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Research Article

Prevalence of obligate anaerobes and aerobes in uterine lumen of subfertile crossbred cows in relation to physical appearance of cervicovaginal mucus

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ABSTRACT

Aerobes causing bacterial endometritis have been investigated under Indian condition but involvement of obligate anaerobes have not been studied so far. In the present study, 30 subfertile pleuriparous crossbred cows with the history of not conceiving within 5-18 months of calving and had received ≥3 AI with fertile semen and lacked genital abnormalities; were selected from the university dairy farm. These cows were gred on the basis of colour of cervico-vaginal mucus viz.. gr 1 (clear mucus, n=12). gr 2 (muco-purulent, n=10) and gr 3 (purulent mucus n=8). The uterine swabs taken during estrus with the help of guarded Neilson's Swab Catheter, were subjected to bacteriological culture for isolation of obligate anaerobes and aerobes. Out of total 42 bacterial isolates recovered 16 were obligate anaerobes while 26 were aerobes. Two cows harbored only obligate anaerobes, 10 cows only aerobes, 13 cows both obligate anaerobes and aerobes and the remaining 5 cows were bacteriologically sterile. Amongst the gram negative anaerobes (GNA), *Bacteroides* sp. predominated in all the three grs. Amongst aerobes, *E. coli* and/or *Pseudomonas* sp. predominated in Grs 1 and 2 and *Arcanobacter pyogenes* in gr 3. Cows of gr 3 showed the highest prevalence of mixed infection of both GNA and aerobes. Amongst the mixed infections, combination of *Bacteroides* anaerobes with *E. coli* and/or *Pseudomonas* predominated in gr 1 and 2 while that of *Bacteroides* sp with *Arcanobacter pyogenes* in gr 3. Out of total 26 aerobic isolates, 7 were of *Arcanobacter pyogenes,* of which 5 grew in combination with GNA and 2 were in pure growth. To conclude, nearly half of bacteria positive subfertile cross-bred cows had mixed infection of obligate anaerobes and aerobes. Cows with clear and/or mucopurulent discharge harbored mainly GNA occurring in combination with *E. coli / Pseudomonas* sp while those with purulent mucus had gram negative obligate anaerobes coupled with *Arcanobacter pyogenes.*

Key words: Subfertile, crossbred cows, gram negative anaerobes, *Arcanobacter pyogenes,* cervicovaginal discharge

In crossbred cattle subfertility poses a major hindrance to the economics of dairy industry in India as a result of which farmers are shifting towards traditional rearing of buffaloes. Acute/chronic endometritis contribute to a great extent in this regard (Mohanti *et al.,* 1992, Narsimha Rao *et al.*, 1993).

Uterine infections are usually diagnosed at the time of estrus based on the appearance of cervico-vaginal mucus. It is established that as the muco-purulentity of mucus increases, the severity of infection also increases (Dohmen *et al.,* 1995). So far, the isolation of only aerobic bacteria from the uterine lumen of subfertile cows has been attempted under Indian conditions (Verma *et al.,* 1994; Singh 1996; Baishya *et al* 1998 and Arora *et al.,* 2000). However, recent research worldwide suggests that gram negative obligate anaerobic bacteria (GNA) also play a significant role in the occurrence of bacterial endometritis (Bekana *et al.,* 1994; Dohmen *et al.,* 1995; Cohen *et al.,* 1996 and Huszenicza *et al.,* 1999). It has been well established that aerobic bacteria such as *Arcanobacter pyogenes* act synergistically with Gram negative anaerobes such as *Bacteroides* sp. and *Fusobacterium* sp. in exaggerating the pathogencity of infection (Ruder *et al.,* 1981; Olson *et al.*, 1984 and Huszenicza *et al.,* 1999).

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Consequently, the present study aims to explore the prevalence of GNA as well as aerobes in the uterine lumen of subfertile cows and to find their relationship vis-a-vis physical appearance of cervico-vaginal mucus.

