Aims

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4. To publish occasional publications such as an inventory of ongoing research projects

Buffalo Bulletin is published quarterly in March, June, September and December. Contributions on any aspect of research or development, progress reports of projects and news on buffalo will be considered for publication in the bulletin. Manuscripts must be written in English and follow the instruction for authors which describe at inside of the back cover.

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The artificial insemination (AI) technique plays an important role in improvement of conception rate, prevention of sexually transmitted diseases and transmission of genetic material to the next generation. The breeding soundness evaluation (BSE) plays an important role in selection of male buffalo for the said purposes. This evaluation is an effective, inexpensive and easy method for selection of breeding buffalo bulls. In buffalo breeding management programmes, seasonal variation, nutrition, congenital defect, hormonal changes and hereditary play critical roles in determining the reproductive efficiency in buffalo bulls. The information regarding the reproductive health of buffalo bulls for breeding soundness evaluation is meager. The venereally transmitted infection causes early embryonic death, infection in the female reproductive tract and infertility or sterility in female animals and also infertility and disease condition in the male animals. To avoid such infective conditions, proper and careful investigation of all parts of the reproductive tract and evaluation of semen and treatment are important. So, a male buffalo calf being purchased for breeding purpose must be evaluated for breeding soundness.

Keywords: buffalo bull, Bubalus bubalis, infertility, infectious diseases

INTRODUCTION

The buffaloes are in the order of Artiodactyla, the cloven-hooved mammals, genus Bubalus and species bubalis. Two main species of buffalo are found in the world: the Asiatic (water) buffalo (Bubalus bubalis) and the African buffalo (Syncerus caffer). The two buffalo types are have different habitats and chromosome numbers. There are about ~170 million buffaloes in the world (Perera et al., 2005). Out of this 97 percent of them are water buffaloes and are mainly found in the Asian region. Riverine buffaloes are characterized by black colour and have long curled horns (e.g. Murrah Breed) and the Swamp buffaloes are dark grey, but may also be black, black and white, or even all white, have long, gently curved horns. Riverine buffaloes (70 percent of the total world population) are reared in high numbers in South Asia, especially in India and Pakistan. The name ‘swamp’ has probably arisen from their preference for wallowing in stagnant water pools and mud holes (Subasinghe et al., 1998). Swamp buffaloes are found mainly in southern China Sri Lanka, and the South-East Asian
countries of Thailand, the Philippines, Indonesia, Vietnam, Burma (Myanmar), Laos, Cambodia and Malaysia (Chantalakhana and Falvey, 1999). Riverine buffaloes are predominantly used for milk production and they are also used for meat, fuel and fertilizer production, as well as for draught power, whereas swamp buffaloes are traditionally kept as draught animals and are also (to a lesser extent) used for meat production. The river-type buffalo has a diploid number of 50 as compared to 48 for the swamp-type buffaloes. The Murrah has two extra acrocentric chromosomes, which are presumably translocated onto the short arms of the number 1 autosome of the swamp buffalo. Differential staining revealed that Egyptian water buffaloes, with a diploid number of 50, are closely related to the Murrah buffalo (Cribiu and Obeidah, 1978). Hybridization between Murrah and swamp buffaloes is possible and the hybrids are fertile. Improvement of buffalo reproduction helps in production and enhances significantly the economy and living standards of many rural communities throughout the world. The common defects affecting the fertility of the male are congenital defects in the male reproductive tract, hormonal imbalances between different glands, infectious diseases of the reproductive system or any other system, hereditary defects, imbalanced nutrition, psychological defects and abnormal climatic conditions. The purpose of this paper is to describe the different types of infectious disturbance which affect the reproductive performance of the buffalo bull.

GENERAL VIEW OF INFECTIOUS DISEASES IN BUFFALO BULLS

Infectious microorganisms affect fertility in two ways: they directly affect the reproductive system and the quality of semen, and they affect other systems, and this interferes with semen production, affects the libido and mating ability and prolongs reaction time. Lagerlof (1934) established a relationship between semen characteristics and testicular pathology. The condition of the testis is clearly reflected in the type of semen produced. Semen characteristics and fertility have a close relationship. Bacterial contaminants of the semen lead to the production of number macrophages and polymorphonuclear granulocytes as the first line of defence against bacteria in the semen, and this in turn leads to the generation of ROS (Ochsendrof, 1998) and also the production of a greater number of dead sperm, which will enhance the production of ROS in semen (Aitken, 1995). Increased ROS generation impairs sperm function and fertilizing capacity (Aitken, 1995; Griveau et al., 1995). Various types of microorganism cause conditions like balanitis, posthitis, seminal vesiculits, prostatitis, urethral inflammation, testicular degeneration, orchitis, epididymitis, and ampulitis which result in male infertility. Under practical conditions, it is not possible to produce semen free from microorganisms as contamination with few nonpathogenic organisms is unavoidable (Binda et al., 1994). Commensal microorganisms also infect the reproductive tract and affect sperm motility and viability when increasing stress to the animal; such organisms include E. coli (Schirren and Zander, 1966; Huwe et al., 1998) and Streptococcus faecalis (Bisson and Czyglick, 1974; Makler et al., 1981; Huwe et al., 1998). The organisms present in semen may be bacteria, viruses, protozoa, chlamydia, rickettsia and fungi and generally they are classified as pathogenic, potentially pathogenic, and not pathogenic (Gangadhar et al., 1986). Their
The presence in the semen may be due to from the bull’s systemic or local specific infections and they may also come from the normal prepuce flora or from the contamination that follows the semen collection because of inappropriate manual procedures or contaminated equipment. Many of these microorganisms may survive during storage carried out at low temperatures and they may represent a real danger for the spread of diseases if we consider that the semen is inserted directly into the uterus without exposure to the bactericidal action of the vaginal and cervical secretions produced during estrus.

The mean microbiological load was increased by four fold in the second ejaculate (Gangadhar et al., 1986), hence use same artificial vagina for two successive ejaculates is not accepted under clean semen production. The bacterial contamination of semen is major concern for semen production laboratories as the pathogens adversely affect the semen quality (Diemer et al., 1996). Bacteria contaminate approximately 50 percent of frozen semen doses as reported by Wierzbowski et al. (1984). Moreover it has been reported that the frozen semen of the buffalo bulls has a higher microbial load (Shukla, 2005 (4.860 ± 0.73 X 10^2 ml^-1); Rathnamma et al. (1997) (5.05 - 171.4 X 10^3 ml^-1); Jaisal et al. (2000) (1.0 - 50.0 X 10^2 ml^-1)) and there is a highly significant negative (P<0.01) correlation of standard plate count with progressive sperm motility (Shukla, 2005), live sperm (Shukla, 2005; Ahmad and Mohan, 2001) and HOS % (Shukla, 2005) both in neat as well as cryopreserved semen, whereas a positive correlation of sperm abnormalities with standard plate count was also recorded. The SPC and type of microorganism vary among different bulls (Shukla, 2005). The common microorganisms isolated from buffalo bull semen are Pseudomonas sp., Streptococcus sp., Staphylococcus sp., E.coli, Bacillus sp., Aeromonas sp., and yeast (Gangadhara et al., 1986; Ramasamy et al., 2002). Sensitivity (chloramphenicol (100%), ciprofl oxacin (100%) gentamicin (100%), neomycin (100%), streptomycin (86.62%), tobramycin (80%), co-trimoxazole (73.33%), erythromycin (53.33%), polymixin-B (13.33%), cephalaxin (6.67%), nitrofurantoin (6.67%), tetracycline (6.67%) and resistant penicillin-G (100%), Oxytetracycline (100%), carbenicillin (100%), amoxicillin (100%), ampicillin (100%) to microorganisms has also been reported for buffalo semen in fresh and frozen-thawed semen (Singh et al., 1992; Ramasamy et al., 2002). Microorganisms Balakrishnan et al. (2006) and Prabhakar et al. (1993) isolated from frozen buffalo semen were Staphylococcus sp. (56%), Bacillus sp. (45%), Micrococcus sp. (5%), E. coli (5%), Klebsiella sp. (3%), Proteus sp. (3%) and Pseudomonas sp. (10%). Balakrishnan et al. (2006) reported that the sensitivity test for buffalo semen were perfl oxacin (95.2%), ciprofl oxacin (94.4%), gentamicin (93.60%), enrofl oxacin (92.8%), penicillin (31.2%), streptomycin (16.8%), Oxytetracycline (40.8%), ampicillin (34.8%), amoxicillin (38.4%), chloramphenicol (36.8%) and triple sulph (27.2%). Sharma et al. (1994) reported that addition of gentamicin sulphate (500 mcg per ml), chloramphenicol sodium succinate (500 mcg per ml) or ampicillin sodium (500 mcg per ml) in Tris egg yolk glycerol proved to be more beneficial rate than combination of streptomycin sulphate (500 mcg per ml) and penicillin G sodium (500 IU per ml) at every stage of cryopreservation. Gentamicin sulphate was most effective in controlling bacteria in semen at all stages of deep freezing. Gentamicin, being poorly soluble in lipids, has limited ability to cross cell barriers (Prescott and Baggot, 1988). Consequently, its distribution in the extended semen is primarily extra spermatozoal and the whole of
the drug is available for killing bacteria and this is might be the reason for its better bactericidal effect. The better efficacy of chloramphenicol sodium succinate and ampicillin sodium than that of streptomycin sulphate and penicillin sodium G might be due to their higher sensitivity to bacterial flora of semen (Sharma et al., 1994).

