Evaluating a murine model of endometritis using uterine isolates of *Escherichia coli* from postpartum buffalo

Dar, S. H.[1]; Qureshi, S.[2]; Palanivelu, M.[3]; Muthu, S.[4]; Mehrotra, S.[5]; Jan, M. H.[1]; Chaudhary, G. R.[6]; Kumar, H.[1]; Saravanan, R.[7] and Narayanan, K.[1*]

[1]Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India; [2]Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India; [3]Divsion of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India; [4]Division of Temperate Animal Husbandry, Indian Veterinary Research Institute, Mukteswar, Uttarakhand, 263138, India; [5]Division of Livestock Production and Management, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India; [6]Ph.D. Scholar, Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India; [7]Immunology Section, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India

[*]***Correspondence:*** *K. Narayanan, Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India. E-mail: vetnanny@gmail.com*

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Summary

Ascending infection of the uterus with Gram-negative bacteria is responsible for postpartum endometritis in cattle and buffalo and can adversely affect fertility. Development of a laboratory animal model for bovine endometritis would facilitate the understanding of the pathogenesis as it is difficult to conduct controlled experimentation in the native host. In the present study, 30 virgin Swiss Albino mice (5-8 weeks old) were used to evaluate the pathogenic potential of *Escherichia coli*, isolated from the normally calved postpartum buffalo to induce endometritis. Mice in the diestrus phase of the estrous cycle were randomly allotted to one of the following four intravaginal inoculation (100 µL) treatments: EG (experimental group)-1: sterile normal saline; EG-2, -3 and -4: *E. coli*[@] 1.5×10^4, 10^5 and 10^6 CFU/ml, respectively. The animals were then scarified 36 h post-inoculation to study gross and microscopical lesions. Gross changes were confined to EG-4. Acute endometritis was recorded in 50% of the EG-3 and 66.7% of the EG-4. The rate of acute endometritis development was significantly higher in EG-4 ($P<0.05$) as compared to the other groups. The present study demonstrated that the animal model for bubaline endometritis can be developed in mice by intravaginal inoculation of *E. coli*[@] 1.5×10^6 CFU/ml at diestrus. Ease of intravaginal inoculation, apparent absence of systemic involvement and high infective rate are the advantages of the model over other studies.

Key words: Buffalo, *E. coli*, Endometritis, Mice model, Postpartum

Introduction

Ascending uterus infections caused by a wide range of bacteria are common in cattle and buffalo after parturition (Dohmen *et al*., 2000; Sheldon *et al*., 2002), often leading to endometritis or metritis (Runciman *et al*., 2009). Infection of the endometrium with *Escherichia coli* is the first step towards the development of endometritis as it paves the way for *Arcanobacterium pyogenes* and *Fusobacterium necrophorum* (Williams *et al*., 2007; Sheldon *et al*., 2009). Essential pathogenicity traits of *E. coli* include adhesion to epithelial cells (Torres *et al*., 2005), motility mediated by flagella (identified by the H serogroup) (Lane *et al*., 2007), and toxins such as shiga toxin, heat stable and labile toxins, and lipopolysaccharide (LPS, identified by the O serogroup) (Wolf, 1997).

Laboratory models have been proposed for ruminant diseases like brucellosis (Baldwin *et al*., 2002), campylobacteriosis (Pei and Blaser, 1990) and trichomoniasis (Van Andel *et al*., 1996; Monteavaro *et al*., 2008). A murine model has also been developed to investigate infection induced by preterm pregnancy losses in normal and IL-1β deficient mice (Reznikov *et al*., 1999). In humans, murine models were developed for various reproductive tract pathogens including *Chlamydia trachomatis* (Bernstein-Hanley *et al*., 2006), *Neisseria* gonorrhoeae (Kita *et al*., 1981) and Trichomonad infection (Agnew *et al*., 2008). Alternatively, pathogen-associated molecules such as lipopolysaccharide have been used *in vivo* to establish metritis and endometritis in mice (Deb *et al*., 2005; Aisemberg *et al*., 2007).

