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Vaginal Bacterial Profile of Buffalo-Cows in Relation to Ovarian Activity

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Abstract: Ovarian inactivity is the most predominant cause of reproductive failure and economic losses in buffaloes. Two experiments were carried out on mature buffalo cows reared at Lower Egypt during the period from July 2004 to June 2006 to investigate the relationship between ovarian activity and vaginal bacterial profile in buffalo-cows. The first experiment was a field survey carried out on 258 head of mature buffalo cows, either having inactive (192) or active (66) ovaries. The second experiment was carried out to monitor vaginal bacterial profile before and after treating of 20 buffalo cows raised in a private farm using Pregnant mare serum gonadotrophin and dibasic sodium phosphate. All animals were clinically examined and blood samples and vaginal swabs were collected. Rose Bengal test was used to check all animals for brucellosis. Progesterone level was determined by ELISA to confirm ovarian activity. Bacterial flora was isolated and identified using the standard techniques. Some isolates from *E. coli* were serologically typed and tested for some toxic genes using PCR (Verotoxin-II) and multiplex PCR (Shiga toxin-2 and intemin). Results indicated that, in the first experiment, buffalo-cows suffering from inactive ovaries showed higher rate of bacterial isolation from their vagina with non detectable serum progesterone level as compared with normal cyclic animals. In the same time, *S. aureus*, *S. pyogenes*, *P. vulgaris*, *P. aregenosa*, *M. haemolytica*, C. *freundii*, *Salmonella* spp. and *Shigella* spp. were isolated from buffalo-cows suffering from ovarian inactivity, meanwhile they were not isolated from animals showing active ovaries. In the second experiment, all anoestrous buffalo-cows showed estrous activity 7 -10 days post treatment and become pregnant 2-3 months later. The number of bacteria isolated from vagina is reduced after as compared with before treatment. The predominant isolates were *E. coli*, *S. aureus*, *A. pyogenes* and *S. pyogenes* before treatment and *E. coli*, *C. diversus*, *Micrococcus* spp. and *S. epidermidis* after treatment. Serotyping and PCR (Verotoxin-II) and multiplex PCR (Shiga toxin-2 and intemin) revealed that the examined *E.coli* collected from animals with active ovaries (O28, O126 and untypable strains) were negative for the tested toxins. While, *E.coli* isolated from animals suffering from ovarian inactivity (O157 and O119) were positive for the tested toxin genes (VT-II, stx-2 and eae-A). It was concluded that there is a tight relationship between ovarian activity and vaginal bacterial profile in buffalo-cows. Genital tract infection is influenced by the presence of a suitable vaginal environment, genetic factors and the animal's immunity. *E.coli* are among the predominant strains, especially VT-II, stx-2 and eae-A positive O157 and O119 strains.

Key words: Buffaloes · inactive ovaries · genital tract · bacteria *E. coli* · serology · toxic genes

INTRODUCTION

Buffaloes represent an integral part of the agricultural economy in Egypt and some other developing countries. According to the last FAO census, the world buffalo's population is 160 million heads. Among this, 3.920 million heads are found in Egypt, producing 3,300,000 ton of milk and 270,000 ton of meat [1]. However, in most of developing countries, buffaloes are mainly reared in small holder farms and suffer from a lot of stressful conditions such as malnutrition, bad hygiene, parasitic infestation and pollution [2]. Also, this species tends to have seasonal breeding with relatively slow rate of reproduction and more reproductive problems such as late maturity, silent heat, inactive ovaries, uterine infection and long calving interval [2-6].

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Ovarian inactivity is the most predominant cause of reproductive failure and economic losses in buffaloes. Among buffalo-cows suffering from reproductive problems, the reported percentages are 44.42 [4], 49.35 [7] and 71.43 [8].

Every system in the body has its own normal bacterial flora which play an important role in its protection against infection upset and enhance the host ability to compete pathogens [9,10]. However, it was reported that the presences of some bacterial species in the uterus disturb its function and delay the post partum uterine involution [4-11]. Moreover, this uterine bacterial infection or bacterial products perturb ovarian function [12-14]. Therefore, this work was designed to investigate the relationship between ovarian activity and vaginal bacterial profile in buffalo-cows.

MATERIALS AND METHODS

Two experiments were carried out on mature buffalo cows reared at Lower Egypt during the period from July 2004 to June 2006 to investigate the relationship between ovarian activity and vaginal bacterial profile. The first experiment was a field survey carried out as a part of the National Research Center Project No-7120106. The second experiment was carried out on buffalo cows in a private farm.

