Original article

Utility of the oestrogen-dependent vaginal candidosis murine model in evaluating the efficacy of various therapies against vaginal *Candida albicans* infection

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Summary

The efficacy of yogurt treatment against vaginal candidosis (VC) was examined using an oestrogen-dependent vaginal candidosis (EDVC) murine model. The EDVC mouse model was constructed by inoculating mice with viable *Candida albicans* cells under pseudo-oestrus conditions. Vaginal fungal burden in the various mouse groups was evaluated at several time points following the induction of VC. Untreated and yogurt-treated naïve mice exhibited background levels of VC (<6000 CFU per mouse). *Candida albicans* colonisation in untreated EDVC mice was significantly higher ($P < 0.05$) than that in yogurt-treated EDVC mice at days 20–30. Metronidazole-treated naïve mice developed persistent *C. albicans* vaginal colonisation at significantly lower levels ($P < 0.05$) than that in untreated or metronidazole-treated EDVC mice. *Lactobacillus* was only detected in the reproductive tracts of yogurt-treated naïve and EDVC mice. These findings suggest that the presence of *Lactobacillus* in the reproductive tract can suppress *C. albicans* growth and the antibiotics may predispose to VC.

Key words: *Candida albicans*, oestrogen, *Lactobacillus*, metronidazole, vaginal candidosis, yogurt.

Introduction

The various forms of vaginal candidosis (VC) represent a significant health problem to women of childbearing age.[1, 2] *Candida albicans*, a commensal that resides in the intestinal and reproductive tracts, is believed to be the causative agent in 85–90% of cases.[2] VC has been attributed to several predisposing factors including compromised immunity, diabetes mellitus, antibiotics and elevated levels of oestrogen in the reproductive tract.[2–5] VC is treated with imidazole in ointment or vaginal suppository form, terconazole in topical form or fluconazole in tablet form.[6, 7] The unease of applying these drugs, the persistence and/or recurrence of candidosis and the possible side effects that may result because of overdosing or chronic use have caused therapists and women to turn to alternative therapeutic approaches.

Treatment with yogurt or exogenous *Lactobacillus* species (mainly *Lactobacillus acidophilus*) has been reported to alleviate the symptoms of VC.[8–10] Yogurt applied once or twice daily for 7–14 days was reported to slow or prevent the growth of *C. albicans*.[7] Lack of a suitable animal model of VC represents a major hindrance before any effort to examine the efficacy of yogurt or exogenous *Lactobacillus* species usage. Previous studies, which have addressed this issue by using women as experimental subjects were received with scepticism. They were criticised for small sample size, high attrition rates, dose inconsistencies and lack of detailed parameters of treatment efficacy.[7] This is in addition to increased cost, extended time frames and difficulties related to sample randomisation and timely follow ups. Here, an oestrogen-dependent vaginal candidosis (EDVC) murine

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model[6, 11, 12] was used to evaluate the capacity of yogurt to suppress *C. albicans* growth, the model was also used to evaluate the effect of antibiotic use on the progression of VC.

Materials and methods

Mice and micro-organisms

Throughout this study 8- to 12-week-old female Balb/c non-germ free mice raised at the Hashemite University vivarium were used. *Candida albicans* ATCC strain 36082 used in this study is from Dr M. A. Ghannoum (Mycology Reference Laboratory, University Hospital of Cleveland, OH, USA).

Construction of the EDVC murine model

The mouse model was constructed as previously described.[5, 12] Briefly, *C. albicans* was maintained on Sabouraud's dextrose agar (SDA; Difco, Detroit, MI, USA), stored at 4 °C and subcultured at 3-month intervals. For inoculation, overnight cultures of *C. albicans* were grown at 37 °C in SDA broth as described previously.[13] Immediately before use, cells were harvested and washed twice in sterile physiological saline (SPS). Oestrogen was administered subcutaneously by injecting 0.5 mg of estradiol valerate (Schering AG, Berlin, Germany) dissolved in 0.1 ml of sesame oil 72 h prior to *C. albicans* inoculation and at weekly intervals thereafter. Vaginal *C. albicans* inoculate consisted of 100 μl containing 2×10^7 viable stationary-phase blastoconidia.

Unpasteurised yogurt (DJD Products, Amman Jordan) was applied to the vaginal area of naïve mice and EDVC mice 7 days after the construction of the model, this treatment regimen continued on every-other-day basis for 3 weeks. Hundred microlitres of yogurt was directly injected to the vagina using a Hamilton syringe and 200 μl of yogurt loaded to a sterile cotton swab was applied to the vaginal area. Metronidazole (Drug Ltd, Maharashtra, India), an antibiotic commonly used against Gram-negative bacteria, was injected intravenously at an every other day dose of 200 μg per mouse for a period of 3 weeks.