Uterine infections in cattle and buffaloes inflict high economic losses as they adversely affect the subsequent fertility (Azawi, 2010). Host-pathogen interactions at the endometrium level are yet to be deciphered due to the lack of an appropriate laboratory animal model. Despite the differences between the reproductive physiology of laboratory animals and ruminants, a laboratory animal model for bubaline endometritis will be of use for the following reasons: first, endometrial experimentation in buffalos is expensive, ethically restricting, logistically demanding, laborious and time consuming. Second, in a laboratory animal model, the pathophysiology of bubaline endometrial pathogens can be studied in controlled settings with a large sample size and high statistical power. Third, the efficacy of the novel

intrauterine therapies intended to eliminate the bubaline pathogens can be screened in the event of having a characterized lab animal model. Developing a murine model will be, therefore, advantageous as rabbit (Dombroski *et al*., 1990) and monkey (Gravett *et al*., 1994) models are technically difficult and expensive. In addition, genetically defined mice can be used to ascertain the role of different genes.

Considering the importance of *E. coli* in the genesis of postpartum (PP) endometritis in cattle and buffalo and the absence of a laboratory animal model, the present study was envisaged to evaluate the intravaginal inoculation of *E. coli* isolated from postpartum buffalo uterus to induce endometritis in virgin mice.

Materials and Methods

Animals

Virgin female laboratory Swiss Albino mice aged 5-8 weeks (n=30) were obtained from the Laboratory Animal Section of Division of Animal Breeding and Genetics, IVRI, Izatnagar, Bareilly U.P. The animals were given an acclimatization period of one week; were kept in poly propylene cages and fed on semisolid mash feed supplied by the Feed Processing Unit, IVRI, Izatnagar[@] 10 g/day/mouse. The experiment was approved by the Institute's Animal Ethics Committee.

Determining the stage of the mice estrous cycle

The estrous cycle of mice lasts 4 to 5 days unless interrupted by pregnancy, pseudopregnancy, or anestrus. Changes occurring in the mouse's estrous cycle are evident in the animal's physiology, anatomy and vaginal cytology which help determine the stage of estrous cycle (Byers *et al*., 2012; McLean *et al*., 2012). A sterile 200 µL tip fixed to a micropipette was used to draw approximately 100 µL sterile phosphate buffer saline (PBS). The mice were firmly grasped by the scuff and tail with the help of the left hand. A 200 µL tip loaded with PBS was placed at the opening of the vaginal canal taking care not to penetrate the orifice as vaginal (and cervical) stimulation can induce pseudopregnancy in mice. Gently, the pipette plunger was pressed to release 50-70 µL at the opening of the vaginal canal. The fluid was withdrawn back into the tip and placed on two glass slides, one for wet mount and the other for giemsa staining.

Wet mounts were used to identify the stage of the cycle. Giemsa staining was also performed to corroborate wet mount results. Once the smears became dry, they were fixed in methanol for 5 min, stained with giemsa (1:6 dilutions) and examined under the microscope at ×400 to confirm the stage of the estrus cycle.

Isolation and characterization of *E. coli* from postpartum Murrah buffalo

Custom designed doubled guarded endometrial swab was prepared. Briefly, the assembly consisted of an autoclaved ear bud plastic swab, sterile AI (artificial insemination) plastic sheaths (45 cm length) 4 mm and 5 mm in diameter (Imv Technologies, France) and a polythene sheath. The swab was mounted at one end of the 4 mm AI sheath. The assembly was guarded in the 5 mm AI sheath which formed the first insulation over the swab. The assembly was covered with a sterile polythene cover and left under ultra-violet light for 30 min. Sterility of the swab was confirmed by dipping in autoclaved peptone water as the tube showed no bacterial growth upon incubation for 24 h.