INFECTIOUS BOVINE RHINOTRACHEITIS (IBRT)

The disease is caused by Bubaline Herpes Virus 1 (BuHV-1) and Bovine Herpes Virus 1 (BoHV-1), which are members of the Alphaherpesvirinae. BHV-1 (Gibbs and Rweyemamu, 1977) and which infect the respiratory and genital tracts of cattle and buffaloes, causing various diseases, such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious pustular balanoposthitis, abortion, mastitis, infertility, tracheitis, conjunctivitis-keratoconjunctivitis, encephalitis and fatal disease in newborn calves (Gibbs and Rweyemamu, 1977) and are pathogens responsible for causing significant losses in the livestock industry, through the failure of reproduction and increased mortality in cattle (Cortez et al., 2001; De Carlos et al., 2004). Phylogenically, this virus is closest to Bovine Herpes Virus-5 (BoHV-5), which causes encephalitis in cattle. On the basis of current knowledge, therefore, it cannot be claimed that BuHV-1 is responsible for abortion in buffalo cows. By contrast, BuHV-1 is suspected of being responsible for cross reactions with the IBR virus when Bovine Herpes Virus-1 (BoHV-1) or its parts (glycoproteins) are used as an antigen in serological tests (Galiero, 2007). But the pathogenic role of the BuHV-1 and BoHV-1 has not been clarified yet and the isolations and the serologic test results lead us to believe that this infection is particularly spread among buffaloes (De Carlos et al., 2004). This disease is transmitted through respiratory contact and ocular and reproductive secretions, with the latter route seeming to be the most important for entry into herds (Afshar and Eaglesome, 1990). In all probability, the viruses are eliminated through the semen of bulls both during the acute phase of the disease and during latent infections in clinically normal animals, and bulls may shed virus in semen during both clinical and subclinical infections (van Oirscholt et al., 1993). Viral reactivation from the latent state is generally thought to be stress-induced but can also be induced by the injection of corticosteroids (Pastoret et al., 1982). The excretion may occur even in lack of antibody response; therefore, only sero-negative and viruses-free bulls can be used for artificial insemination. There are differences in susceptibility to the disease among breeds. It has been reported that the Murrah breed (85.8%) is more susceptible (p = 0.0004) than the Mediterranean type (67.7%). Moreover, age is also a major factor for susceptibility to this disease: rates of infection increase progressively with advancing age (Ferreira et al., 2010). Diagnosis is carried out through ELISA, fluorescent antibody tests (FAT) of tissues, serum neutralization test, viral isolation or direct demonstration of the viral DNA through PCR (Polymerase Chain Reaction).

FOOT AND MOUTH DISEASES (FMD)

Foot and mouth disease virus could be transmitted in bull semen before the infected bull shows any signs of disease. The virus carrier stage has been shown to persist in some cattle for many months following recovery from the disease or
following prophylactic immunization. The disease has been shown to be transmissible when susceptible females were inseminated with semen from virus shedding bulls. It is found that FMD virus can survive in semen in freezing procedures in liquid nitrogen. Foot and mouth disease causes severe degeneration of germinal epithelium of the testes of bulls. The semen picture was severely adversely affected. The motility was poor and the total sperm abnormalities range from 34-62%. Significant FMD virus type specific antibody titers (IgG1, IgG2 and IgA) were detected in milk and serum of female buffaloes and serum of male buffalo. FMD virus type specific IgG1 was found to be the predominant subclass as compared to IgG2 and IgA both in milk and serum of vaccinated buffaloes. Milk and serum IgG1, IgG2 and IgA antibody titres were positively correlated with values of regression coefficient (R) as 0.506, 0.434 and 0.396, respectively (Yadav et al., 2007).

BOVINE VIRAL DIARRHOEA (BVD)

This disease is caused by Bovine Viral Diarrhoea Virus (BVDV) and the virus belongs to genus Pestivirus and family Togaviridae (Andrews et al., 1978). Although cattle are the primary hosts, BVDV can infect most even-toed ungulates. The main mode of transmission is through the oral route, but the disease may also be transmitted by inhalation and artificial insemination using semen of persistently infected bulls. Interspecies spread of this virus has been demonstrated but the epidemiological significance of this is uncertain. Seroepidemiological studies seem to indicate that these viruses circulate to some extent within the buffalo population in Italy (Galiero, 2007). To date, however, there is no conclusive scientific proof that the same viruses isolated in cattle are not also present in the buffalo, nor that they act through the complex pathogenic mechanisms that have long been known to operate in cattle (Galiero, 2007). Analysis of the molecular characteristics of the two strains made it possible to classify the isolates as BVDV-1, sub-genotype 1b. Evidence suggests that this virus is present within the buffalo population, and is associated with abortion, thus bringing to light a previously unknown health problem for the buffalo (Galiero, 2007). There are too little data to clarify the pathogenic role of BVDV in buffalo. Further research could shed light on the role of this virus and make it possible to determine pathogenesis, clinical characteristics and lesions.

BRUCELLOSIS

Buffaloes are susceptible to infection with Brucella abortus and Brucella melitensis. B. melitensis biovar 3 and B. abortus biovars 1 and 6 predominates in Italian buffalo herds. Brucella infection in bulls may affect the testicles, the epididymis, the seminal vesicle, and the ampulla. The infected males eliminate the Brucella spp., with the semen, so they play an active role in the spread of the disease (Eaglesome and Garcia, 1992). Brucellosis is a contagious infectious disease that affects bovines of traditional domestic and nondomestic species such as the buffalo. It is characterized by the production of abortions in the last third of gestation, retention of placenta, metritis, infertility, stillbirth, mastitis, poor production and quality of milk. Affections of the male are arthritis, orchitis, and epididymitis. Brucellosis is a zoonosis of world importance (Radostits et al., 2000). In this regard, artificial insemination represents a good tool to control this pathology on condition
that the donors are carefully monitored. Since infected bulls may give serologically negative results, bacteriological tests are necessary. The tests have to be applied to the semen and carried out in successive stages. The seminal plasma has to undergo the serum-agglutination test. Freezing procedures for the preservation of embryos cause a 64% decrease of the Brucella abortus vitality (Martinez et al., 2007). The administration of the appropriate antibiotics causes a 99% inactivation of the microorganism and the national eradication schemes are more beneficial in eradicating this organism when based on the detection and slaughter of infected buffaloes.

