P<0.05) **(Figure 3)**.

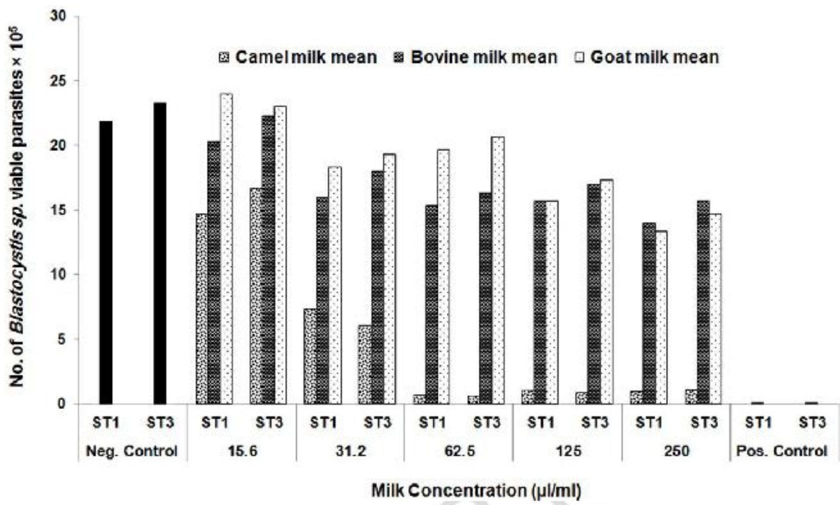

Figure 3: *In vitro* antiparasitic activity against ST1 and ST3 *Blastocystis sp.* subtypes of camel, cow and goat raw milk at a concentration of 62.5 µl/ml, in parallel with positive (0.1 mg/ml Metronidazol) and negative controls.

Both, cow and goat raw milk did not show a noticeable *in vitro* killing effect at the highest concentration (250 µl/ml). No significant difference of antiparasitic effects of raw milk types were observed between *Blastocystis sp.* subtypes ST1 and ST3.

4. DISCUSSION

Previous investigations have shown the predominance of *Blastocystis sp.* ST3 subtype in Makkah region, especially among symptomatic patients [26]. Accordingly, in the current study, 5 out of 7 (71%) *Blastocystis sp.* positive cases were determined as ST3 subtype and 2/7 (29%) as ST1 subtype.

Antiparasitic activity of milk from humans and different animals, in particular cow, goat and camel have been investigated by many authors [14 15]. This is the first reported study concerning antiparasitic activity of raw bovine, goat and camel milks against *Blastocystis sp.* parasites *in vitro*. Camel raw milk showed significant *in vitro* killing activity against *Blastocystis sp.* ST3 and ST1 isolates from patients with gastrointestinal symptoms. It has been reported that camel milk have *in vivo* anti-schistosomal activity on *Schistosoma mansoni* due to an immuno-modualatory effect at a dose of 200 µl/day in mice [19]. More recently, Alimi *et al.* [27] demonstrated *in vitro* ovicidal activity of raw camel milk against *Haemonchus contortus* at a concentration of 100 mg/ml as well as adult worm paralysis and/or death, differently from other animals' milk that did not show perceptible antiparasitic activity. Likewise, in our study goat and cow raw milks did not show *in vitro* antiparasitic activity against *Blastocystis sp.*

UNDER PEER REVIEW

Furthermore their antiparasitic activity, a number of studies have reported antibacterial, antifungal, and antiviral effects of camel milk constituents such as lysozymes and lactoferrin which levels were indicated to be at least two and three times higher than those of cow's milk, respectively [17 28]. Alimi *et al.* [27] found that lactoferrin amount was 6-fold higher in camel milk than cow and goat milk. Lactoferrin is a multifunctional protein that has been analyzed thoroughly; its antiparasitic effect is mainly associated with iron sequestration and destabilization of the membrane of parasites such as *Pneumocystis carinii* and *Toxoplasma gondii* [29 30]. Lactoferrin showed amoebicidal effect against *Entamoeba histolytica* trophozoites by membrane binding leading to lipid disruption and cell damage [31]. Bovine lactoferrin peptides caused the formation of pores and substantial membrane disruption and apoptosis in *Giardia intestinalis* trophozoites *in vitro* [32]. Oral treatment with Lactoferricin has prevented death in 100% of mice challenged with *Toxoplasma gondii* cysts compared to 80% mortality in untreated group by acute toxoplasmosis within 14 days post challenge [33]. Additionally, lactoferrin was confirmed as a potent antiviral [34], antifungal [35] and most significantly anti-cancer [36]. The prophylactic therapy with recombinant human lactoferrin improved defences against invasive *E. coli* in the nascent small intestine [37].

5. CONCLUSION

Raw camel milk revealed a substantial dose-dependent *in vitro* antiparasitic activity against *Blastocystis sp.* ST1 and ST3 subtypes, opening a promising perspective for its use in the control of this wide spread gastrointestinal parasite. In contrast, cow and goat raw milks did not show noticeable anti-*Blastocystis sp.* activity against both subtypes. Further *in vitro* and *in vivo* investigations are needed to explore most effective antiprotozoal components of camel raw milk.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Rowida A. B. designed the study, and wrote the first draft of the manuscript. Raafat T. M. performed parasites culture, DNA extraction and genotyping experiments, while Alharthi O.A., Hushlul S.M. and Elshehry A. performed *in vitro* susceptibility assays and statistical analysis. Finally Elbali M.A. wrote the protocol, shared in molecular experiments and manuscript writing. All authors read and approved the final manuscript.

CONSENT

All participants who joined this research had signed an informed consent.

ETHICAL APPROVAL

Ethical approval for this project was obtained from the Medical Research Centre and Research Committee at the Faculty of Medicine, Umm Al-Qura University, Saudi Arabia

UNDER PEER REVIEW

(Research protocol# 43409049). All participants who joined this research had signed an informed consent.

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UNDER PEER REVIEW

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