A normally calved postpartum Murrah buffalo of 2nd parity on day 20 PP was selected from the Institute's Animal Farm. After cleansing the perineum, the guarded uterine swab was guided through the cervix using the rectovaginal technique. At the level of inner os, the outer polythene sheath was retracted to expose the tip of the 5 mm AI sheath. At the uterus level, the inner AI sheath (4 mm) was pushed forward so that the swab was exposed to the endometrium. The exposed swab was scrapped against the endometrial lining and retracted back into the outer sheath (5 mm). The endometrial swab was immersed in peptone water and transported to bacteriological laboratory within 1 h. The buffalo was found to have subclinical endometritis on postpartum day 30 through the cytology of the uterine lavage.

The swabs were incubated for 1-2 h at 37°C in glucose peptone water, streaked on MacConkey agar and kept at 37°C overnight for incubation. Selected (pink) colonies were picked and streaked on eosin-methylene blue (EMB) agar to isolate and identify *E. coli*. Upon incubation for 24 h, greenish metallic sheen on EMB agar was suggestive of *E. coli* which was further confirmed biochemically by methyl red, motility indole lysine, triple sugar iron and citrate utilization tests. The colonies were identified as *E. coli* when they were positive for methyl red, motility indole lysine and triple sugar iron tests and negative for the citrate utilization test. Further confirmation was obtained by a PCR based detection of the *E. coli* (*fimH*) virulent factor after the isolation of bacterial gDNA, using *fimH* specific primers (Bicalho *et al*., 2012). The PCR product was cloned in pTZ57R/T and sequenced using outsourcing. The *fimH* gene sequence showed 98.6% homology to *E. coli* ETEC H10407 (NCBI Reference Sequence: gi|309700213).

Evaluation of the pathogenic potential of pure *E. coli* culture to induce acute endometritis in mice

A discrete colony of *E. coli* was picked from a brain heart infusion (BHI) agar plate and inoculated into peptone water broth and incubated at 37°C overnight. The broth culture was serially diluted and 100 µL of each dilution was plated. After overnight incubation, the colonies from two plates of each dilution were counted and their average values were taken. The pure culture concentration was 1.5×10^7 CFU/ml. Inocula containing *E. coli*[@] 1.5×10^4, 10^5 and 10^6 (CFU/ml) were prepared from the pure culture peptone water broth of *E. coli* with sterile pyrogen free normal saline.

On the day of the experiment, mice that were at

diestrus were randomly allotted to one of the following four experimental groups: experimental group 1 (EG-1) was administered 100 µL normal saline intravaginally, serving as negative control. Group 2, 3 and 4 (EG-2, -3 and -4) were inoculated (100 µL/mice) with *E. coli* intravaginally[@] 1.5×10^4, 1.5×10^5 and 1.5×10^6 CFU/ml, respectively.

Histopathology

The experimental mice were sacrificed 36 h post-inoculation and a cross section of the uterine tissue from the middle part of either left or right horn was preserved in 10% neutral buffered formal saline (1:10 v/v) for histopathology. The haematoxylin and eosin stained endometrial slides were read by a veterinary pathologist. Diagnosis of acute endometritis was based on the PMN leukocyte influx into the uterine lumen and infiltration in the stratum compactum, sloughing of epithelia and vascular changes.

Statistical analysis

Presence or absence of acute endometritis in the experimental groups was cross-tabulated and analyzed by Fisher's exact Chi-square test using Graphpad Prism software version 5. Significance was set at 95%.

Results

After inoculation, no apparent change in feed intake or general behaviour was noted in any mice till the time of sacrifice. Mild congestion of the uterus was observed in the EG-1 (Fig. 1A), EG-2 and EG-3. Edema of the uterus and exudation into the uterine lumen were found in EG-3 and -4. However, gross changes were more consistent in EG-4 than that of EG-3. The diameter of the uterus and uterine horns had almost increased five times in EG-4 as compared to the mice in the control group (Fig. 1B). In some of the EG-4 animals, the uterus was distended with fluid giving a balloon like appearance.

Histology of the endometrium in the control group mice (EG-1) showed intact endometrial lining of columnar epithelia with invaginating endometrial glands. Practically, no PMN leukocyte was found either in the lumen of uterus or in the endometrial glands.