**ARCANOBACTERIOSIS**

Arcanobacterium pyogenes is commonly present on the nasopharyngeal mucosa of buffalo; in bulls. The usual habitat is the preputial mucosa (Radostis et al., 2003). Arcanobacterium pyogenes is a common cause of suppurative lesions in buffalo. This bacterium has been associated with mastitis, metritis, pyometra and abortion in buffalo cows. In bulls Arcanobacterium pyogenes is an important cause of orchitis, epididymitis or seminal vesiculitis, so the organism can be eliminated with semen. Specimens suitable for diagnostic laboratory procedures include exudates, aspirates, tissue samples and semen. Arcabacterium pyogenes is a Gram positive pleomorphic rod and produces a characteristic haemolytic pin-point colony in 48 hours of incubation. The diagnosis is based on bacteriological examination of the organs. Arcanobacterium pyogenes develops in 24-48 h, forming haemolytic colonies on agar supplemented with 5% sheep erythrocytes. Biochemical tests yield definitive microbial identification (Galiero, 2007).

**CAMPYLOBACTERIOSIS**

The infection of buffaloes with Campylobacter foetus may be widespread. Campylobacter fetus subsp. venerealis causes a type of venereal disease in the cow that is transmitted through natural service or artificial insemination. The disease is characterized by infertility, prolonged estrous, premature embryo death and in some instances, untimely abortion with placental retention (Das and Paranjape, 1987). The use of communal bulls and the use of males that have not been tested for Campylobacter foetus at artificial insemination centers are important factors in spreading infection. It has been reported that there are seven strains of Campylobacter sputorum subsp. Bubulus (Modulo et al., 1997) and Campylobacter fetus, Campylobacter fetus subsp. venerealis and Campylobacter fetus subsp. fetus (Joshi et al., 2006) have isolated from the prepuce of buffalo bulls. The disease has been recorded in India, Malaysia and the former U.S.S.R. (Eaglesome and Garcia, 1992). The animals used for artificial insemination have to undergo quarantine and have negative results in three consecutive cultural tests carried out on preputial scraping. Afterwards their sanitary conditions have to be checked every six months.

**LEPTOSPIROSIS**

The microorganisms belonging to this genus are mobile, helical bacteria, the terminal part of the bacterial body being hooked-shaped. Although cytochemically Gram-negative, they do not stain
well with the conventional bacterial stains, and are normally observed under a dark-field microscope. In the past, leptospires were subdivided on the basis of serological reactions into two species: *L. interrogans* and *L. biflexa*. In nature, leptospires survive in ponds, puddles and wet earth. They can be hosted by animals and humans, causing diseases of the urinary and genital apparatus or serious systemic diseases. In the animal reservoir, the micro-organism is hosted in the renal tubules or genital tract. The pathogenic role of *Leptospira hardjo* has long been known in cattle, in which it causes abortion, stillbirth and agalactia. Serological studies and the sporadic isolations described in the literature seem to suggest that various serotypes of *Leptospira* spp. are present in many buffalo herds. Many serologic researches demonstrate that the buffalo population has antibodies against several *Leptospira* spp. (Eaglesome and Garcia, 1992). Since these microorganisms cause hypofertility and abortion and survive in the frozen semen, particular attention has to be paid to bulls involved in the artificial insemination. The seminal vesicles of the bull are considered to be a major site for the localization of *Leptospira interrogans* serovar *hardjo*. The *L. pomona*, *L. canicola* and *L. hardjo* serotypes have also been found in several foetal buffalo kidneys (Galiero, 2007). The isolation of *Leptospira* spp. from the semen is not easy; therefore, the micro-agglutination test has to be used even if it does not allow distinguishing the vaccinated animals from the infected ones. Because of these problems bulls should not be vaccinated.

**CHLAMYDOPHILOSIS**

Chlamydiae are members of the family *Chlamydiaceae*, a group of obligate intracellular bacteria. Their developmental cycle comprises two forms: infecting elementary bodies and non-infecting reticular bodies. The former are small and metabolically inert, and penetrate the host cell by means of endocytosis. The reticular bodies are metabolically active and replicate by means of binary fission inside an endosome. Two genera are recognized on the basis of ribosomal RNA analysis: *Chlamydyphila* spp. and *Chlamydia* spp. Some of the species that cause chlamydiosis are zoonotic: *Chlamydyphila abortus*, *Chlamydyphila psittaci*, *Chlamydyphila felis* and *Chlamydyphila pneumonieae*, while others are not: *Chlamydyphila caviae*, *Chlamydyphila pecorum*, *Chlamydia suis*, *Chlamydia muridarum* and *Chlamydia trachomatis*. Ruminants can be infected by two species: *Chlamydyphila abortus* and *Chlamydyphila pecorum*. *Chlamydyphila abortus* causes abortion in small ruminants. This pathogen is also deemed to be responsible for abortion in buffaloes. Abortion, which may even become epidemic, occurs in the second half of pregnancy. The *Chlamydyphila abortus* infection may cause abortion and hypofertility. *Chlamydyphila pecorum* has long been recognized as the etiological agent of encephalomyelitis in buffalo calves. Recent studies conducted by means of molecular biology techniques on positive foetal tissues from archives have enabled the species involved to be typed as *pecorum*. It can therefore now be claimed that *Chlamydyphila pecorum* is the main agent responsible for abortion in buffalo cows, as well as for encephalomyelitis (Galiero, 2007). The microorganism is eliminated through the semen of sick bulls that appear clinically normal, even if, sometimes, their semen has a large number of leukocytes and a low concentration of sperm with poor motility and high percentage of sperm cell abnormalities. To isolate or demonstrate
Chlamyphila abortus from the semen, preputial or urethral swabs, ELISA, PCR, embryonated eggs or culture tissue are the techniques generally used.

**MYCOBACTERIOSIS**

*Mycobacterium bovis* can be responsible of orchitis in the buffalo male. Therefore, the donor buffalos’ semen has to be tested before using it and then once a year. The tests applied are the single intradermal test and, if necessary, the comparative intradermal test. Blood based assays which have been developed for use in conjunction with the tuberculin test include the gamma interferon test, ELISA and PCR.

**TRICHOMONIASIS**

Trichomoniais is caused by *Trichomonas foetus*, a pyriform protozoan that causes premature abortions, pyometra and infertility. This flagellate is transmitted to the female buffalo during her mating with an infected bull and vice versa. *Trichomonas foetus* infection of the genital tract of buffaloes was recorded only in India and Egypt (Eaglesome and Garcia, 1992). This suggests that the buffalo is an unusual host for this parasite and is not generally susceptible to infection. The infected animals may be carriers for their whole life. The parasite persists in diluted semen and is resistant to freezing; therefore, donors have to be tested frequently either through a microscopic examination or through a cultural test in order to exclude any infection.

**MYCOPLASMOSIS**

Various workers have reported the recovery of mycoplasma from bovine semen. It is established fact that mycoplasma is common in the bull and buffalo bull semen and survives during semen processing, freezing and storage. The survival of mycoplasma in frozen semen at -196°C for 18 months has been reported. Mycoplasma cause granular vulvo-vaginitis and damage the inner lining of the oviduct in females while impairing fertility in males. Mycoplasma especially the *bovigenitalicum* and *agalactiae* species are frequently associated with seminal vesiculitis in the male and metritis and salpingitis in the female.

**FUNGI (MOULD AND YEAST) INFECTIONS**

Several genera of fungi have been cultured from raw and extended semen and preputial washings. These fungi may contribute to reproductive failure under certain conditions. Their source may be semen or anatomical loci within the male or female reproductive tract or contaminated semen collection equipments. *Candida trapisalis, Candida stellatoidea, C. albicans, Torulopsis femata* and *Aspergillus fumigatus* have been isolated primarily by culture isolation and *Aspergillus* sp., *Fuzerium sp., Penicillum* sp., *Mucor* sp., by staining examination from buffalo semen and found to be associated with reduced fertility (Kodagali, 1979). Management practices leading to lowered resistance, feed fortified with antibiotics, and nutritional disorders are supposed to make animals liable for mycotic infection.
**Escherichia coli** INFECTIONS

*E. coli* is a gram-negative, non-endospore producing, facultative aerobic bacillus. Its antigenic structure comprises the lipopolysaccharides of the cell wall (O antigen), the polysaccharides of the capsule (K antigen) and the flagellar and fibrillar proteins (H and F antigens, respectively). Although about 50,000 serotypes have been identified, only a limited number of strains are able to cause disease. The pathogenic action is linked to the ability of the clone to produce so-called virulence factors, which may be either structural (flagellae, capsule, lipopolysaccharides, adhesins or secreted (cytotoxic and cytotoxic toxins, haemolysins). A wide variety of different serotypes of *E. coli* can be found in buffalo herds. Many of these are pathogenic in newborns, such as enterotoxaemic and enterohaemorrhagic *E. coli*, which produce heat-stable toxins, verocytotoxins and necrotising cytotoxic factor. The buffalo is an important reservoir of verocytotoxic *E. coli* serotypes, especially O157. *E. coli* may also cause abortion, albeit sporadically. To date, it is not certain whether abortion is caused by the bacterium and its structural antigens or by the cytolytic action of its toxins. The diagnosis is based on the serological examination of the foetal organs. *E. coli* develops in MacConkey agar medium, fermenting lactose and producing reddish-pink colonies. Any haemolytic activity can be evaluated by means of blood agar. PCR is a useful tool in detecting, from isolated strains, the gene sequences responsible for coding virulence factors or toxins (Galiero, 2007).