Evaluation of vaginal colonisation with *C. albicans* and *Lactobacillus* species

The vaginal fungal burden was evaluated following previously published methods.[5, 12] Mice were killed at different time points after *C. albicans* inoculation. Vaginas were isolated, examined for the presence of white lesions characteristic of *C. albicans* infection, pooled, trimmed and homogenised in 10 ml of SPS in a sterile glass homogeniser (Ystral GmbH, Gottingen, Germany). Serial 10-fold dilutions were prepared from the homogenate; 1 ml of aliquots of the appropriate dilution were added into culture plates containing 10 ml of SDA and chloramphenicol at 50 mg l^{-1}, plates were left to solidify and then incubated at 37 °C; each sample dilution was cultured in triplicate. Yeast colonies were counted 48 h after plating and colonisation results were expressed as the mean colony-forming unit (CFU) per mouse based on data from five to six animals per group. The presence of *Lactobacillus* species was qualitatively tested by microscopic examination of Gram-stained colonies growing on blood agar streaked with homogenised vaginal tissue suspension. Blood haemolysis and catalase tests of colonies isolated from the blood agar cultures were also carried out.

Statistical analysis

Using experimental mean values, analysis of variance (one-way ANOVA) was employed to determine levels of significance between different experimental groups at $P < 0.05$.

Results

Consistent with previous studies,[5] *C. albicans* vaginal colonisation levels in naïve mice were minimal or undetectable (Fig. 1). EDVC mice began to show significant and persistent levels of *C. albicans* vaginal colonisation starting from day 1 following the induction of VC onwards (data for days 1–8 not shown). This is also consistent with previous reports, which have shown that oestrogen is capable of maintaining significant levels of *C. albicans* colonisation for up to 8 weeks.[12] Up to day 20, levels of colonisation fluctuated between around >36 000 CFU at day 12 and <4000 CFU at day 18 (Fig. 1). Although this pattern of fluctuation is consistent with previous findings[12] it cannot be readily explained at the present time. The level of *C. albicans* vaginal colonisation in untreated EDVC mice was significantly higher ($P < 0.05$) than that in yogurt-treated EDVC mice at days 20–30 of the experiment. This is consistent with the general notion that treatment with yogurt can resolve VC.[7]

To validate that this significant difference between treated and untreated EDVC mice was due to yogurt treatment; vaginal tissue suspensions prepared from the same mouse groups were tested for the presence of *Lactobacillus* species. Untreated naïve mice and

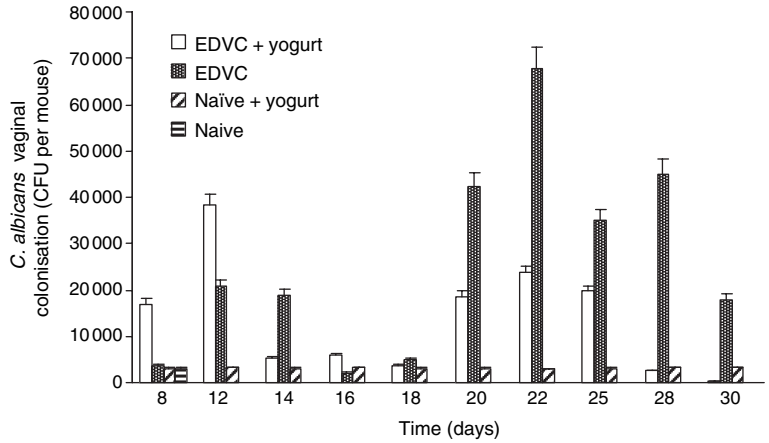

Figure 1 The effect of yogurt treatment on vaginal fungal burden during experimental vaginal candidosis. Levels of *Candida albicans* colonisation in untreated naïve and oestrogen-dependent vaginal candidosis (EDVC) mice as well as yogurt-treated naïve and EDVC mice were assessed at days 8, 12, 14, 16, 18, 20, 22, 25, 28 and 30 post infection. Yogurt treatment commenced at day 7 following the construction of the animal model and continued on every other day basis until the termination of the experiment. Mean *C. albicans* CFU count per vagina ±SEM was calculated based on the average of five to six mice per group per time point using data from three separate experiments.

untreated EDVC mice were free of *Lactobacillus* species (Table 1, columns 2 and 5). However, vaginal tissue suspensions of yogurt-treated naïve and yogurt-treated EDVC mice were replete with *Lactobacillus* species (Table 1, columns 3 and 6) at all time points tested.