Stromal hyperplasia and vascular congestion which were frequently observed, were regarded as a cyclic change (Fig. 2A). Similar changes were noticed in the mice inoculated with *E. coli*[@] 1.5×10^4 CFU/ml (EG-2). Typical histopathological changes that are the hallmark of acute inflammation were observed in the mice inoculated with *E. coli*[@] 1.5×10^6 CFU/ml (EG-4) and include: endometrial epithelial discontinuity and sloughing (Fig. 2B), copious influx of PMN cells into the uterine lumen (Fig. 2C), stromal hyperplasia (Fig. 2D), PMN infiltration in stratum compactum (Fig. 2E), glandular degeneration, glandular and periglandular infiltration of PMN cells (Fig. 2F), glandular obliteration (Fig. 2G) and perivascular infiltration of PMN cells (Fig. 2H). However, histological changes in EG-3 mice were inconclusive as only three out of six mice had lesions that were suggestive of acute endometritis. The pathological changes were largely confined to the mice in EG-4 and to some extent in EG-3.

The effect of three different doses of *E. coli* on the occurrence of acute endometritis at 36 h post-inoculation showed significant difference ($P<0.05$, Table 1). The infection rate was highest (66.7%) in EG-4, followed by EG-3 (50%).

Discussion

Endometritis was induced by infusion of *E. coli* through intrauterine infusion in the vagina (Sheldon *et al*., 2010) or laparotomy (Nishikawa *et al*., 1985), and intracervically (Hasan *et al*., 2013). The intravaginal route was chosen in the present study as it mimics the typical ascending infection of the postpartum uterus and eases delivery. Corbeil (1980) laid out certain criteria for

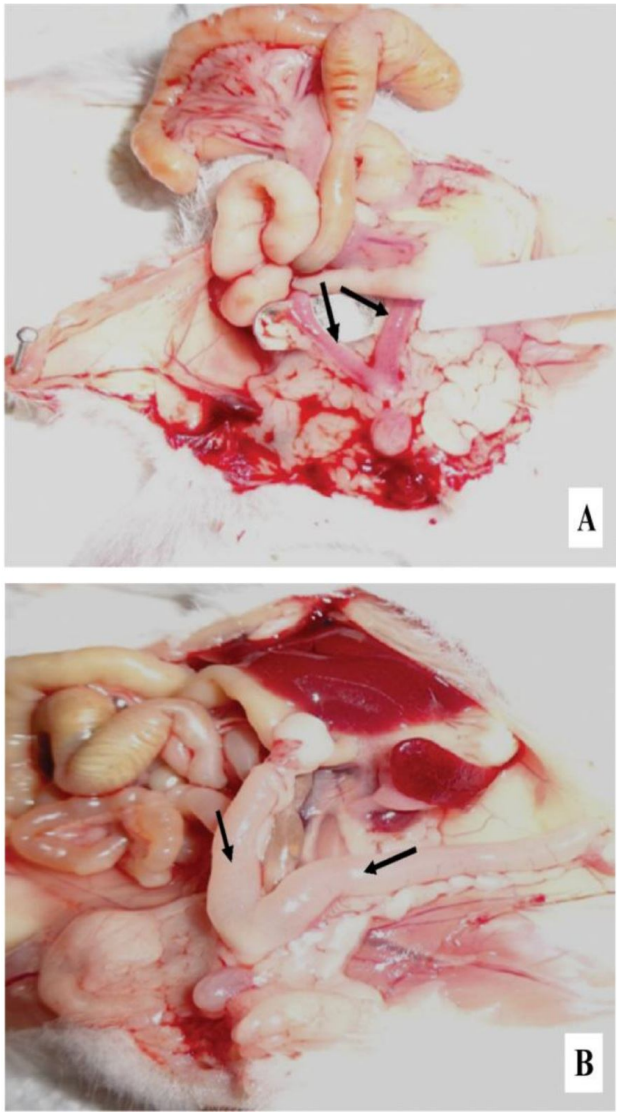

Fig. 1: Representative images on gross changes in the mice uterus at 36 h post intravaginal inoculation of either sterile normal saline or *E. coli*[@] 1.5×10^6 CFU/ml. **A**: Mild congestion in the uterine horns (arrows), and **B**: Distention ofuterine horns (arrows) with inflammatory exudates

