Original Research Article

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

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ABSTRACT

Aims: the aim of the current study was to investigate in-vitro anti-protozoal activity of camel, cow, and goat milks against *Blastocystis sp.* strains isolated from symptomatic patients.
Study design: experimental research study
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 PCR using sequence-tagged-site primers. *Blastocystis sp.* parasites susceptibility assays were performed in 2ml final volumes seeded with $2x10^5$ parasites and incubated for 48h at 37°C. 500µl, 250µl, 125µl, 62.5µl, 31.25µl, and 15.6µl of bovine, goat and camel milk were tested in duplicate for their antiparasitic activity against two *Blastocystis sp.* isolates. Metronidazole was used at 0.1mg/ml as positive antiparasitic control in all assays.
Results: Out of the eight 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 milk at minimal concentration of 62.5µl/2ml culture media compared to cow milk (P>0.007) and goat milk (P>0.002), on both subtypes. Both, cow and goat raw milk did not show a noticeable in-vitro killing effect at the highest dose of 500ul/2ml.
Conclusion: Whole 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., SSUrDNA STS sub-typing, camel 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. Its incidence had exhibited a remarkable increase in the past few years, with nearly 60% prevalence documented in tropical, subtropical and developing nations [2]. *Blastocystis sp.* display varied morphological forms; they may appear as

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vacuolar, granular, ameboid, cystic, avacuolar or multivacuolar [3]. The pathogenic potential of *Blastocystis* is debatable; several reviews discussed the controversy of its capability to cause disease [4]. *Blastocystis* parasites have been identified in patients with various gastrointestinal or even allergic skin symptoms, but also in evidently healthy people. It has been suggested that genetically diverse genotypes or subtypes may be linked to its pathogenic potential [5]. Different molecular approaches such as PCR by SSUrDNA Sequence-tagged-site primers are used to study genetic variation among *Blastocystis sp.* isolates [6-9].

Antiparasitic activity of milk from humans and different animals has been investigated by many authors. Bovine, goat and camel milks are the most investigated ones [10 11]. Milk includes numerous compounds such as lacto-peroxidase, lactoferrin, immunoglobulin G, secretory immunoglobulin A, and Lysozymes [12]. The protective effect of these proteins had been screened against several bacterial strains like *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Lactococcus lactis* and rotavirus [13]. Camel milk lacto-peroxidase had been identified as bacteriostatic and bactericidal against Gram-positive and Gram-negative strains, respectively. Its high content in anti-viral antibodies are protective against rotavirus [13].

Camel milk lactoferrin showed anti-cancer effect by reducing colorectal cancer cells proliferation in vitro [14]. Mature and colostral camel milk have proven to be anti-schistosomal against *Schistosoma mansoni* in infected mice [15].
The present study is the first report on antiparasitic activity of bovine, goat and camel milk against *Blastocystis sp.* isolates from symptomatic patients.

2. MATERIAL AND METHODS

2.1 Samples collection and parasites identification:

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 [16]: 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.

2.2 *Blastocystis sp.* in vitro culture:

The samples had been cultured in 11×100-mm sterile screw-capped tubes containing 2 ml of media and incubated 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 [17]. A drop of culture was examined after 24, 48, and 72 by direct microscopy. After several passages of positive cultures, parasites were counted in a Neubauer chamber and cryo-preserved 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 cultures by using QIAmp DNA extraction kit (QIAmp, QIAGEN Inc, Germany) according to manufacturer's protocol. Concentration and purity of isolated DNA were measured by a spectrophotometer (SpectraDrop, SpectroMax, life technology, USA). *Blastocystis sp.* subtyping was performed by PCR using sequence-tagged-site primers according to [18] **(table 1)**. 2 µl of DNA extracts were amplified in PCR reactions of 25µl with AmpliTaq Gold 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 57°C for 30 s, and extension at 72°C for 1 min,

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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 SSUrDNA identification by PCR.