The capacity of antibiotics commonly used to treat against Gram-negative bacteria to initiate or enhance the growth of *C. albicans* was evaluated. This was based on the above-noted findings that yogurt treatment, which results in the introduction of *Lactobacillus* species, is capable of suppressing the growth of *C. albicans*. As shown in Fig. 2, naïve mice treated with metronidazole started to develop significant and persistent levels of *C. albicans* vaginal colonisation by day 16 (>37 000 CFU) onwards. However, these levels were lower than that in the EDVC mouse group. *Candida albicans* vaginal colonisation was significantly greater ($P < 0.05$) in metronidazole-treated naïve mice when compared with that in untreated naïve mice at days 16 and 30 in particular (Figs 1 and 2). In EDVC mice treated with metronidazole, *C. albicans* vaginal colonisation fluctuated between days 8 and 22, then started to stabilise in the upward direction. Overall, *C. albicans*

Table 1 The presence (+) or absence (–) of *Lactobacillus* species in the vaginal tract of various naïve and oestrogen-dependent vaginal candidosis (EDVC) mouse groups.

	Presence (+) or absence (–) of *Lactobacillus* species					
Day	Untreated-naïve mice	Yogurt-treated naive mice	Metronidazole-treated naive mice	Untreatred-EDVC mice	Yogurt-treated EDVC mice	Metronidazole-treated EDVC mice
0	–	–	–	–	–	–
8	–	+	–	–	–	–
12	–	+	–	–	+	–
14	–	+	–	–	+	–
16	–	+	–	–	+	–
18	–	+	–	–	+	–
20	–	+	–	–	+	–
22	–	+	–	–	+	–
25	ND	ND	–	–	+	–
28	ND	ND	–	ND	+	–
30	ND	ND	–	ND	+	–

ND, not determined.

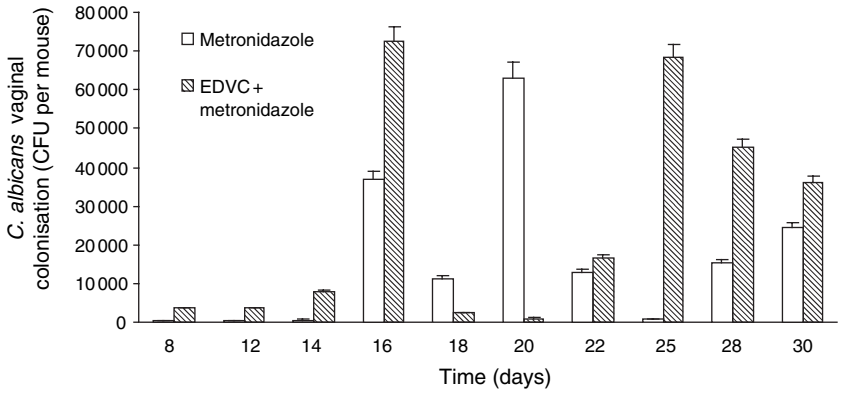

Figure 2 The effect of metronidazole treatment on vaginal fungal burden during experimental vaginal candidosis. Levels of *Candida albicans* colonisation in metronidazole-treated naïve and oestrogen-dependent vaginal candidosis mice were assessed at days 8, 12, 14, 16, 18, 20, 22, 25, 28 and 30 post infection. Treatment with metronidazole commenced at day 7 following the construction of the model and continued on every other day basis until the end of the experiment. Mean *C. albicans* CFU count per vagina ±SEM was calculated based on the average of five to six mice per group per time point and three separate experiments per group.

colonisation in this group persisted throughout the experiment. Although VC was persistent in both EDVC mice and metronidazole-treated EDVC mice, kinetics of *C. albicans* colonisation between the two groups was different. Whereas vaginal fungal burden in untreated EDVC mice peaked at day 22 (68 000 CFU per vagina), it peaked at day 16 (74000 CFU per vagina) in metronidazole-treated EDVC mice. This was consistently noted – in qualitative terms but slightly different time frame – in three separate experiments.

To validate the effectiveness of metronidazole application, the presence of *Lactobacillus* species in treated and control mouse groups was qualitatively evaluated. Tissue homogenates prepared from the vaginas of metronidazole-treated naïve and EDVC mice were free of *Lactobacillus* species (Table 1, columns 4 and 7) at all time points tested.

Discussion

It is evident that the EDVC murine model can be effectively utilised to study various aspects of VC. First, the model maintains persistence of vaginal *C. albicans* infection, a prerequisite to the utility of the model in VC pathogenesis and therapy studies. Our findings show that weekly injections of 0.5 mg oestrogen can maintain vaginal *C. albicans* infection persistent for 4 weeks. In previously published reports,[5, 11, 12] it was noted that persistence of vaginal *C. albicans* infection could last for up to 8 weeks under pseudo-oestrus conditions. Secondly, expansion of sample size and randomisation of the various experimental and control groups is possible. Here, we used 400 mice grouped into six different age-matched experimental and control groups. A follow-up time of 3 weeks was sufficient to detect statistically significant differences between the various groups included in the study; mainly, those with persistent VC in the presence or absence of yogurt treatment. Treatment efficacy parameters were objective, in that the progression or lack, thereof, of VC was established based on the accurate measurement of vaginal fungal burden at 10 time points within the treatment period. The capacity of yogurt used in the treatment to introduce *Lactobacillus* species was also directly examined. Inclusion of additional parameters like the use of antibiotics and evaluation of its effect to enhance or suppress VC was also possible in this preliminary study.