**OTHER MISCELLANEOUS MICRO ORGANISMS**

The other micro organisms that are involved in infectious infertility in buffaloes are *Usteria monocytogenes*, *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Streptococcus* spp., *Staphilococcus* spp., *Proteus* spp., *Aeromonas* spp., yeast and moulds. Fungal infections may develop during the processing of semen for preservation. Fungal infections of the prostate and other accessory sex glands may result in the presence of fungal infections in semen, which reduces quality of semen.

The principal part of contamination of semen consists of saprophytic or opportunistic organisms from the prepuce and upper parts of the genital organs. Since the greatest amount of the bacteria present in semen comes from the prepuce, from the skin and from the equipment used, semen collecting procedures have to be carried out using sterilized tools and materials and the bull’s preputial cavities must be cleaned constantly. In spite of these preventive measures, sometimes the ejaculate presents a non-specific microbial flora (5 x 10^6 CFU/ml). These concentrations may be reduced by administering antibiotics and diluting the semen, while lowering the number of viruses and bacteria about 100 times, and freezing until the following count is obtained: 8 x 10^2 CFU/ml (considered normal) (Visintin et al., 1997). A permissible count of 500 bacteria per dose of semen straw is the international standard. Accordingly, it is recommended that preputial washings (maximum 50,000 count) and neat semen (maximum 1000 to 5000 count) should carry lowest minimum bacterial load with no count for AI equipment and dilutors as they are to be used only after strict sterilization (Visintin et al., 1997). Liquid nitrogen used for
storage of frozen semen doses may act as vehicle for contaminant pathogens with variety of organisms and at various degrees. The same thus serves as source of infection to cows and buffalos during AI. Even fresh liquid nitrogen carries Staphylococcus aureus. Certain bacterial contaminants acquire a level of resistance to antibiotics and they are able to survive at -190°C in liquid nitrogen (Ranold and Prabhakar, 2001).

**FUTURE PROSPECTS**

The future prospects to improve the reproductive traits and eliminate the reproductive disorders in buffalo bulls are mentioned below.

There is a need to study to a greater extent the sexual development, attainment of puberty, sexual maturity, sex libido, semen production, reproductive performance of buffalo bulls. Study of molecular marker (DNA/gene) assisted selection and cytogenetic studies linked to genes of interest with major effects on reproduction need to be strengthened for selection of breedable males in bubaline species at an early age. A cytogenic marker would help to cull/eliminate a particular bull from the herd to prevent the spread of a genetic/congenital defective gene from that male to future offspring and to prevent reproductive disorders.

Breeding soundness evaluation technique would help to improve the breeding efficiency of buffalo bulls.

Realistic remedial measures for reducing infertility and enhancing fertility need to be emphasized for the effective control of various reproductive disorders.

Biosafety measures for production disease-free germplasm and registration of all A.I. bulls by a national society to initiate a certified disease-free semen service for the whole nation need to be addressed.

**REFERENCES**


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ABSTRACT

This communication reports a case of dystocia due to foetal anasarca coupled with amelia of one foetus in a twin pregnancy and its successful per-vaginal management in a Mehsana buffalo.

Keywords: dystocia, foetal anasarca, amelia, twin pregnancy, Mehsana buffalo

INTRODUCTION

Dropsical conditions viz. fetal ascitis, fetal anasarca, edema of the allantochorion, hydrops of the amnion or allantois or both etc. are reported to be causes of dystocia (Roberts, 1971). Twin pregnancy with foetal anasarca of one foetus has also been observed as an occasional cause of dystocia in bovine. Therefore, a rare case of dystocia due to foetal anasarca coupled with amelia of one foetus in twin pregnancy (Figure1) and its successful per-vaginal management in a Mehsana buffalo is placed on record.

CASE HISTORY AND OBSERVATIONS

A five-year-old full term pregnant Mehsana buffalo was presented with the history of dystocia. Further, the water bags had ruptured three hours before and there was no progression in foetal delivery. The animal was straining repeatedly with expulsive efforts and exhibiting all the external signs of approaching parturition. Per-vaginally, the cervix was completely relaxed and jam-packed with a large, soft and smooth oedematous mass leading difficulty in advancing the palpation. Accordingly, the case was diagnosed to be the dystocia of foetal origin and it was decided to manage per-vaginum.

TREATMENT AND DISCUSSION

Following cleaning of the perineum and achieving caudal epidural analgesia using injection lignocain HCL 10 ml between the last sacral and first coccygeal vertebra; the lubricated hand was introduced into the uterus through vagina by pushing back the jam-packed foetal parts. Eventually, it became possible to palpate the atrophied jaws of the foetus, while the limbs

1College of Veterinary Science and Animal Husbandry, Navsari Agricultural University (NAU), Navsari, Gujarat - 396 450, India, E-mail: vsdabas@yahoo.co.in
2Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University (SDAU), Deesa, Gujarat, India
3Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University (NAU), Navsari, Gujarat, India
could not be traced. Subsequently, two obstetrical hooks were separately placed between the mandibles and in the oedematous mass. While applying progressive gentle traction to each of the hooks, one hand was kept inside the genital tract for giving requisite force and the directions to the foetal parts. Ultimately, a dead oedematous foetal monster was delivered. While the genital tract was examined to rule out the possibilities of injury and removal of the afterbirths, one more normal sized dead foetus was palpated inside the uterus and could be easily removed. Subsequently, both the easily detachable placentas were removed manually and four Oriprim-U boluses were put in each uterine horn. Injection calcium-borogluconate 450 ml intravenously was given once and injections oxytetracycline 40 ml, chlorphenermine meleate 10 ml and ketoprofen 15 ml, intramuscularly were given for three consecutive days. Soon, the animal recovered uneventfully. The majority of monstrosities are described as single with deviated morphology of typical monster (Arthur et al., 2001). However, two defects viz. anasarca and amelia all together were observed in the present case.

REFERENCES


Figure 1. Fetal anasarca coupled with amelia of one foetus in twin pregnancy.
ABSTRACT

The present investigation was carried out to study the various pathological conditions occurring spontaneously in the livers of buffaloes. A total number of 2,119 buffalo liver samples was collected irrespective of sex, breed and age of the animal from slaughterhouses located in and around Bikaner, Rajasthan. Among the various pathological conditions of the livers, fatty change was found to be the highest followed by cirrhosis, congestion, cell swelling, abscess, RES response, haemorrhage, pigmentation, necrosis, apoptosis and telangiectasis.

Keywords: buffalo, fatty changes, liver, prevalence, spontaneous lesions

INTRODUCTION

The buffalo is one of the key animals in the agricultural economy, contributing substantially to the gross national income (GNP) by way of good quality milk, meat, export quality leather and physical power. The buffalo is a part of the property, possession and profession of rural farmers. Not only that, it is an easily ‘convertible currency’ and a reliable ‘living bank’ to serve the immediate needs of the rural masses in several communities. Of all domestic animals, the Asian buffalo holds the greatest promise and potential for production (Cockrill, 1994). India has world’s best buffalo dairy breeds and provides superior buffalo germplasm to several countries of the world (Kaikini, 1992). The liver is one of the vital organs of the body, susceptible to various affections which influence the total health status of the animal. Further, considerable liver damage may be present before clinical signs are apparent largely due to the high degree of reserve functional capacity. The present investigation was carried out on the livers of buffaloes to investigate the various spontaneous lesions of the liver.