MATERIALS AND METHODS

A total of 50 crossbred cows (Holstein Friesian X Sahiwal) with the history of not conceiving within 5-18 months of calving were examined at the dairy farm of the Punjab Agricultural University, Ludhiana. Twenty cows with genital abnormalities (as diagnosed on rectal examination) and/or having not received 3 AI were left out. The remaining thirty cows were categorized as subfertile (i.e. failure to conceive after $\geq$ 3 AI's with good quality semen and with no apparent abnormalities of genitalia) and were included in the study. These cows were in their 3rd-5th parity and yielded on an average 15 liters of milk per day. All animals were kept in loose housing system, fed concentrate and green fodder as per nutritional recommendation for dairy animals in the tropics. Selected crossbred cows were divided into three grs on the basis of appearance of the cervico-vaginal mucus, aspirated into sterilized glass pipette at the time of standing estrus (Dohmen *et al.*, 1995), The cows with clear mucus formed gr 1 (n=12), cows having mucus with flecks of pus (muco-purulent /cloudy) formed gr 2 (n=10) and cows with thick white purulent/foul odour mucus formed gr 3 (n=8). Majority of the gr 3 cows had the history of dystocia and/or retention of placenta in their recent calving and had least postpartum period (5-8 months) at the time of sampling as compared to that of gr 1 and 2 (9-18 months).

Collection of swabs

The uterine swabs were collected at the time of standing estrus with the help of sterilized Neilson's Swab Catheter having guarded cotton swab (Bekana *et al.*, 1994). In brief, after restraining, rectum was emptied of the feces and perineal region was cleaned and disinfected. With an assistant holding the vulvar lips apart, the examiner passed one gloved and well lubricated hand into rectum to hold the cervix. Subsequently catheter was introduced through vagina into the uterus with the other hand. Thereafter, the swab was advanced into the uterus, rotated 4-5 times and withdrawn into outer tubing prior to removal of catheter. Further, the catheter was cleaned with spirit swab and uterine swab was immediately transferred into transport media viz. Cary Blair Media. The swabs were cultured both for aerobic and anaerobic bacteria.

Bacteriological culture (Aerobic culturing)

For culture of aerobic bacteria, swabs from transport media were inoculated onto Blood Agar with 5-10% sterile defibrinated sheep blood and MacConkey Agar and then incubated aerobically upto 48 hours at 37°C. Colonies were identified using standard bacteriological culture techniques (Quinn *et al.*, 1999). Cultivation of obligate anaerobic bacteria

MacIntosh anaerobic jar with vents and gas pack system (Hi-gas pack, Hi-media Ltd., Mumbai) for anaerobiosis was used for the culturing of obligate anaerobic bacteria. The vents were sealed with parafilm. After inoculating the Anaerobic Brewer's Agar by streaking the swab (till then placed in Cary Blair Media) over the agar surface, the plates were transferred to the anaerobic jar and made airtight by closing the lid and sealed with parafilm. Jar was then placed in an incubator at 37°C for seven days. An indicator tablet (Hi-media Ltd., Mumbai) was kept in jar (3-5 litre capacity) to keep a check on anaerobic conditions.

To avoid killing of any obligate anaerobic bacteria by exposure to oxygen, the cultivation of anaerobes was carried out using a reducing media such as Anaerobic Brewers Agar (Hi-media Ltd., Mumbai). For sub culturing, liquid media, such as cooked meat broth or Thioglycolate medium supplemented with vit K (10 Hg/ml) and Haemin (5 p-g/rni) were used. Anaerobic Brewer's Agar was always prepared fresh, approximately 2-3 hours before its use. The media was made selective for Gram negative anaerobes by the addition of antibiotics i.e. vancomicin (7.5 Ug/ml) and kanamicin (100 u.g/ml). All anaerobic isolates obtained were characterised morphologically and bio-chemically as per Lennette *et al.* (1985) and Holt *et al.* (1994).

A culture was designated as 'pure', if it had only one or more pathogenic species of either aerobic or anaerobic bacteria following examination of uterine

swabs. A culture was designated as 'mixed', if it had both the aerobic and anaerobic pathogenic species. For preliminary confirmation, all anaerobic organisms isolated on anaerobic Brewer's Agar were further sub-cultured aerobically; those organisms failed to grow aerobically were considered as 'true anaerobes'. Categorization of bacterial isolates by anaerobic and aerobic culture of uterine swab, based on their potential pathogencity (Laven *et al.*, 2000) was done as under: Category I (Most important): Bacteria mainly responsible for bacterial endometritis *(E. coli, Arcanobacter pyogenes, Bacteroides* sp. and *Fusobacterium* sp.) Category II (Important): Bacteria rarely responsible for bacterial endometritis *(Bacillus* sp., *Staphylococcus* sp. *Streptococcus* sp.). Frequency of mixed infection in different grs was compared by non-parameteric Fischer's exact test using 2X2 contingency table of two independent samples.