First experiment: A total number of 258 mature buffalo-cows reared in small holder farms were used. Case history, the general health condition and owner complain were recorded. Blood samples were collected and serum was separated (X 1500 g, 15 minutes at 4°C), checked for brucella antibody and kept frozen (at –20°C) for assaying progesterone levels. Rectal examinations were carried out at least for two times (7 days interval) for palpating the ovarian size, texture and physiological structures. Also, uterine and size, texture was recorded as outlined by [4-15]. Ovarian activity was confirmed later on by determination of serum progesterone level to avoid misdiagnosis [16]. Vulva was thoroughly dry cleaned using tissue paper and vaginal mucus membrane was examined. Swabs were collected under aseptic conditions from the anterior vagina using the rectovaginal technique [15] and inoculated into a tube containing 10 ml Tryptic soy broth for isolation and identification of the present bacteria.

Second experiment: In a separate experiment, 20 buffalo-cows reared at a private farm and suffered from ovarian inactivity during the breeding season (September - March) were used. These animals were treated using pregnant mare serum gonadotrophin (2000 IU of Folligon®; Intervet, the Netherland; injected intramuscularly once) as well as sodium dibasic phosphate (Adwia Co., Egypt; 20 g per head per day for 10 successive days). Animals were observed twice daily for estrous detection and blood samples and vaginal swabs were collected before and after treatment. Animals come in heat were naturally mated by proven fertile bulls, then after rectal palpation were carried out monthly to diagnose pregnancy.

Table 1: The diagnostic *E.coli* "O" sera

Polyvalent	Pathogenic *E. coli* Antisera						
1	O1	O26	O86	O111	O119	O127	O128
2	O44	O55	O125	O126	O146	O166	
3	O18	O114	O142	O151	O157	O158	
4	O6	O27	O78	O148	O159	O168	
5	O20	O25	O63	O153	O167		
6	O8	O15	O115	O169			
7	O28	O11	O124	O136	O144		
8	O29	O143	O152	O164			

Table 2: Oligonucleotide primer sequences used for detection of *stx2*, *eae-A* and (*VT-II*) genes by PCR

Primers	Sequence (5'-3')	Specificity	Amplicon size	Annealing Temp
stx2-F	GGC ACT GTC TGA AAC TGC TCC	Shiga toxin type-2 (including *stx-2* variants)	255	65°C
stx2-R	TCG CCA GTT ATC TGA CAT TCT			
eaeA-F	GAC CCG GCA CAA GCA TAA GC	Intemin gene (encoded by *eae- A*)	384	65°C
eaeA-R	CCA CCT GCA GCA ACA AGA GG			
VT-IIF	TTA ACC ACA CCC ACG GCA GT	Vero toxin type -II (encoded by *VT-II*)	346	55°C
VT-IIR	GCT CTG GAT GCA TCT CTG GT			

Serum examination: Serum samples were screened for *Brucella* antibodies using Rose Bengal test [17].

Progesterone level was determined by ELISA by kits (DIMA, Germany) using the micro- well method. The kit had a sensitivity of 2.0 pg/ml with the inter- and intra-run precision coefficient of variations of 2.9 and 4.85, respectively [18].

Bacterial profile: Swabs were incubated at 37°C for 24 hrs and then subcultures were streaked from the enriched broth onto Nutrient, Mannitol, Blood and MacConkey

agar plates. Swabs were also inoculated into Selenite-F-broth for 16 hrs then subcultured onto SS agar medium then plates were incubated at 37°C for 48 hrs [19].

Individual colonies were picked and separately inoculated onto slope agar and semisolid agar for further investigations. Films from pure, suspected, fresh, young cultures were smeared, fixed and stained with Gram's Method [20]. Biochemical identifications were carried out as outlined by [19]. Also, serological tests were carried out using *E. coli* diagnostic antisera for pathogenic types (Table 1). The diagnostic "O" Sera included 51 vials (8 polyvalent and 43 monovalent; Denka Seiken Co., Ltd.]

Extraction of genomic DNA from selected *E. coli* isolates was done and 2 primer pairs were used for the identification of shiga toxin type 2 (*stx2*, encoded by *stx2*) and intimin gene (encoded by *eae-A*) by multiplex PCR [21]. Also, 2 primer pairs were used for detection of *VT-II* genes encoded by VTEC [22]. The primer sequences, the specificity, the amplicon size and the annealing temperature were summarized in Table (2).