MATERIALS AND METHODS

The materials for the present study comprised livers obtained from buffaloes slaughtered at slaughterhouses located in and around Bikaner, Rajasthan. A total of 2,119 livers of slaughtered buffaloes were examined irrespective of age, breed and sex for spontaneous liver lesions. Gross study was performed during collection of samples from the slaughterhouses primarily and then during trimming of the samples for histopathology. Out of these, 476 livers exhibiting
gross alterations were cleaned with normal neutral saline and the changes were recorded. The lesion containing tissue samples were collected and preserved in 10% neutral formalin solution for histopathological study. After 48-72 h, these tissue pieces were washed overnight in running tap water, dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax (60-62°C melting point). Sections of 4-5 micron thickness were cut and stained with haematoxylin and eosin as per the standard procedure recommended by Lille (1954).

RESULTS AND DISCUSSION

The various spontaneous liver lesions observed during the present study were as shown in Table 1. Examination of the livers of 476 buffaloes revealed various types of lesions in 180 animals, amounting to 37.82%.

Among the various pathological lesions of the livers, fatty changes were found to be the highest (6.09%), akin to the observation of Gupta (1983). However, Kulkarni (1992) and Dhote et al. (1992) reported much lower incidences, being 2.45% and 1.5%, respectively. While Purushotam and Rajan (1985) noticed the condition in a much higher number of cases (21.7%). Grossly these livers appeared pale, soft, and greasy with rounded edges. Histopathologically, the hepatic cells showed presence of fat droplets as clear round spaces and granular cytoplasm. Large clear cavities were seen indicating that the scattered cells with fatty changes coalescence (Figure 1). Sinusoides were often reduced by swollen fatty cell cords (Figure 2). Focal, diffuse, pericentral and periportal types of fatty changes noticed presently were comparable to those described by Jubb et al. (1993) and Dhote et al. (1992). Ketosis may be one of the possible causes of fatty changes.

A total of 5.25% cases showed partial or complete cirrhosis. The liver was constricted in many cases. Thickened capsule, finally granular surface, hard to cut and numerous newly formed bile ducts in cirrhotic liver were observed in the present

Table 1. Spontaneous liver lesions in slaughtered buffaloes (n = 476).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spontaneous lesions</th>
<th>No. of Cases (180)</th>
<th>Percentage of affected animals (37.82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fatty change</td>
<td>29</td>
<td>6.09%</td>
</tr>
<tr>
<td>2.</td>
<td>Cirrhosis</td>
<td>25</td>
<td>5.25%</td>
</tr>
<tr>
<td>3.</td>
<td>Congestion</td>
<td>24</td>
<td>5.04%</td>
</tr>
<tr>
<td>4.</td>
<td>Cell swelling</td>
<td>19</td>
<td>4.00%</td>
</tr>
<tr>
<td>5.</td>
<td>Abscess</td>
<td>18</td>
<td>3.78%</td>
</tr>
<tr>
<td>6.</td>
<td>RES response</td>
<td>18</td>
<td>3.78%</td>
</tr>
<tr>
<td>7.</td>
<td>Haemorrhage</td>
<td>14</td>
<td>2.94%</td>
</tr>
<tr>
<td>8.</td>
<td>Pigmentation</td>
<td>13</td>
<td>2.73%</td>
</tr>
<tr>
<td>9.</td>
<td>Necrosis</td>
<td>9</td>
<td>1.90%</td>
</tr>
<tr>
<td>10.</td>
<td>Apoptosis</td>
<td>7</td>
<td>1.47%</td>
</tr>
<tr>
<td>11.</td>
<td>Telangiectasis</td>
<td>4</td>
<td>0.84%</td>
</tr>
</tbody>
</table>
study as described by previous researchers (Gupta, 1983; Moorty et al., 1984; Dhote et al., 1992; Vegad and Katiyar, 1998). Microscopically considerable proliferation of fibrous connective tissue was marked mainly in the portal areas replacing hepatic cells. There was little lymphocytic infiltration of fibrous strands. Portal veins and sinusoids were dilated and irregular in shape. Cirrhosis was frequently noticed concomitant with parasitic condition; however, in many cases contributory factors could not be incriminated.

Congested liver 24 (5.04%) showed the character of “nutmeg” liver and mottled appearance. Microscopically, sinusoids were dilated and central veins were engorged with erythrocytes. Slight periductal infiltration of round cells was noticed. Hepatic cells around the central veins were degenerated and atrophied. Moderate proliferation of fibrous tissue was also seen in portal spaces, corroborating the descriptions of Cohrs (1967) and Gupta (1983).

Cell swelling were found in 19 (4.00%) of the cases. Grossly, the affected livers were enlarged and of lighter color with rounded edges. Microscopically, the enlarged rounded hepatocytes containing eosinophilic granular cytoplasm which obliterated sinusoidal space.

Abscess was found in only 2.94% cases. Grossly, whitish foci on the surface of the liver were found (Figure 3). Their size varied from 0.5 to 1.5 cm in diameter. Polymorphonuclear leukocytes at the center surrounded by a thin fibrous capsule were seen microscopically (Figure 4). Similar histopathological changes have been reported in earlier studies (Gupta, 1983 and Dhote et al., 1992).

RES response was observed in 3.78% cases. Grossly and microscopically the livers with RES response showed variable extent of diffusely distributed small foci in the form of Kuffer’s cell hypertrophy and hyperplasia, degeneration, necrosis and granulomas being comparable to the descriptions of Cohrs (1967). These apparently were consequent to the body defense against underlying subtle infections.

In the 14 cases of haemorrhage, the liver showed a dark brown petechial and/or ecchymotic nature and superficial or deeper location microscopically variable extents of degeneration, necrosis and neutrophilic and lymphocytic infiltrations in the involved area in conformity with Dhote et al. (1992).

Pigmentation was seen in 13 (2.73%) cases. The darker colored liver showing yellowish brown granules of haemosiderin in the Kuffer’s cells macrophages and free in the sinusoids in H&E stain (Figure 5) being blue in Perls’ (Figure 6) were similar to the reports of Gupta (1983) and Tripathi and Chattopadhyay (1989).

In hepatic necrosis (1.90%), pale patches were found on the surface of liver presenting a mosaic appearance. White, opaque necrotic foci were uniformly distributed throughout the liver. Microscopically, the hepatic cells were very much swollen and granular with pyknotic or lytic nuclei, infiltration with neutrophils and lymphocytes, engorged veins, fibroplasias and Kuffer’s cell proliferation were observed. This presumably may be due to involvement of some toxins in feed (Jubb et al., 1993) possibility fungal, bacterial or plant toxins or chemicals playing some role in causing the condition.

Apoptosis was seen in seven (1.47%) cases. Occurrence of apoptic cells and apoptic bodies in the hepatocytes were similar to Kumar et al. (1992) and Bhel and Tripathi (1999). These apparently pointed to be the result of the mechanism regulating the number of cells since these were
Figure 1. Photomicrograph of liver section showed presence of fat droplets as clear round space and granular cytoplasm of hepatic cells. H&E X 200.

Figure 2. Photomicrograph of liver section showing fatty changes with coalescence forming large sized cavities, sinusoidal congestion and edema is also seen. H&E X 400.

Figure 3. Photomicrograph of centrilobular hemosiderosis showing golden brown pigment in the Kuffer cells and in the sinusoids. H&E X 200.
Figure 4. Photomicrograph showing blue haemosiderin pigment in the Kuffer cells. Pearls’ prussian blue staining X 200.

Figure 5. Surface and cut surfaces of liver pieces showing abscesses (a,b) and congestion (d).

Figure 6. Photomicrograph showing within outside area of calcification, detritus and pyogenic membrane. H&E X 200.
neither frequent and nor abundant.

Telangiectasis was observed in 0.84% cases. This was comparable to Gupta (1983) and Hassib et al. (1995), who reported 1% and 0.58%, respectively. The gross and microscopic character of liver showed as dark reddish depressed single or multiple areas of variable size and dilatation of group of sinusoids and/or larger cavernous spaces lined by endothelium and containing erythrocytes with or without connections were in line with the descriptions of Runnels (1976).