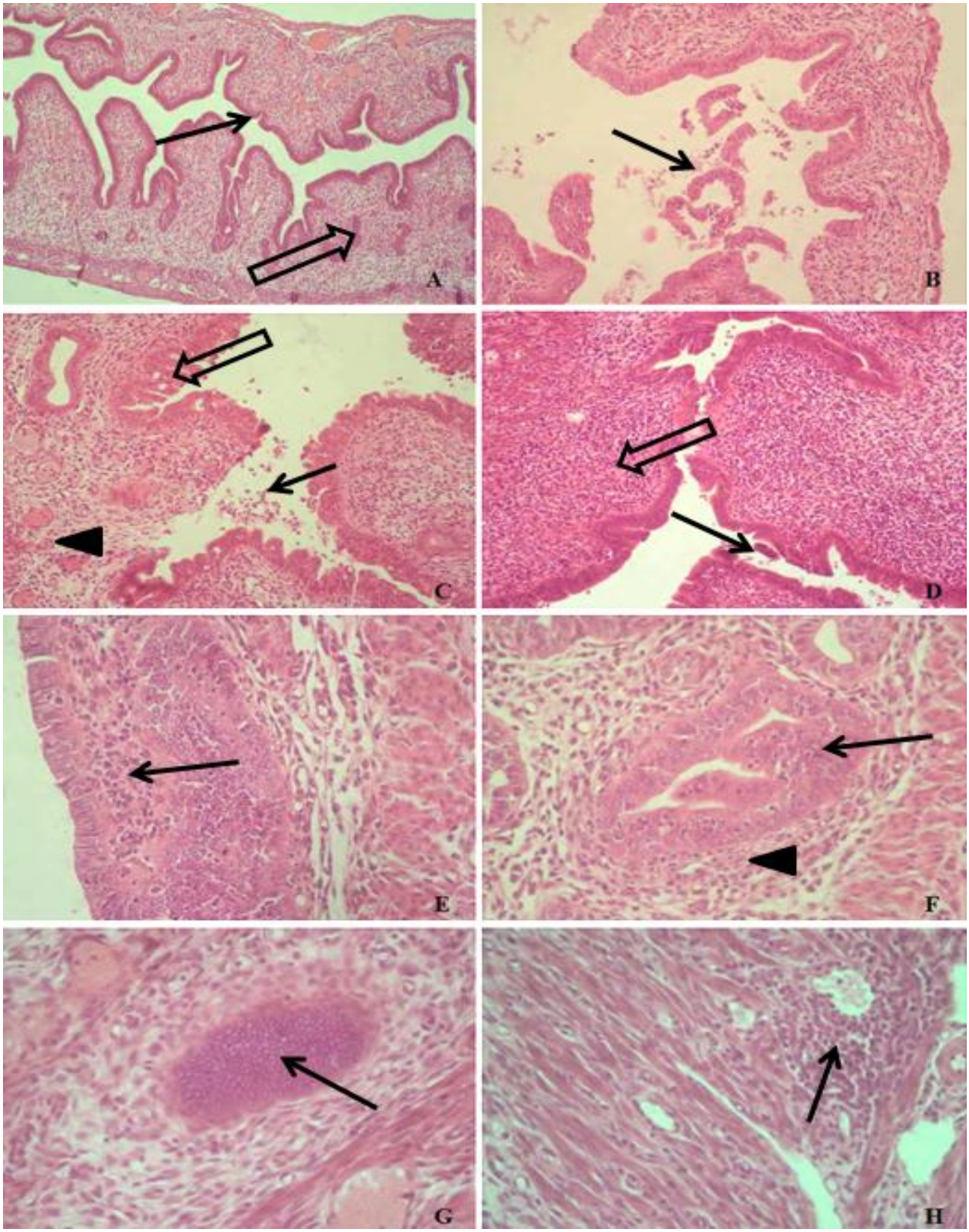

Fig. 2: Hematoxylin and eosin stained representative images of histopathological changes in the mice uterus at 36 h post intravaginal inoculation of either NSS (**A**) or *E. coli*[@] 10^6 CFU/ml (**B** through **H**). **A**: Intact endometrial lining (arrow) and vascular congestion (open arrow) (×200), **B**: Sloughing of endometrial epithelium (arrow) (×200), **C**: Influx of PMN leukocytes into uterine lumen (arrow), epithelial vacuolization (open arrow), and vascular congestion (diamond arrow) (×200), **D**: Stromal hyperplasia (open arrow), and epithelial sloughing (arrow) (×200), **E**: Focal aggregate of PMN leukocytes (arrow) in the stratum compactum of endometrium (×400), **F**: Glandular degeneration (arrow), glandular and periglandular infiltration of PMN leukocytes (diamond arrow) (×400), **G**: Glandular occlusion (arrow) (×400), and **H**: Perivascular infiltration of PMN leukocytes (arrow) (×400)

Table 1: Effect of intra-vaginal inoculation of *E. coli* at different doses during diestrus on the development of acute endometritis 36 h post-inoculation in mice

Experimental group [$]	Inoculum of *E. coli* (CFU/ml)	Endometritis		Rate of infection (%)
		Present	Absent	
EG-1 (n=6)	Sterile normal saline	0	6	0
EG-2 (n=6)	1.5×10^4	0	6	0
EG-3 (n=6)	1.5×10^5	3	3	50
EG-4 (n=12)	1.5×10^6	8	4	66.7

[$] At diestrus, the mice were allotted to one of the following four experimental groups: experimental group 1 (EG-1) was administered 100 µL sterile normal saline intravaginally and served as negative control. EG-2, -3 and -4 were inoculated (100 µL) with *E. coli* intravaginally[@] 1.5×10^4, 1.5×10^5 and 1.5×10^6 CFU/ml, respectively. At 36 h post-inoculation, mice were sacrificed and a cross section of the uterus was preserved in 10% neutral buffered formal saline (1:10 v/v) for histopathological diagnosis of acute endometritis. Data was analyzed by Fisher's exact test ($\chi^2 = 12.06$, P=0.0072)