Statistical analysis: Data were statistically analyzed according to [23].

RESULTS

The first experiment: Rectal and vaginal examinations of mature buffalo cows showed that 192 cases suffered from ovarian inactivity and 66 showing normal cyclic ovarian activity. Animals having inactive ovaries did not show oestrous signs during the breeding season (September-March) for a period of time not less than 6 months after calving and have small sized, hard texture ovaries with neither corpus luteum nor Graafian follicles and small sized flaccid uterus with pale dry vaginal mucous membrane. The undetectable level of progesterone (< 0.002 ng/ml) confirmed the state of ovarian inactivity. While, in normal cyclic animals, the level (ng/ml) was significantly (P<0.01) higher during the luteal phase (4.62±0.95) than the follicular phase (0.52±0.15) of the estrous cycle.

Rose Bengal test reveled that a single case was positive for brucellosis with an incidence of 0.003%. This buffalo-cow suffered from ovarian inactivity and had a history of retained placenta.

In buffalo-cows suffering from inactive ovaries, the rate of bacterial isolation was significantly (P<0.001) higher (3.48±0.25) as compared with the normal cyclic animals (2.70±0.33). The most predominant isolates were *E. coli*, *S. aureus*, *S. pyogenes*, *A. pyogenes* and *Klebsiella* spp. (Table 3).

Table 3: Vaginal bacterial profile of buffalo cows in relation to ovarian activity

	Ovarian activity			
	Average of normal cyclic animals (66)		Animals having inactive ovaries (192)	
Bacterial isolates	No	%	No	%
Gram negative bacteria				
Y.enterocolitica	25	37.88	36	18.75
E. coli	47	71.21	152	79.17
Shigella spp.	0	0.00	4	2.08
Salmonella spp.	0	0.00	8	4.17
C. diversus	17	25.75	20	10.42
C. freundii	0	0.00	12	6.25
Klebsiella spp.	5	7.58	56	29.17
M. haemolytica	0	0.00	12	6.25
P. multocida	6	9.09	28	14.58
P.vulgaris	0	0.00	28	14.58
P.mirabilis	3	4.55	12	6.25
P. aeruginosa	0	0.00	12	6.25
E.aerogenes	0	0.00	4	2.08
Gram positive bacteria				
C. bovis	3	4.55	8	4.16
Micrococcus spp.	27	40.91	24	12.50
A.pyogenes	0	0.00	53	27.60
S. aureus	2	3.03	88	45.83
S. epidermidis	5	7.58	8	4.16
S. pyogenes	0	0.00	59	30.73
Bacillus spp.	12	18.18	24	12.50
E. faecalis	26	39.39	20	10.42
Mean of isolates/case±SE	2.70±0.33		3.48±0.25	

No = number of positive samples.
% = was calculated according to number of examined samples ( ).

Table 4: Vaginal bacterial profile of buffalo-cows before and after treatment of ovarian inactivity

	Treatment of ovarian inactivity (20)			
	Before		After	
Bacterial isolates	No	%	No	%
Gram negative bacteria				
Y.enterocolitica	4	20.00	3	15.00
E. coli	17	85.00	16	80.00
C. diversus	2	10.00	8	40.00
C. freundii	1	5.00	0	0.00
Klebsiella spp.	6	30.00	0	0.00
M. haemolytica	3	15.00	0	0.00
P. multocida	1	5.00	0	0.00
P. vulgaris	3	15.00	0	0.00
Ps. aeruginosa	1	5.00	0	0.00
Gram positive bacteria				
S. bovis	1	5.00	2	10.00
E. faecalis	1	5.00	2	10.00
A.pyogenes	9	45.00	0	0.00
S. aureus	12	60.00	0	0.00
S. epidermidis	2	10.00	4	20.00
S. pyogenes	6	30.00	0	0.00
Bacillus spp.	2	10.00	1	5.00
Micrococcus spp.	1	5.00	5	25.00
Mean of isolates/ case ± SE	3.60±0.80		2.05±0.46	

No = number of positive samples.
% = was calculated according to number of examined samples ( ).