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EVALUATION OF CYSTOURETHROPLASTY VIS-A-VIS CAECOURETHROPLASTY TECHNIQUE IN BUFFALOES (Bubalus bubalis)

Md. Moin Ansari1 and S.P. Sharma2

ABSTRACT

The present experimental urothroplasty was undertaken in sixteen male buffalo calf models apparently in good health status using urinary and caecal grafts. The surgical technique was evaluated on the basis of clinical observation, determination of blood urea nitrogen, histopathological and post-operative complication for a period of 60 days post-operatively. Urethroplasty using formalin preserved urinary bladder and caecal grafts acted as a scaffold around which there was gradual regeneration of urethral tissue and resolution of grafted material by 60th post-operative day. However, both these techniques were worthy and feasible in buffalo calves, yet caecourethroplasty was preferred, because it was easier and safer than the cystourethroplasty.

Keywords: buffalo, cystourethroplasty, caecourethroplasty, urinary bladder, caecal graft

INTRODUCTION

Urethroplasty is an open surgical procedure for the repair of an injury or a defect in the walls of the urethra. Different grafts such as gut segments, free facial grafts, polyvinyl silicone patch, peritoneum, omentum, gelatine sponge, OMS membrane, Teflon felt, free autologous preputial tissue, autogenous skin grafts, pedicle, autogenic, allogenic, xenogenic, synthetic and processed grafts, seromuscular graft, lyophilized human dura and formalin preserved buffalo duramater, urinary bladder grafts and intact caecum have been used for surgical repair of bladder and urethral defects both experimentally and clinically in man and animals (Kelami, 1971; Kudale and Hattangady, 1971; Prasad et al., 1973, Sharma and Khan, 1978, 1980; Prasad et al., 1980; Sharma and Agrawal, 1997 and Sengupta et al., 1998).

It is obvious from the available literature that the clinical and experimental replacement of the urethra and ureter is not a new concept for human beings and canine but it has not been studied further in large animals. The paucity of literature on large animals as well as the high incidence of urethral calculi leading to rupture of the urethra have focused attention upon urethral substitution, which can be achieved with a suitable substitute if needed in many of the conditions: to repair the wall of the urethra when it is excessively damaged or badly replaced due to urethral calculi, violence or non-specific reasons; concurrent papilloma and / or carcinoma of the urethra; any anatomical defects

1 Division of Surgery and Radiology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-E-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Shuhama, Alesteng Srinagar - 190 006, India
2 Department of Surgery and Radiology, Bihar Veterinary College, Patna - 800 014, Bihar, India
of the urethral wall, like irreparable urethral fistula; urethral stenosis, stricture or ill development, congenital anomalies like hypospadia, epispadias, urethral diverticulum, urethrocele. Success attained with vesiculoplasty in humans, canine and bovine initiated this study on urethroplasty using urinary bladder and caecal grafts. Based on a research and experienced gained in urological surgery of buffalo calves either experimentally or clinically, the study was designed (Sharma and Khan, 1978; Sharma, 1995; Sharma and Agrawal, 1997, Ansari, 2002).

The damaged, diseased or congenital urethral defects have been corrected and substituted by preputial tissue. Various workers like Vyas et al., 1987; Wessells and Mc Anich, 1996; Kocvara and Dvoracek, 1997 and Sengupta et al., 1998 have replaced urethra partially or completely with preputial graft. Uroepithelium is known for its regenerating property (Bohne et al., 1955). Therefore, the primary objective of urethroplasty is to provide an environment for regeneration of normal urethral tissue for urinary passage.

MATERIALS AND METHODS

Cystourethroplasty and caecourethroplasty were conducted in 16 healthy male buffalo calf models weighing about 80-115 kg. The animals were divided into two groups consisting of eight animals each. Formalin-preserved urinary bladder and caecal allografts were used as urethral prosthesis in Groups 1 and 2, respectively. Pre- and post-operative temperatures, pulse, respiration and blood urea nitrogen (Levine, et al., 1961) using a photo-calorimeter were recorded in all the buffalo calves.

Urinary bladder and caecum obtained from buffalo calf cadavers were preserved in 10% formalin solution for 15 to 30 days. The formalin preserved urinary bladder/caecal grafts were kept in running tap water for 24 h prior to grafting to remove formalin from them. Then the graft was kept in Povidine Iodine*** (Win-Medicare Ltd. N. Delhi.) solution for 2 h prior to grafting. The margin of the graft material was trimmed; mucosa and sub-mucosa were scrapped to prepare a graft of 3.5 X 1.5 cm. It was stored in normal saline before urethroplasty.

Xylazine* (Astra IDL Ltd. Bangalore) 0.05 to 0.2 mg/kg body weight was given intramuscularly. Ten milliliters of 2% xylocaine** (Astra IDL Ltd. Bangalore) was infiltrated on the line of incision. Post-scrotal urethroplasty was done in routine manner giving about 10 cm incision. The ventral wall of the urethra was excised for about 3.5 cm length and about 1.5 cm breadth. Bleeding vessels were ligated. A polythene tube about 2.5 mm diameter was passed inside the urethra towards the ischial arch into the urethra and the lower end of the polythene tube was passed through the urethra in anterior direction, to take out through the pre-preputial opening. The formalin-preserved seromuscular urinary bladder/caecal graft was placed over the excised urethra in such a position that the serous layer faced outside. Then four stay sutures were applied at the corners. Simple interrupted sutures were applied using chromic catgut no. 2/0 and Ethicon black braided silk no. 3/0 in both the groups. The protruding polythene tube was anchored with the silk thread at the preputial area. The cutaneous incision was closed giving Halsted suture using black braided silk no. 2. The cutaneous stitches were removed on the 8th post-operative day while the polythene catheter was allowed to remain in the urethra for 2 weeks. The operated buffalo calves were sacrificed on day 15, 30, 45 and 60 and materials were collected for
RESULTS AND DISCUSSION

In cystourethroplasty, seven out of the eight buffalo calves survived subjected to urethroplasty. An initial rise of temperature, pulse and respiration was marked in almost all the animals after surgery, which might be due to tissue reaction. The same was observed by earlier studies in goats using caecal graft (Mukherjee, 1988) and PTFE, caecal and bladder grafts Shivaprakash (1990) and in dogs using peritoneal graft (Nair et al., 1988) following urinary bladder reconstruction.

Estimation of blood urea nitrogen on days 1, 2, 3, 7, 14, 21 and 28 after reconstruction showed slight elevation from the pre-operative level. During the post-operative period clinical observation and the pattern of blood urea nitrogen level proved that there was no obstruction to the normal passage of urine. These findings on the blood urea nitrogen levels in operated buffalo calves were in agreement with the observation made in human beings, canine and bovine (Shoemaker and Marucci, 1955; Prasad et al., 1973; Prasad et al., 1979; Sharma and Khan, 1978, 1980; Nair et al., 1988; Sharma, 1995) after partial reconstruction of the urinary tract experimentally and clinically. Gross observations, which were made during the study, could not detect untoward pathological changes in the wall of grafted urethral tissue except in one buffalo calf which died showing tympany. Histological examination of the tissue taken from the operated area of the urethra revealed gradual regeneration of the urethral tissues and resolution of the grafted material. There was lack of distinct epithelium lining on the 15th post-operative day (Figure 1) but it was evident on the 30th post-operative day (Figure 2) and the epithelium of the urethra was creeping towards the graft side. On the 45th post-operative day (Figure 3), lymphocytic cells were moderate in number and there was gradual regeneration of uroepithelium and underlined connective tissues at the operated area. On the 60th post-operative day (Figure 4), all the inflammatory changes were minimal and there was complete regeneration of the uroepithelium over grafted tissue from the host side. This observation was in agreement with the results of Sharma (1995), who tried formalin preserved urinary bladder and terylene lined hemispherical hollow plastic balls as bladderprosthesis in experimental buffalo.