animal models of human diseases suggesting that the animal disease produced should have a similar route of infection as the human disease. Intravaginal inoculation of *E. coli*[@] 1.5×10^6 CFU/ml at the diestrus phase of the mouse estrous cycle was associated with typical changes of acute endometritis with an infection rate of 66.7%. Analysis of different doses to induce acute endometritis revealed a significant difference. Of all the histo-pathological indicators, typical changes of acute endometritis were found mainly in EG-4. However, 33.3% mice did not develop acute endometritis, which may be attributed to the heterogeneity of the genetic background of mice that can resist colonization of *E. coli* in the endometrium following inoculation. The rate of infection may possibly be improved by a higher inoculum dose. Sheldon *et al*. (2010) induced pelvic inflammatory disease in C57BL6 mice following intrauterine inoculation of endometrial specific *E. coli*[@] 10^4 CFU/ml 24 h post-inoculation. In their study, one animal died and one group developed toxaemia. This is in contrast with the findings of the present study, where none of the mice died and all were active with apparently no change in feed and water intake. Further, no apparent depression was seen in any mice till the time of sacrifice. Recently, Hasan *et al*. (2013) demonstrated the development of endometritis in the mice following intracervical inoculation of *E. coli*[@] 10^4 CFU/ml after 24 h of treatment; however, the source of *E. coli* or the phase of the mice's estrous cycle were not reported.

In summary, the present study has provided evidence supporting a laboratory animal model for bubaline endometritis in mice by intravaginal inoculation of *E. coli*[@] 1.5×10^6 CFU/ml at diestrus. Ease of intravaginal inoculation, apparent absence of systemic involvement and high infective rates are the advantages of this model over other studies. However, the possibility of improving the rate of acute endometritis and establishing a model of chronic endometritis needs to be further explored.

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Conflict of interest

None of the author has any conflict of interest to declare.

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