RESULTS AND DISCUSSION

A total of 42 bacterial isolates were recovered from 30 subfertile crossbred cows. Out of these, 31 (74%) isolates (*E. coli, Arcanobacter pyogenes, Bacteroides* sp. and *Fusobacterium)* were categorized as most important (Category-1), while 11 (26%) isolates *{Pseudomonas* sp., *Streptococcus* sp., *Staphylococcus* sp., *Bacillus* and *Peptostreptococci)* were categorized as important (Category-II). Greater isolation of category I bacteria from subfertile cows matches well with the conclusion drawn by Laven *et al.* (2000). Except *E. coli,* no other coliform bacteria like *Proteus* sp. or *Klebsiella* sp, were recovered from uterine lumen of subfertile cows in the present study, as reported in previous studies (Rahman *et al.*, 1984, Sirohi *et al.*, 1989, Khan *et al.*, 1990, Ramaswamy *et al.*, 1992). Recently it had been suggested that *Proteus, Klebsiella* and *Micrococcus* were never responsible for endometritis and they were just the fecal contaminants from vestibular, vulvar and outer perineal area (Laven *et al.*, 2000). Greater isolation of non-specific bacterial infection like *E. coli, A. pyogenes* is in agreement with the earlier opinion that these pathogens were significantly correlated with infertility, whereas *Staphyhcoccus, Streptococcus, Micrococcus, Proetus* sp. and *Klebsiella* were not significantly correlated with infertility (Bondurant, 1999).

Frequency and type of anaerobic/aerobic bacteria isolated from the bovine uterus of different grs are presented in (Table 1). Out of 12 samples collected from subfertile cows of gr-1 (clear mucus), 8 samples had bacterial growth and 4 were bacteriologically sterile. Amongst the bacteriologically positive samples, 4 had pure growth, of which 2 samples yielded pure culture of GNA and another 2 yielded pure culture of aerobes. Remaining four samples had mixed growth of GNA and aerobes. In cows of Gr-2 (n=10), 9 uterine swabs showed bacterial growth and one was bacteriological sterile. Within the positive samples, 4 samples had mixed infection of GNA and aerobes while 5 samples had pure culture of aerobes only. All the samples from gr 3 cows (n=8) revealed the presence of bacteria.

In both Grs 1 and 2, *E. coli* and *Pseudomonas aeruginosa* were the predominant aerobic bacteria while *Bacteroides* sp. was the predominant GNA. In the g3, *Arcanobacter pyogenes* was the predominant bacteria. In addition, anaerobic bacteria viz., *Bacteroides* sp. was isolated from 4-5 samples from each of the three groups.

The findings of the present study are in concordance with the earlier ones (Pandey *et al.*, 1983 and Saini 1993) that a shift in the colour of cervicovaginal mucus from a clear towards purulent discharge indicates uterine /cervical bacterial infection, which is responsible for subfertility in cattle, due to altered uterine environment (Arthur *et al.*, 1989).

The isolation of pathogenic obligate anaerobes and aerobes in gr I cows having clear mucus, suggest that majority of these animals may be suffering from subclinical bacterial endometritis. Singla (1999) reported that *E. coli* causes subclinical bacterial endometritis in crossbred cows. Nearly 66% of gr I animals showed bacterial growth, suggesting that all bacteria are not pus forming. Isolation of *Bacteroides* spp. alone in 2 of these cows suggests that the presence of *Bacteroides* sp. alone may not be sufficient to produce and maintain putrid endometritis and hence only cause subclinical endometritis. High recovery rate of *Bacteroides* spp. from the uterus of clinically cured cows after treatment in a previous study supports the above hypothes

Table 1: *Frequency and type of mixed isolates (obligate anaerobe + aerobes) / pure isolates in the uterine lumen of subfertile crossbred cattle in relation to colour of mucus diacharge*

Table 1: Frequency and type of mixed isolates (obligate anaerobe + aerobes) / pure isolates in the uterine lumen of subfertile crossbred cattle in relation to colour of mucus discharge

