COMPARISON OF BACTERIAL ORGANISMS INSIDE THE UTERUS FOLLOWING TIMELY AND UNTIMELY INSEMINATIONS AND TWO TECHNIQUES OF AI GUN INSERTION IN CATTLE

IBRAHEEM KUTTY C[1]., ABDUL AZEEZ C. P. AND CHINTU RAVISHANKAR.

Department of Animal Reproduction, Gynaecology and Obstetrics,
College of Veterinary and Animal Sciences,
Kerala Veterinary and Animal Sciences University
Pookode P.O. Wayanad District, Kerala

Received:10-02-2021 Accepted: 12-11-2022

ABSTRACT

Various factors predisposing high incidence of infertility caused by non-specific uterine infections among AI (artificial insemination) bred cattle include inadequacy of aseptic practices, faulty techniques and untimely insemination. The present study was taken up to compare bacterial organisms present inside the uterus following two techniques of AI gun insertion (M1 and M2) during and after estrus period. Out of the 32 samples 43.75% showed bacterial growth and among the 8 samples from cows, 62.5% had microbial growth as against 37.50 % from heifers (n=24). Gun insertion contacting the vaginal fornix resulted more samples of bacterial growth (62.50%) as against direct insertion (25.00%). Comparing methods of insertion together with stages of cycle, chances of bacterial contamination was significantly higher (P ┐ 0.01%) during post estrual phase insertion with more contact on vaginal fornix (21.88%) as against direct cervical insertion during proper estrus (3.13%). It is concluded that untimely AI forms the major reason for increased occurrence of endometritis leading to infertility among AI bred cattle.

Key words: Insemination; Cattle; Bacteria; Artificial insemination; Uterine infection**.**

INTRODUCTION

Incidence of infertility among farm animals bred through artificial insemination (AI) are on the rise and is mostly due to reproductive tract infection (RTI) especially endometritis (Kather *et al.*, 2012; Kutty and Ramachandran 2003). Various factors predisposing occurrence of RTI consequent to AI are inadequacy of aseptic practices at semen processing, inappropriate techniques of semen deposition and untimely insemination (Bas *et al.* 2011). Lot of abnormal variations in the duration of estrus has also been reported making assessment of the right time for insemination very difficult (Kutty 2004, Kutty and Ramachandran 2000, Van-Eerdenburg *et al.* 1996). As the result, confirmation of estrus in animals presented for AI has become very crucial and demanding high levels of professional skill (O'Connor and Peters, 2003).

Observation of aseptic precautions during AI including semen handling and preparations for the deposition does not guarantee freedom from contamination of uterus since there is every possibility of carrying the vaginal flora and depositing in utero by the insemination process (Bas *et al.* 2011). Such contamination leads to establishment of RTI unless tubular defense mechanisms are maximum (Luthje *et al.* 2013) or if the manipulations associated with insemination are done judiciously (O'Connor and Peters 2003). In this respect semen deposition beyond the time of proper heat and increased manipulations associated with AI are two potential factors favoring RT infection. Hence objective of the present study was to compare the possibility of uterine contamination by two methods of AI gun insertion during estrus and the period beyond proper estrus in cattle.

[1] ***Address for correspondence*** *: Cholakkal House, Mattathur P.O., Malappuram Dist., Kerala, Pin code 676528; Mobile 95624 97320; Email: ibraheemkutty50@gmail.com*

MATERIALS AND METHODS

The study was carried out at instructional livestock farm complex, Kerala Veterinary & Animal Sciences University located at Pookode. Six heifers of 12 to 15 months and two cows not bred postpartum were used for the study. The animals were managed under semi-intensive system of rearing and detection of estrus was done by frequent observation by the workers. Animals detected in estrus were examined clinic-gynecologically to confirm the stage of heat and soundness for breeding. Animals chosen for AI were subjected to thorough cleaning of the external genitalia using sterile tissue paper. AI gun loaded with empty semen straw and covered with sterile protective sheath was used for sample collection. Insertion of the AI gun was performed by adopting the two methods one after the other and withdrawn for sample collection as follows

Method 1. Palpated and located the cervical opening using the thumb per rectally. The AI gun was inserted to reach middle of the vagina, pushed the cervix forward to straighten the vagina and moved the gun directly into

the cervical canal without touching the vaginal fornix at any time during the insertion (Wiki-how, 2018).