Subtype	Primers set name	PCR products size (bp)	Accession N° in GenBank	Sequences
ST 1	SB83	351	AF166086	F: GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAGGCAGC
ST 2	SB340	704	AY048752	F: TGTTCTTGTGTCTTCTCAGCTC R:TTCTTTCACACTCCCGTCAT
ST 3	SB227	526	AF166088	F:TAGGATTTGGTGTTTGGAGA R:TTAGAAGTGAAGGAGATGGAAG
	SB228	473	AF166089	F: GACTCCAGAAACTCGCAGAC R: TCTTGTTTCCCCAGTTATCC
	SB229	631	AF166090	F: CACTGTGTCGTCATTGTTTTG R: AGGGCTGCATAATAGAGTGG
ST4	SB337	487	AY048750	F: GTCTTTCCCTGTCTATTCTTGCA R:AATTCGGTCTGCTTCTTCTG
ST5	SB336	317	AY048751	F:GTGGGTAGAGGAAGGAAAACA R:AGAACAAGTCGATGAAGTGAGAT
ST6	SB332	338	AF166091	F: GCATCCAGACTACTATCAACATT R:CCATTTTCAGACAACCACTTA
ST7	SB155	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 [19] in 2ml final volumes seeded with $2x10^5$ parasites and incubated for 48h at 37°C. 500µl, 250µl, 125µl, 62.5µl, 31.25µl, and 15.6µl of bovine, goat and camel milk were tested in duplicate for their antiparasitic activity against two *Blastocystis sp.* isolates. Metronidazole was used at 0.1mg/ml as positive antiparasitic control in all assays. Milk and Metronidazol free cultures were used in parallel of each assay as parasites growth controls. After 48h, 1.5ml of supernatant media were carefully aspirated out. Sediments were then agitated to distribute evenly the parasites in the remaining media before counting in Neubauer chambers. Counting was performed by two investigators in triplicate for each assay.

2.5 Statistical analysis:

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

3. RESULTS

During the two 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)**.

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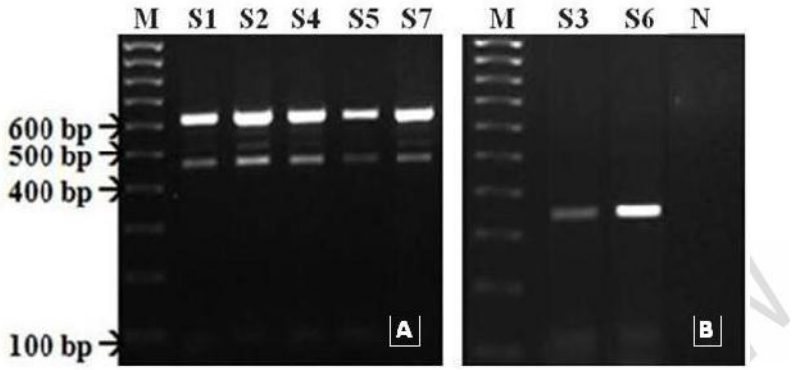

Figure 1: Sequence-tagged Sites (STS) SSUrDNA primer-based PCR analysis of *Blastocystis sp.* subtypes of positive samples from symptomatic patients (S1-S5) and asymptomatic individuals (S6 and S7) using: SB227 (ST3-526bp), SB228 (ST3-473bp), and SB229 (ST3-631bp) combined primer pairs as a multiplex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

Two isolates, S1 (ST3 subtype) and S3 (ST1 subtype), from GIT symptomatic patients were used for milk susceptibility in vitro assays. A significant in vitro killing effect was obtained with camel milk at minimal concentration of 62.5µl/2ml culture media compared to cow milk (**P>0.007) and goat milk (**P>0.002) (**Table 2**).

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Table 2: Camel milk antiparasitic effectiveness against *Blastocystis sp.* compared to cow and goat milks at different concentrations:

Concentration of milk (µl/2ml)	Parasites' count (mean±SD)x10^5		P-value
	Camel Milk	Bovine Milk (B) Goat Milk (G)	
31.2	16.67±4.16	(B) 22.33±6.81 (G) 23.00±5.19	0.286 0.175
62.5	6.00±1.00	(B) 18.00±4.00 (G) 19.33±3.21	0.007 0.002
125	0.57±0.31	(B) 16.33±4.51 (G) 20.67±5.03	0.004 0.002
250	0.83±0.15	(B) 17.00±3.61 (G) 17.33±4.16	0.001 0.002
500	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 125µl/2ml culture with camel milk **(Figure 2)**.