In caecourethroplasty, all the operated animals survived after the reconstruction. An initial rise of temperature, pulse and respiration for the first few days were noticed in almost all the animals, but these all became normal in due course. All the operated animals were taking feed normally after second post-operative day. Gross observation in few cases showed echymotic haemorrhage in the urethral mucosa and peelable necrotic foci on the caecal graft even up to the 45th post-operative day. This type of haemorrhage could be harmless as none of the animals showed any complications up to the 60th post-operative day. Necrotic foci which were present on caecal graft up to the 45th day were also observed by Prasad et al. (1973) and Sharma and Khan (1978, 1980) during intestinocystoplasty in canine and bovine. Histological examination on the 15th post-operative day (Figure 5), showed epithelial lining was not clearly discernible at the junctional zone. At the end of the 30th post-operative day (Figure 6), epithelium of the urethra was creeping towards the graft side and trying to overlap the grafted
Figure 1. Photomicrograph from the grafted urethral junction using urinary bladder on 15 POD (H & E-150), showing not clearly discernible epithelium on of the urethral either side of the zone of junction and marked infiltration of lymphocyte.

Figure 2. Photomicrograph from the grafted urethral junction using graft on 30 POD (H&E-150), showing the epithelium creeping towards the graft side.

Figure 3. Photomicrograph from the grafted urethral junction using urinary bladder graft on 45 POD (H&E-150), showing gradual regeneration of the regenerated uroepithelium and underlined connective tissues.

Figure 4. Photomicrograph from the grafted urethral junction using graft on 60 POD (H&E-150), shows completely uroepithelium and surrounding tissue.

Figure 5. Photomicrograph from the grafted urethral junction using caecal graft on 15 POD using caecal graft on (H&E-150), showed epithelial lining was not clearly discernible at the regeneration of the junctional zone.

Figure 6. Photomicrograph from the grafted urethral junction 30 POD (H&E-150), showing the gradual urethral tissues.

Figure 7. Photomicrograph from the grafted urethral junction using caecal graft on 45 POD using caecal graft on (H&E-150), showed gradual regeneration of transitiona uroepithelium.

Figure 8. Photomicrograph from the grafted urethral junction 60 POD (H&E-150) showed well developed transitional uroepithelium.
tissue at the junctional area. On the 45<sup>th</sup> post-operative day (Figure 7), gradual regeneration of transitional epithelium was observed. The lymphocytes and the blood vessels were fewer in number. On the 60<sup>th</sup> post-operative day (Figure 8), the transitional epithelium of the urethra had completely developed and lymphocytes and blood vessels were scare in number. Thus, the result of the study was agreement with the work of Sharma and Khan (1980) who described complete regeneration of uroepithelium over the caecal graft in buffalo on the 75<sup>th</sup> post-operative day during caecocystoplasty. Although both the techniques were successful in buffalo calves, caecourethroplasty was preferred because it was easier and safer to perform.

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*sContinued from page 82*


ABSTRACT

A study was carried out to find out various buffalo management practices adopted by the farmers in Khammam district of Andhra Pradesh. More than half of the farmers under survey (58%) were able to provide insemination or natural service to their animals in heat at the right time, while 42% of farmers were unable to inseminate their animals in time due to various reasons. Only 16.66% farmers utilized the facility of artificial insemination, while 37.51% farmers opted for natural service. About 85.85% farmers fed green and dry roughage in combination and provided clean drinking water to their animals, but none of the farmer practiced silage making or other special treatments like chaffing, soaking or urea treating paddy straw. Most of the farmers collected fodder from the fields. Only 3% farmers fed additional concentrates to pregnant animals; the rest did not do this. As a part of summer management, 51% farmers allowed their buffaloes to wallow in the village tanks during the hotter parts of the day and 49.16% farmers washed their animals by splashing water manually. Washing of the animals and of their udders before milking were practiced by 15.68 and 98.40% farmers, respectively. Non-descript buffaloes were predominant in this region and the majority of the farmers were poor in certain aspects of scientific feeding, breeding, housing, milking and health care practices and needed to be educated. Artificial insemination should be made available to all the farmers, which in turn would result in the upgrading of local buffaloes and improving their performance.

Keywords: buffaloes, breeding, feeding, housing, management, milking

INTRODUCTION

Buffaloes are the backbone of rural economy in many developing countries of the Asian region including India. Buffaloes occupy a prominent place in the social, economic and cultural life of Indian rural communities and are useful as a triple purpose animal for milk, meat and draft power. Dairying with buffaloes in India is a closely interwoven integral part of agriculture. India possess 283 million dairy bovines and stands first in milk production with more than 100 million metric tonnes but the productivity of dairy animals is very much less than in the developed countries.
The performance and productivity of buffaloes in the region appears to be at low level and the factors responsible need greater attention. Productivity of an animal is primarily the product of interaction of its genetic makeup and the environment in which it develops. Therefore, a study was undertaken to assess various management practices such as breeding, feeding, housing, milking practice etc., in Khammam district of Andra Pradesh.

MATERIALS AND METHODS

Two revenue divisions in Khammam district of Andra Pradesh were selected purposively, having highest buffalo population. From each revenue division two mandals and from each mandal five villages were selected. Thus, 20 villages from four mandals of two revenue divisions were selected. From each village six farmers who were rearing buffaloes were selected randomly, giving a sample size of 120 farmers for the study. While conducting survey, the assistance of the local Veterinary Assistant Surgeon was sought.

The selected farmers were interviewed by contacting them at their doorstep utilizing a pre-tested interview schedule developed for the purpose. While collecting data sufficient time was given to the farmer to arrive at values by the memory recall method. The family members of the farmers were also involved in collection of the data so as to get accurate information as far as possible. The information regarding management practices such as breeding, feeding, housing, milking, health care including calf management were collected and data were subjected to appropriate statistical analysis (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Results of buffalo management practices adopted by the farmers in the villages under study are appended in Tables 1, 2, 3, 4 and 5.

General observations

Occupation: It was observed (Table 1) that agriculture was the main occupation among 84.5% of the farmers and dairying was the secondary occupation of 15.5% of farmers, whereas dairying was the main occupation of 15.5% and agriculture was the secondary occupation of 84.5%. Prasad et al. (2001) reported that dairying was the main occupation among 64% of the milk producers and a subsidiary occupation for 36% in urban areas of Andhra Pradesh. Similarly 75% farmers had dairying as secondary occupation in the North east zone of Tamil Nadu (Balusami, 2004). The present study indicated that the majority of the farmers depended on agriculture as the main source of livelihood and also maintained livestock, especially buffaloes, as these enterprises are interdependent on each other. They maintain buffaloes for secondary source of income i.e. through sale of milk or utilizing milk for their home needs. These farmers utilized their agricultural byproducts like paddy, jowar and maize straws and green grasses, either cultivated or natural fodders for their buffaloes, which converted these poor roughages into valuable milk.

Land holding: In the present investigation, it was found that 43.3% farmers possessed between one to three acres of land, 47.5% possessed above three acres of land and 9.2% were landless. Singh and Thomas (1992) and Yadav and Yadav (1995) observed that the land holding was positively associated with the level of adoption of dairy innovations among the respondents. It was observed that, all the farmers irrespective of size
of land holding maintained buffaloes as source of income. The majority of the dairy farmers held land as major source of income.

Fodders cultivation: Among the selected farmers, 45.0% grew fodders and 55% did not cultivate fodder but depended on natural grasses from their own fields, common lands, bunds and other sources. Among the fodder growing farmers, 18.3% grew fodders on an area of about 0 to 0.5 acre and 26.6% on an area of about 0.5 acres and above. These findings are in agreement with the results of Garg et al. (2005) who reported, about 48.43% farmers grew fodders. The results indicated that almost 55% farmers in this study did not grow any fodders and were dependent on either sole grazing or cut natural grasses for feeding of buffaloes. Most of the farmers grew sorghum as a source of fodder, which may have been due to utilisation of agricultural land for growing regular crops during kharif season and sorghum as fodder crop during rabi season because of suitable agro-climatic conditions.