Method 2. Cervix was grasped and pushed the cervix forward to straighten the vagina per rectally and inserted the AI gun to reach the vaginal fornix. Withdrawn and redirected the gun into cervical canal by forward and backward movements of the gun together with tilting the direction of cervix being held by the palm (DeJarnette and Nebel, 2013).

Irrespective of the methods, insertion through anterior rings of the cervix was achieved by working on the cervix until tip of the gun becomes palpable at the uterine body (DeJarnette and Nebel, 2013). The AI gun was then withdrawn, external aspect of the sheath beyond half a centimeter from the tip was cleaned by sterile tissue and the tip immersed in 1 ml of nutrient broth for mixing the RT secretions adhered to the tip of AI sheath within the media. The insertion of the gun was carried out by the same person to ensure uniformity of the technique on all the animals being studied and the time taken for insertion was recorded for comparison of the two methods. The animals were inseminated thereafter by loading the semen straw and a new sheath on the same AI gun.

The same methods for sample collection were repeated two days after the estrus phase of same animals. In animals with tightly closed anterior cervix, gun insertion into the uterine body was not done to avoid damage to cervical tissue and some of them being already inseminated. Collected samples were cultured aerobically and also under the anaerobic jar. Defined volume of the broth was used for culturing in brain heart infusion (BHI) agar by spread plate method to detect the presence of bacteria introduced into the uterus by the two methods /stages of sample collection and findings are summarized.

The data were analysed for descriptive information and Chi square values using the online interactive tool provided by Preacher (2001) and culture growth values were compared between stages and methods of gun insertion and the two animal groups involved.

RESULTS AND DISCUSSION

A total of 32 samples were collected from the six heifers (n=24) and two cows (n=8) being 6 each by the two methods during estrus and post estrus phases as shown in table 1. On culturing 43.75% showed bacterial growth under aerobic conditions and no growth was observed under anaerobic jar. Only 4 (25.00%) samples collected during estrus showed bacterial growth while the proportion was 62.50 % among the post estrus phase samples, the difference being statistically significant (P<0.05).

Table 1. Details of bacterial growth from culturing of samples collected from the tip of AI sheath withdrawn after insertion to the site of insemination

Stage	Method	Samples	Growth	Percentage
Estrus	M1	8	1	12.50
	M2	8	3	37.50
	M1 + M2	16	4	25.00[a]
Post estrus	M1	8	3	37.50
	M2	8	7	87.50
	M1 + M2	16	10	62.50[b]
Total	M1	16	4	25.00[c]
	M2	16	10	62.50[d]
	M1 + M2	32	14	43.75

Values with superscripts a and b as well as c and d varied significantly (P<0.05)

Vaginal cavity being opened outward at the vulva has more chances of contamination from outside, in addition to the microbial flora already present there in (Zambrano-Nava *et al.* 2011). Enhanced tubular defense mechanisms of the oestrogen dominant phases of the oestrous cycle minimize the chances of contamination into the uterus together with suppressing the growth of invading organisms (Bas *et al.* 2011, Williams *et al.* 2005). Such protective mechanisms are not fully operational during non estrus phase since tight closure of the cervix takes over the protective role to prevent contamination into the anterior segments. For the same reason, invasion of uterus beyond estrus phase favors increase of bacterial load at least from the natural flora of vaginal cavity (Luthje *et al.*, 2013).

During the estrus, intromission / natural mating causes entry of large number of spermatozoa into the uterus together with introduction of microbes as well into the vagina and even the uterus in some cases. However, establishment of the infection and other adverse biological reactions are prevented by the augmented local defense mechanisms of the tubular tract during oestrogenic phase (Bas *et al.* 2011, Panchal 2017). Thus, deposition of microbes into the reproductive tract is unavoidable during natural mating but limited to vaginal cavity. At the same time AI is more prone to contribute infection (Kather *et al.* 2012) owing to more anterior sites of semen deposition unless proper care is exercised to ensure the congeniality of the phase having adequacy of the tubular mechanisms for infection prevention.

Comparison of the bacterial growth in samples from cows and heifers are shown in Table 2. Maximum samples (75%) were collected from un-bred heifers to rule out the chances of existing infection (Wang *et al.* 2013) and only few samples (25%) were collected from cows for comparison. While only 37.5% of the samples from heifers were showing microbial growth, the proportion was significantly high (P<0.01) in cows (62.50 %) in agreement with the studies by Kather *et al.* (2012) and Zambrano-Nava *et al.* (2011).