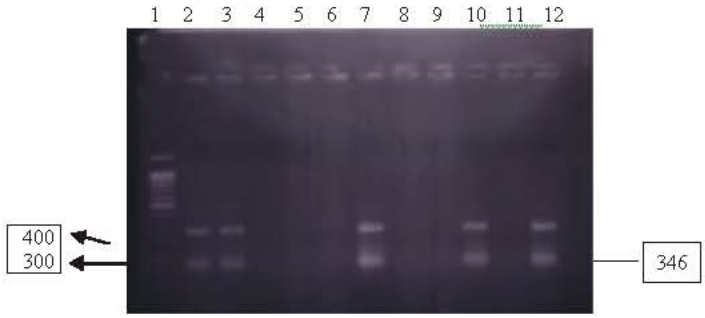

Fig. 1: Agarose gel electrophoresis showing amplification of 346 bp of *VT-II* gene lanes 2, 3, 7 and 10 while lanes 4,5,6,8,9 and 11 are negative. Lane 1 is 1.5 bp ladder and lane 12 is a control positive

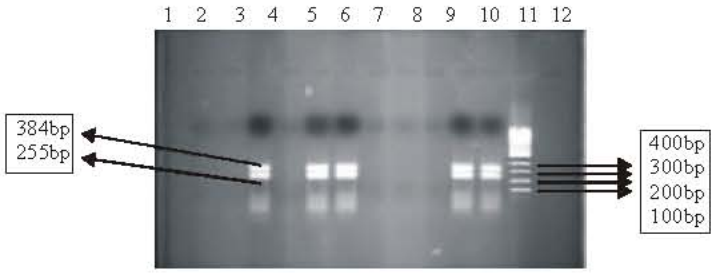

Fig. 2: Agarose gel electrophoresis showing multiplex PCR amplification of 384 and 255 bp of Intimin (*eae-A*) and shiga toxin-2 (*stx-2*) genes in lanes 3, 5, 6 and 10 while lanes 1, 2, 4, 7, 8 and 9 are negative. Lane 12 is 100 bp ladder and lane 11 is a control positive

Table 5: Serotyping and toxigenic genes of *E.coli* strain

		Toxigenic genes		
Animal condition	Serotyping (Monovalent)	*VT-II*	*Stx-2*	*eae-A*
Active ovaries	O28	-	-	-
Active ovaries	Untypable	-	-	-
Active ovaries	O126	-	-	-
Active ovaries	Untypable	-	-	-
Active ovaries	O28	-	-	-
Inactive ovaries	O157	+	+	+
Inactive ovaries	O119	+	+	+
Inactive ovaries	O119	+	+	+
Inactive ovaries	O78	-	-	-
Inactive ovaries	O157	+	+	+

The second experiment: In this experiment, all buffalo-cows suffered from ovarian inactivity (Serum progesterone levels <0.002ng/ml) come in estrus within 7-10 days post treatment (Serum progesterone levels >1 ng/ml on days 7-10 after appearance of oestrous signs). Rectal palpation 2-3 months post mating revealed occurrence of pregnancy. Bacteriological examination indicated that the number of isolates was reduced after (2.05±0.46) as compared with before (3.60±0.80) treatment. The predominant isolates were *E. coli*, *S. aureus*, *A. pyogenes* and *S. pyogenes* before treatment and *E. coli*, *C. diversus*, *Micrococcus* spp. and *S. epidermidis* after treatment (Table 4).

Serotyping of 10 *E.coli* isolates revealed O78 (1 case), O157 (2), O119 (2), O28 (2), O126 (1) and 2 untypable strains (Table 5).

PCR (Verotoxin-II) and multiplex PCR (Shiga toxin-2 and intemin) revealed that the examined *E. coli* collected from normal cyclic animals (O28 and untypable strains) were negative for the tested toxins. On the other hand, *E. coli* isolated from animals suffering from ovarian inactivity of serotype: O157 (2 cases) and O119 (2) were positive for the tested toxigenic genes (*VT-II, stx-2* and *eae-A*); while serotype O78 was negative for all the tested toxigenic genes (Table 5; Figures 1 and 2)

DISCUSSION

Ovarian inactivity is the most predominant cause of reproductive failure and economic losses in Egyptian buffaloes. The reported percentages are 33.80% among mature buffalo-heifers [24] and 44.42% [4] and 49.35% [7] of the total genital disorders in buffalo-cows and cause great economic losses [25]. Moreover, animals with inactive ovaries have great affinity for infection due to their lower immune response [26-27].