Although the focus of this preliminary study was the utility of the EDVC as an animal model in VC pathogenesis studies, few findings regarding yogurt treatment *per se* are worth mentioning. It is clear that yogurt can significantly slow down the growth of *C. albicans* via introducing *Lactobacillus* species into the reproductive tract milieu. These findings corporate numerous previous studies, which have indicated that yogurt treatment ameliorates the symptoms of VC.[7, 8–10, 14] However, yogurt does not completely eradicate the fungus as evidenced by the finding that the infection remained persistent in yogurt-treated EDVC mice throughout the experiment.

Establishing – in quantitative terms – the difference between the symptomatic of *C. albicans* as a VC disease state and the innocuous presence of the fungus in the reproductive tract is paramount for determining treatment efficacy. In other words, can the difference between yogurt-treated mice and untreated mice in

terms of vaginal fungal burden be equated with VC vs. the normal homeostatic state. Although our findings show a statistically significant difference between yogurt-treated and untreated-EDVC in terms of vaginal fungal burden, it is impossible to interpret this difference as the difference between health and disease. Possible side effects, although not seen in general terms as yogurt-treated EDVC mice looked healthy and survived for months after treatment (data not shown), were not exhaustively studied. Several groups have proposed the use of exogenous *Lactobacillus* species as means of treating VC.[7] However, the therapeutic dose, *Lactobacillus* species and strain to be used and the appropriate route of administration are issues yet to be settled. The EDVC mouse model could prove useful in this regard.

Acknowledgments

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References

1 Ferrer J. Vaginal candidosis: epidemiological and etiological factors. *Int J Gynaecol Obstet* 2000; **71**: 521–7.
2 Odds FC. *Candida* and Candidosis, 2nd edn. London: Bailliere Tindall, 1988.
3 Kinsman OS, Pitblado K, Coulson CJ. Effect of mammalian steroid hormones and luteinizing hormone on the germination of *Candida albicans* and implications for vaginal candidosis. *Mycoses* 1988; **31**: 617–24.
4 White S, Larsen B. *Candida albicans* morphogenesis is influenced by estrogen. *Cell Mol Life Sci* 1997; **53**: 744–9.
5 Hamad M, Abu-Elteen KH, Ghaleb M. Estrogen-dependent induction of vaginal candidosis in naive mice. *Mycoses* 2004; **47**: 304–9.
6 Abu Elteen KH, Hamad M. Anti fungal compounds (Chapter 8). In: Cavanaugh K (ed.), *Fungi: Biology and Applications*. New York, NY, USA: Wiley & Sons, 2005: 191–217.
7 Jeavons HS. Prevention and treatment of vulvovaginal candidiasis using exogenous Lactobacillus. *J Obstet Gynecol Neonatal Nurs* 2003; **32**: 287–96.
8 Hughes VL, Hillier SL. Microbiologic characteristics of *Lactobacillus* products used for colonization of the vagina. *Obstet Gynecol* 1990; **75**: 244–9.
9 Hilton E, Rindos P, Isenberg HD. *Lactobacillus* GG vaginal suppositories and vaginitis. *J Clin Microbiol* 1995; **33**: 1433.
10 Williams AB, Yu C, Tashima K, Burgess J, Danvers K. Evaluation of two self-care treatments for prevention of vaginal candidiasis in women with HIV. *J Assoc Nurses AIDS Care* 2001; **12**: 51–57.
11 Fidel PL, Sobel JD. Protective immunity in experimental *Candida* vaginitis. *Res Immunol* 1998; **149**: 361–73.
12 Ghaleb M, Hamad M, Abu-Elteen KH. Vaginal T lymhocyte population kinetics during experimental vaginal candidosis: evidence for a possible role of CD8+ T cell in protection against vaginal candidosis. *Clin Exp Immunol* 2003; **131**: 26–33.
13 Abu-Elteen KH, Abdul-Malek AMM, Abdul-Wahid NA. Prevalence and susceptibility of vaginal yeast isolates in Jordan. *Mycoses* 1997; **40**: 179–85.
14 Hilton E, Isenberg HD, Alperstein P, France K, Borenstein MT. Ingestion of yogurt containing *Lactobacillus acidophilus* as prophylaxis for candidal vaginitis. *Ann Intern Med* 1992; **116**: 353–7.