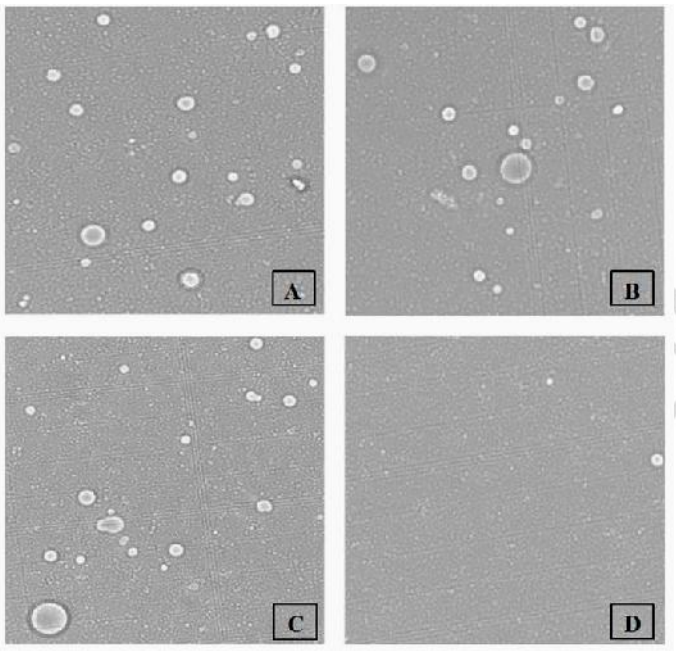

Figure 2: *Blastocystis sp.* parasites counting in Neubauer chambers after 48h culture incubation of negative control (A), and susceptibility assays using 125µl milk of cow (B), goat (C) and camel (D) milks.

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115 At this concentration, camel milk showed the highest significant killing effect compared to
116 cow milk (**P>0.004) and goat milk (**P>0.002) **(Figure 3)**.

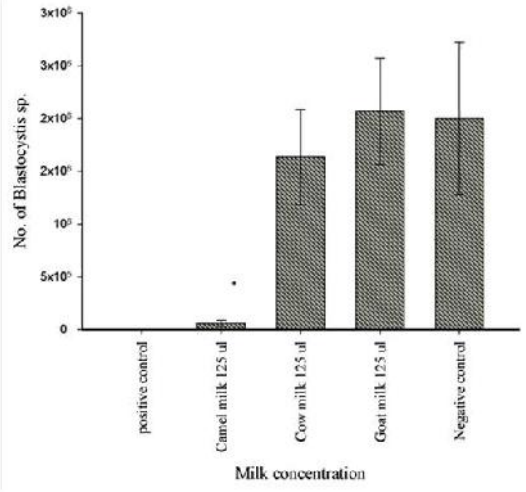

Figure 3: In vitro antiparasitic activity against *Blastocystis sp.* of camel, cow and goat

milks at a concentration of 125µl/2ml, in parallel with Metronidazole (positive control) and negative control.

Both, cow and goat raw milk did not show a noticeable in vitro killing effect at the highest dose of 500ul/2ml culture. 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 [20]. 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 [10 11]. This is the first reported study concerning antiparasitic activity of raw bovine, goat and camel milks against *Blastocystis sp.* parasites in vitro. Camel milk showed significant in vitro killing activity against *Blastocystis sp.* ST3 and ST1 isolates from patients with gastrointestinal symptoms. It has been reported that both mature and colostoral camel milk have in vivo anti-schistosomal activity on *Schistosoma mansoni* due to an immuno-modualatory effect at a dose of 200µl/day in mice[15]. More recently, Alimi *et al.* [21] demonstrated in vitro ovicidal activity of raw camel milk against *Haemonchus contortus* at a concentration of 100mg/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 milk did not show in vitro antiparasitic activity against *Blastocystis sp.*

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 [13 22]. Alimi *et al.* [21] 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* [23 24]. Lactoferrin showed amoebicidal effect against *Entamoeba histolytica* trophozoites by membrane binding leading to lipid disruption and cell damage [25]. Bovine lactoferrin peptides caused the formation of pores and substantial membrane disruption and apoptosis in *Giardia intestinalis* trophozoites in vitro [26]. 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 [27]. Additionally, lactoferrin was confirmed as a potent antiviral [28], antifungal [29] and most significantly anti-cancer [30]. The prophylactic therapy with recombinant human lactoferrin improved defences against invasive *E. coli* in the nascent small intestine [31].

5. CONCLUSION

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Whole 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 milk.

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