Table 2. Comparison of bacterial growth in samples collected from cows and heifers

	Total samples		Samples having microbial growth			
	Heifers	Cows	Heifers		Cows	
Total	24	8	9	37.50 %[a]	5	62.50 %[b]
Estrus phase	12	4	3	25.00 %	1	25.00 %
Non estrus phase	12	4	6	50.00 %[a]	4	100.0 %[b]
Insertion method M1	12	4	2	16.67 %[a]	2	50.00 %[b]
Insertion method M2	12	4	7	58.33 %[c]	3	75.00 %[d]

Values with superscript a and b (P<0.01) and c and d (P<0.05) varied significantly

Compromised immune response of the lactating cows under the influence of various stress factors and persistence of mild vaginal infection from previous calving explain for the increased bacterial growth in samples collected from cows (Rensis *et al.* 2017, Schuller *et al.* 2017, Williams *et al.* 2005). Even though same proportion of samples collected from cows and heifers during estrus phase had microbial growth, the pattern was significantly different (P<0.01) during non estrus phase (50% and 100 % among heifers and cows respectively). This high proportion indicates the increased chance of infection (P<0.05) during non-estrus phase irrespective of previous calving as shown in table 1. (Lewis 2004, Luthje *et al.* 2013).

Between the two methods of AI (Table 1), more M2 samples showed bacterial growth (62.50%) as against M1 samples (25.00%) the difference being significant (P ┐ 0.05). In both the methods microbial growth was significantly more (P<0.01) for samples from cows, indicating more prevalence of vaginal infection in them concurring the report by Kather *et al.* (2012). However, the number of samples tested from cows alone was inadequate to make valid conclusions.

Across the type of animals, the pattern was same with increased growth (P<0.01) in M2 samples than M1. Among heifers, the difference between M2 and M1 samples were highly significant (58.33% versus 16.67%), while the difference was non-significant among cows (75.00% versus 50.00%). Among the total samples collected as well as for samples from heifers, bacterial growth indicating chance of contamination into the uterus was more for M2 (P<0.01). This can be attributed to repeated contact of AI gun at the vaginal fornix that might carry more bacteria at the tip of AI sheath to the site of semen deposition (Bas *et al.* 2011, DeJarnette and Nebel 2013) and get deposited into the uterus along with the semen.

Vaginal fornix being a blind pouch formed by the fold of vaginal wall anterior to cervical opening, chances of washing out by the cervical mucus flow is minimum (Kather *et al.* 2012, Solutions 2018). Hence touching the AI gun tip at the fornix may carry the materials retained over there into the uterus and the chance increases with number of times and the different sides of the fornix being touched by the AI gun during the attempts for insertion by M2 (Karunakaran *et al.* 2012, O'Connor and Peters 2003, Panchal, 2017). However, direct insertion of the gun into the cervical lumen (M1), avoiding the contact of the AI sheath tip at the fornix (Wiki-how, 2018), might be the reason for less microbes being carried forward. Even though cervical os is exposed to vaginal cavity and bacterial flora therein (Wang *et al.* 2013), flow of oestrual mucous washes out the cervical lumen and keeps minimum load of contaminant bacteria (Verma *et al.*, 2014).

Time taken for insertion of the AI gun by either of the methods varied from 9 to 31seconds from insertion through the vulval lips until reaching the uterine body, the average taken being 18.25 seconds. Time taken by the method 1 was 15.88 seconds as against 20.38 seconds in method 2. More the time taken, more contact with the vaginal mucosa (Panchal, 2017) and increases the chances of vaginal flora being carried into the uterus (Kather *et al.* 2012, Wang *et al.* 2013). Increase of insertion time by M2 means more number of touches at the fornix, which acts to reduce the chance of conception (Karunakaran *et al.*, 2012) not only by increasing the possibility of contaminating tip of the AI sheath, but also by interfering the neuroendocrine mechanisms leading to conception (Nanda *et al.* 1990, Panchal, 2017).

Even though the time taken for insertion of the gun up to the site of insemination in the study is only 18.25 seconds, less experienced inseminators often take more time (Wiki-how, 2018) causing many times probing at the fornix or even other parts of the vaginal wall together with infliction of pain or irritation. Such manipulations initiates release of adrenalin and prostaglandins, which may act to reduces the chance of conception (Nanda *et al.*, 1990). Ideally, the insemination has to be done within the shortest time possible without causing irritation or injury to soft tissues of the reproductive tract and is better facilitated by adopting the method M1 wherein position of the cervical opening is identified in advance and the AI gun is inserted directly into the cervical canal avoiding entry and repeated probing into the fornix in the effort for cervix insertion.