In this work, vaginas of buffalo-cows suffering from inactive ovaries showed higher rate of bacterial isolation as compared with normal cyclic animals. *In the same time, S. aureus*, *S. pyogenes*, *P. vulgaris*, *P. aregenosa*, *M. haemolytica*, C. *freundii*, *Salmonella* spp. and *Shigella* spp. were isolated from buffalo-cows suffering from ovarian inactivity, meanwhile they were not isolated from animals showing active ovaries. *A. pyogenes, E. coli* and *M. haemolytica* were associated with infertility [28]. *A. pyogenes* is the primarily pathogenic bacteria that secrete a cytotoxic hemolysin for PMN and for kidney cells [29]. Moreover, it produces a growth factor for *F. necrophorum* that produces leukotoxin [30]. Genital bacterial infection or bacterial products suppress pituitary LH secretion and perturb postpartum ovarian follicle growth and function, which disrupts ovulation in cattle [31-32]. Thus, endometritis is associated with lower conception rates, increased intervals from calving to first service or conception and more culls due to failure to conceive [33-34]. Lewis [35] mentioned that nonspecific uterine infections reduce the reproductive efficiency of cows and *A. pyogenes*, either alone or with other bacteria, is often associated with uterine infections.

In the second experiment, the number of isolates is reduced after treatment with PMSG and Dibasic sodium phosphate as compared with before. The predominant isolates were *E. coli*, *S. aureus*, *A. pyogenes* and *S. pyogenes* before treatment and *E. coli*, *C. diversus*, *Micrococcus* spp. and *S. epidermidis* after treatment. Various schedules of gonadotropin treatment, such as PMSG [24-36], tapering doses of FSH [36-37] and single injection of FSH[38] have been adopted to stimulate ovarian function in bovines [39]. Crestar (Synthetic progestagen) alone and in combination with PMSG (Folligon®) effectively induced oestrus in post partum anoestrus buffaloes [40]. PMSG stimulate the maturation of Graafian follicles, secretion of oestradiol and oestrous activity [24]. Dibasic sodium phosphate improves the feeding status and stimulate the secretion of internal gonadotropin. and induced ovarian activity [24].

In the present investigations *E. coli* was the predominant isolates in the vagina of buffaloes. This finding coincides with those reported by many authors who concluded that *E. coli* was the most important pathogen in genital tract of bovine [41-43]. So in the present investigation serological identification in combination with PCR assay using specific primers targeting the *VT-II, stx-2* and *eae-A* genes were used to identify isolated *E. coli* in relation to ovarian activity. O157, O126, O119, O78 and O28 were serologically identified. In this respect, it was reported that bovine act as a reservoir for *E. coli* O157 and shed it into the surrounding. Therefore, attention should be paid for early diagnosis of infection to reduce the transference and the degree of shedding into the environment [44]. PCR (Verotoxin-II) and multiplex PCR (Shiga toxin-2 and intemin) revealed that the examined *E. coli* collected from animals with active ovaries (O28, O126 and untypable strains) were negative for the tested toxins. While, *E. coli* isolated from animals suffering from ovarian inactivity (O157 and O119) were positive for the tested toxin genes (*VT-II, stx-2* and *eae-A*); while serotype O78 was negative for all the tested toxin genes. Yilmaz [43] concluded that *eae-A* gene was detected in all O157 to O157: 117 strains tested, both *VT-II* and *eae-A* genes were detected in 4 (80%) of 5 strains of *E. coli* O157. It was clear from this study that the tested toxigenic genes of *E. coli* were detected in high incidence in cases suffering from ovarian inactivity. This condition could be attributed to the adverse effect of these toxins on the general health condition and it may be reflected on the different body functions including the reproductive one. It was proved that toxigenic *E. coli* (*VT-II, stx-2* and *eae-A*) are associated with different health problems such as mastitis and diarrhea in bovine [45-46] and bloody diarrhea, hemolytic uremic syndrome, hemorrhagic colitis and thrombotic thrombocytopenia purpura [47] as well as central nervous system complications[48] in human. Moreover, it was reported that animals suffering from mal-nutrition are good reservoirs for pathogenic *E. coli* O157:H7 [49].

In conclusion, there is a tight relationship between ovarian activity and vaginal bacterial profile in buffalo-cows. Genital tract infection is influenced by the presence of a suitable vaginal environment, genetic factors and the animal's innate and acquired immunity. However, the qualitative and quantitative bacterial contamination is dependent on the challenge between bacterial contamination and the animal's defence mechanisms. Data obtained from such basic studies may be used to develop and test models for enhancing reproductive efficiency. Thus, effective diagnosis and treatment of genital infection is essential in reproductive control programs. *E. coli* were among the predominant strain and it may represent human hazards, especially the toxigenic strains. The challenge for the future is to design prevention and control programs to reduce the incidence of the disease.

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