Type of mucus discharge	Number of samples	Number positive for bacterial growth	Positive for mixed bacterial growth	Type of bacteria (number)	Positive for pure bacterial growth	Type of bacteria
Clear (gr-1)	12	8	4 (33.3%)	*Bacteroides* sp. + *E. coli* (2)	4 (33.3%)	*Bacteroides* (2)
				Pseudomonas + *Bacteroides* sp. (1) *Bacteroides* sp. +		*E.coli* (1)
				Peptostreptococci + *E. coli* (1)		*Pseudomonas* (1)
Muco-purulent or cloudy (gr-2)	10	9	4 (40%)	*Bacillus* + *Bacteroides* (1)	5 (50%)	*E. coli* (2)
				Pseudomonas + *Bacteroides* (1) *Arcanobacter pyo genes* + *Bacteroides* (1)		*E. coli*+ *Staph* (1) *Pseudomonas* (2)
				E. coli + *Bacteroides* sp. (1)		
Purulent / Foul odorous (gr-3)	8	8	5 (62.5%)	*Arcanobacter pyogens* +	3 (37.5%)	*A. pyogens* (2) *Streptococcus* + *Staphlococcus* (1)
				Bacteroides (4)		
				E. coli + *Bacteroides* (1)		
Total	30	25	13 (43.3%)		12 (40%)	

(Dohmen *et al.*, 1995). Beside this, findings that samples with clear/muco-purulent discharge contained *Bacteroides* anaerobes along with *E. coli* and/or *Pseudomonas* sp. led us to hypothesize that these combination of bacteria may not cause severe endometritis with clinical signs hence might be responsible for either mild or moderate endometritis. However, a comparative study involving normal fertile animals is needed prior to confirmation of this hypothesis.

Arcanobacter pyogenes was found to be the most prevalent aerobe in subfertile cows with purulent / foul odorous mucus discharge, which has previously been reported to be the most frequent cause of bovine bacterial endometritis (Sambyal *et al.*, 1986. Farin *et al.*, 1989 and Cohen *et al.*, 1996) embryonic death (Semambo *et al.*, 1992) and prolonged infertility (Olson *et al.*, 1984, Dohmen *et al.*, 1995, Noakes *et al.*, 1990, Huszenicza *et al.*, 1999). Once *A. pyogenes* establishes in the endometrium, it leads to chronic endometritis, self healing of endometrium becomes almost impossible which causes infertility that lasts well beyond the time after cows overcome this infection (Olson *et al.*, 1984, Huzenicza *et al.*, 1999).

Out of the total 30 uterine swabs samples, 13 (43.3%) yielded mixed growth of GNA in combination with aerobes (Table 1). The frequency of mixed bacterial infection was only 33% in gr I while it increased to 63% in gr III cows. The frequency of isolation of *Arcanobacter pyogenes* and *Bacteroides* sp in discharge of gr 3 cows was higher than Grs 1 and 2, but the differences were statistically non-significant. Mixed infection of GNA and aerobes was found in 43% of swab samples, almost similar frequency (55%) of mixed infection was reported in a previous study of post partal endometritis in which uterine swabs were collected from day 19-231 post partum (Cohen *et al* ., 1996). As the cervical mucus became purulent, the prevalence of *Arcanobacter pyogenes* in combination with gram negative anaerobes also increased, but was not significantly higher from cows with clear and muco-purulent vaginal discharge. It suggested that *Arcanobacter pyogenes* alone or in combination with gram negative anaerobes was associated with clinical abnormalities, which included purulent/foul smelling

cervico-vaginal mucus discharge and hence more severe infection than any other bacterial combination. Similar results were also reported by Miller *et al.*(1980), Olson *et al.* (1984) and Dohmen *et al.* (1995).

In gr 1 and 2, GNA (*Bacteroides* sp.) occurred mostly in combination with *E. coli* and/or *Pseudomonas* sp. However, in gr 3, out of 8 samples, 4 harbored GNA occurring in combination with *Arcanobacter pyogenes* and one sample had gram negative anaerobes in combination with *E. coli.* Out of total 7 isolates of *Arcanobacter pyogenes* recovered from gr-2 and 3, 5 isolates were in combination with GNA and 2 isolates had pure growth. This supports the previous hypothesis (Ruder *et al.,* 1981; Olson *et al.,* 1984; Bekana *et al.,* 1994; Dohmen *et al.,* 1995 and Cohen *et al.,* 1996) that *Arcanobacter pyogenes* and GNA act synergistically in causing putrid endometritis and these two grs of bacteria were isolated more frequently, when cervical discharge contained more pus.

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