Time taken for gun insertion was almost the same during metestrus and oestrous phases (18.68 versus 17.56) and the insertion was done without much difficulty in most of the cases as the cervix was not tightly closed even after many hours post estrus. Anterior cervix was not crossed during metestrus in two of the heifers with tight rings, to avoid possible chances of soft tissue injury. These two heifers were among the four already inseminated during the estrus. Upon subsequent verification, all the inseminated heifers (n=4) were found to have conceived, in spite of gun insertion repeated

during metestrus in two of them. Even though bacterial growth was obtained from 50 % of the samples collected from the AI sheath insertion during metestrus, the removal of the gun without any deposition into the uterus might have avoided the chance for infection and possible damage to the conception.

Among cows and heifers time of gun insertion was 15.25 and 19.08 seconds respectively. The time taken for gun insertion may vary depending upon the experience of the inseminator, breed and temperament of animal (Panchal 2017, Wiki-how 2018). Wider cervical opening might be the reason for easier insertion in cows, even though number of cows included in the study was only two and need more trials.

Considering methods of insertion together with stages of cycle, chances of bacterial contamination through insemination was significantly higher (P ┐ 0.01) for AI during non estrus phase and adopting the method 2 wherein there was more contact with vaginal fornix (21.88%) as against AI during estrus (3.13%) adopting either methods. Thus, AI beyond proper time of heat appears to be the single major reason for uterine infection in AI bred animals (Gill *et al.* 1974, Karunakaran *et al.* 2012, O'Connor and Peters, 2003) and can be attributed to the breach of natural barriers during a phase where tubular defense mechanisms are inadequate (Bas *et al.* 2011, Gunter *et al.* 1955, Lewis 2004).

Even if an animal is mated without proper heat, though it is very unlikely to occur under typical natural situation, the intromission is occurring only in the vagina and chance of contaminating the uterus is very less. However, AI being done intra uterine, insertion of the gun beyond the stage of proper heat forms highly potential reason for uterine contamination and establishment of infection. Owing to many aberrations of estrus signs and difficulty of detection under the prevailing management / breeding system, chances of faulty detection of heat and wrong time insemination is on the increase (Nasir and Kutty 2004). Unless extreme care and adequate skill are exercised on clinical confirmation of the right time of heat and adoption of the best technique for insemination (Wiki-how 2018, O'Connor and Peters 2003), there is every chance of causing reproductive tract infection.

Even if all possible aseptic precautions associated with preparation of the semen dose for AI are observed, AI personnel must be aware of the fact that even the natural flora of the vagina deposited in to the uterus can become potential cause for infection (Kather *et al.* 2012, Wang *et al.* 2013). Maximum concern for prevention of RTI associated with AI should be on ensuring the stage of estrus cycle with maximum tubular defense against microorganisms invading the uterus during the process of semen deposition (Bas *et al.* 2011, Karunakaran 2012, O'Connor and Peters 2003).

CONCLUSION

It is concluded that adoption of AI gun insertion with more contact of its tip within the vagina, especially at the fornix predisposes deposition of more microorganisms into the site of AI and together with untimely AI, these organisms may multiply faster and forms the major reason for increased occurrence of reproductive tract infection among AI bred cattle.

ACKNOWLEDGEMENT

The authors thankfully acknowledge the sincere help by Dr Logeshwaran G and Dr. Nandhakumar D., PG students of the Department of Veterinary Microbiology, and PG students and faculty members of the Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Pookode, in conducting the study.

REFERENCES

Bas, S., Hoet. A., Rajala-Schultz, P., Sanders, D. and Schuenemann, G. M. (2011). The use of plastic cover sheaths at the time of artificial insemination improved fertility of lactating dairy cows. *J. Dairy Sci.* **94**: 793–799.

DeJarnette, M. and Nebel, R. (2013). Artificial Insemination Technique in Cattle. www.selectsires.com/resources/fertilitydocs/ai_technique cattle.

Gill, R. S., Gangwar, P.C. and Kooner, D.S. (1974). Optimum time of insemination for high conception rate in buffaloes. *Indian J. Anim. Sci.*, **43**: 355-357.

Gunter, J., Collins, W. J., Owen, J., Sorenson, A. M., Scale, J. W., Alford, J. A. (1955). A survey of the bacteria in the reproductive tract of dairy animals and their relationship to infertility. *Am. J. Vet. Res.* **16**: 282.

Karunakaran, M., Chakurkar, E.B., Naik, P.K., Swain, B.K. (2012). *Artificial Insemination in Dairy Cows - Procedure and Facts for Improving Conception Rate,* ICAR Research complex for Goa, India, Extension Folder No. **55**.

Kather, A.N.Y., Salih, S.H.W. and Mohammed, D.S.N. (2012). Bacterial flora isolated from genital tract of cows submitted for artificial insemination in Balad district. *Kufa J. Vet. Med. Sci.* **3**: 12- 19.

Kutty, C.I. (2004). Emerging fertility problems in cattle and buffaloes bred through Artificial Insemination. *Intas Polivet.***5**: 269-274.

Kutty, C.I. and Ramachandran, K. (2000). Reproductive performance of cross bred cattle reared by small holders under artificial insemination programme in two districts of Kerala. *Proceedings of the small holder livestock production systems in developing countries,* pp. 101- 104.

Kutty, C.I. and Ramachandran, K. (2003). Bovine infertility – a field oriented categorization based on investigation among cross bred cattle in a district of Kerala. *Indian J. Anim. Sci.* **73**: 155-157.

Lewis, G.S. (2004). Steroidal regulation of uterine immune defenses. *Anim. Reprod. Sci.* **83**: 281-94.

Luthje, P., Brauner, H., Ramos, N.L., Ovregaard, A., Glaser, R., Linden, A., Hirschberg, H., Aspenstrom P. and Brauner, A. (2013). Estrogen supports urothelial defense mechanisms. *Sci. Translation. Med.* **190**: 180-90.

Nanda, A.S., Dobson, H. and Ward, W.R. (1990). Relationship between an increase in plasma cortisol during transport stress and the failure of oestradiol to induce an LH surge in dairy cows. *Res. Vet. Sci*. **49**: 25–28.

Nasir, N. A. and Kutty, C.I. (2004). Disappearance of heat signs in cattle herds continuously bred through artificial insemination. *Proceedings of the sixteenth Kerala science congress, Kozhikode,* pp 336-341.

O'Connor, M., and Peters, J. (2003). Reviewing artificial insemination technique.https://extension.psu.edu/downloadable/download/sample/sample_id/444/

Panchal, M.T. (2017). Improvement in Conception Rate in AI Delivery, *Write up of National Workshop on AI Delivery and INAPH System Implementation.* pp *1-65*.

Preacher, K.J. (2001). Calculation for the chi-square test: An interactive calculation tool for chi-square tests of goodness of fit and independence [Computer software]. Available from http://quantpsy.org/.

Rensis, F.D., Lopez–Gatius, P., Garcia-Isperto, I., Morini, G. and Scaramuzzi, R.J. (2017). Causes of declining fertility in dairy cows during the warm season. *Theriogenology*. **91**: 145-153.

Schuller, L.K., Michaelis, I. and Heuwieser, W. (2017). Impact of heat stress on estrus expression and follicle size in estrus under field conditions in dairy cows. *Theriogenology.* **102**: 48-53.

Solutions, (2018). Reproductive Anatomy and Physiology of Cattle, Select sires, www.selectsires.com/resources/fertilitydocs/reproductive_anatomy.pdf?version...

Verma, K.K., Prasad, S., Kumaresan, A., Mohanty, T.K., Layek , S.S., Patbandha, T.K. and Chand, S. (2014). Characterization of physico-chemical properties of cervical mucus in relation to parity and conception rate in Murrah buffaloes. *Vet. World* **7**: 467-71.

Wang, Y., Ametaj, B.N., Ambrose, D. J. and Ganzle, M.G. (2013). Characterisation of the bacterial microbiota of the vagina of dairy cows and isolation of pediocin producing Pediococcus acidilactici. *BMC Microbiol.* **29**, (13) :19.

Wiki-how. (2018). How to artificially inseminate Cows and Heifers, https://www.wikihow.com/Artificially-Inseminate-Cows-and-Heifers.

Williams, E.J., Fischer, D.P., Pfeiffer, D.U., England, G.C.W., Noakes, D.E., Dobson, H. and Sheldon, I. M. (2005). Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. *Theriogenology*, **63**: 102-117.

Zambrano-Nava, S., Ocando, J.B. and Nava, J. (2011). Normal bacterial flora from vagina of Criollo Limonero cows. *Trop. Anim. Health. Prod.* **43**: 291- 4.