Breeding Management

The present study revealed that, 95% farmers were not able to detect heat in animals (Table 2). This observation was not in agreement with Dwaiapayan et al. (2005), who observed inability of the 6.11% farmers to identify heat symptoms; on the contrary Balusami (2004) reported that 82% farmers were able to detect heat in buffaloes in Tamil Nadu. More than half of the farmers under survey (58%) were able to provide insemination or natural service to their animals in heat at the right time while 42% farmers were unable to inseminate their animals in time due to various reasons. Only 16.66% farmers utilized the facility of artificial insemination, while 37.51% farmers opted for natural service and 45.83% simultaneously had their animals inseminated and also undertook natural service with the expectation that it would increase the conception rate. Breeding bulls or scrub bulls were accessible to all farmers in their villages for natural service. Sawarkar et al (2001) reported that, most of the farmers preferred natural service only due to various reasons. It was also noticed that as many as 75.83% were unable to detect pregnancy diagnosis at 3 months age. Low adoption of artificial insemination by farmers may have been due to the presence of scrub bulls, the distance to artificial insemination centers, lack of faith in artificial insemination, ignorance of farmers, being busy with agricultural operations and non availability of veterinary staff etc.

Feeding Practices

From the present investigation it was observed that only 11.67% farmers fed balanced rations to their animals; the rest of the farmers (88.33%) had not adopted this practice (Table 3). It was found that on an average 9.17, 5 and 85.83% farmers fed only green fodder, dry roughages and both in combination, respectively, and 36.67% farmers fed 1 - 10 kg dry roughages per day while 63.33% farmers fed more than 10 kg. Out of the total selected farmers, the majority (71.67%) fed 1 kg of concentrate mixture as feed supplement, whereas 28.33% of them were feeding 2 kg. Kamboj and Tomar (2000) and Sahu (2001) reported that farmers were not feeding or feeding minimum quantities of concentrates to their buffaloes. About 85.83% of the farmers were feeding green and roughage fodder in combination and provided clean drinking water to their animals, but none of the farmers practiced silage making or any other special treatments like chaffing, soaking or urea treating paddy straw. Most of the farmers collected fodder from the fields. Dwaiapayan et al. (2005) reported that,
59.72% farmers fed poor quality concentrates. The present results clearly indicated that there is a lot of scope for improvement of buffalo performance by better feeding. Only 3.5% farmers fed additional concentrates to pregnant animals; the rest did not. Similarly, 11.25% farmers providing additional concentrates to pregnant animals was reported by Garg et al. (2005) in rural areas of Baran district of Rajasthan. About 13.33% farmers provided a supplementary mineral mixture to their animals while 86.67% did not follow this practice. Garg et al. (2005) also observed that 62.5% farmers did not feed mineral mixture or feed supplements to their dairy animals. The non-feeding of additional supplements to the pregnant animals and of mineral mixture to all animals clearly indicates that productive animals were facing shortages of nutrients which would inhibit their exhibiting their performance. This might have been due to a lack of scientific feeding knowledge among the buffalo farmers, high costs and feed and mineral mixtures or non-availability etc.

**Housing Management**

Among the farmers who provided housing, 60% provided thatched roof sheds, 36.66% provided asbestos roof sheds and 3.34% provided no housing. Among the sheds, 84.17% had _kutchha_ type flooring and 15.83% had _pucca_ type (cement concrete) flooring. The present study is in agreement with the reports of Deoras et al. (2004), who reported similar types of housing for the animals of the majority of farmers. On the contrary, Sohi and Kherde (1980) reported that a large number of dairy farmers had sheds for their animals as they were commercial dairy farmers. The results of the present study do not agree with the findings of the said authors as this study was conducted in rural areas. The majority of the farmers (62.5%) maintained cleanliness in the shed, and the floor space available to the animals of almost all (98.24%) was adequate. Similarly, 94% of the farmers provided proper ventilation for their animals. These findings were similar to the results of Deoras et al. (2004) who reported, 100 percent of the farmers provided adequate floor space and ventilation for their dairy animals in rural areas. Srivastava et al. (2000) also reported that 99.5% the farmers studied kept their animals in well-ventilated houses. It was also found that locally made mangers were used by 83% farmers, and 17% farmers maintained cement mangers. Improper or no mangers may lead to wastage of feed and fodder, which is already in short supply. The majority of farmers (> 94%) provided adequate drainage systems for their animals. It was noticed that application of disinfectants was occasional by most (88.33%) of the farmers, and about 11.67% of them applied disinfectants to their sheds rarely. Similarly, low use of disinfectants was also reported by Lal (1999). One hundred percent of the farmers in the present study had manure pits nearer to their dwellings or farms and dumped solid manure into these pits, which was used by them for agricultural purposes. The reasons for disinfectants were not used might have been a lack of awareness among farmers, a high disinfectant cost, and an additional burden which did not give any immediate return to the farmer. As a part of summer management, 50.84% of the farmers allowed their buffaloes to wallow in village tanks during hotter parts of the day and 49.16% of the farmers washed their animals by splashing water on them manually. Tailor and Pathodiya (2000) reported 4.67% of the farmers they studied in Rajasthan allowed their buffaloes to wallow. Hot and dry climatic conditions and heat intolerance of buffaloes requires summer management like wallowing, sprinkling and splashing of water etc. to improve the performance
of buffaloes during the summer.

**Milking Practices**

In the present study, all the buffalo farmers allowed their calves to suckle their mothers before and after milking twice a day and also used the calf to let down milk from the udder (Table 5). It was observed that 100 percent of the farmers were following a regular milking interval, which is in agreement with the reports of Malik and Nagpaul (1999) wherein 88.88% of the farmers followed a similar practice in Murrah buffaloes in Haryana. In the present study, 88.33, 4.17 and 7.5% of farmers used steel utensils, iron buckets and plastic vessels, respectively. Very few farmers (8%) followed the full hand method of milking; the remainder (92%) followed the knuckling method, which was not a recommended practice. Khupse et al. (1980) reported that 8.18% of the farmers studied had adopted full hand milking, whereas Malik and Nagpaul (1999) reported that 36.11% farmers followed the knuckling method of milking in Haryana. These results indicate lack of awareness among farmers and the urgent need for education on the correct method of milking. Washing of animals before milking and of the udder after milking was practiced by 15.68 and 1.6% farmers, respectively, whereas 76.66% farmers did not follow any sanitary practices before and after milking, and the majority (95%) did not follow any mastitis prevention programme. The majority of the farmers did not wash the entire body of their buffaloes before milking even in the summer (Verma and Sastri, 1994). The practice of not washing the udder after milking might have been due to allowing the calf to suckle after milking, when the calf consumes all the leftover milk and leaves a layer of saliva on the teats, or it might have been a way to save labour.

The present study concludes that management practices had a significant role on the performance of buffaloes in the divisions of Khammam district under study. Non-descript buffaloes were predominant in this region, and majority of the farmers were poor in certain aspects of scientific feeding, breeding, housing and milking practices and need to be educated. Artificial insemination should be made available to all the farmers, and this would result in upgrading of the local buffaloes and improvement in their performance.
Table 1. General information about the selected farmers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Observation</th>
<th>Number of farmers</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Main occupation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agriculture</td>
<td>101</td>
<td>84.50</td>
</tr>
<tr>
<td></td>
<td>Dairying</td>
<td>19</td>
<td>15.50</td>
</tr>
<tr>
<td>2</td>
<td>Subsidiary occupation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agriculture</td>
<td>19</td>
<td>15.50</td>
</tr>
<tr>
<td></td>
<td>Dairying</td>
<td>101</td>
<td>84.50</td>
</tr>
<tr>
<td>3</td>
<td>Land holding (acres)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Landless</td>
<td>11</td>
<td>9.20</td>
</tr>
<tr>
<td></td>
<td>Medium (1-3)</td>
<td>52</td>
<td>43.30</td>
</tr>
<tr>
<td></td>
<td>Large (&gt;3)</td>
<td>57</td>
<td>47.50</td>
</tr>
<tr>
<td>4</td>
<td>Area under fodder crops</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No fodder crops</td>
<td>66</td>
<td>55.10</td>
</tr>
<tr>
<td></td>
<td>0-0.5 acres</td>
<td>22</td>
<td>18.30</td>
</tr>
<tr>
<td></td>
<td>&gt;0.5 acres</td>
<td>32</td>
<td>26.60</td>
</tr>
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</table>

Table 2. Breeding management practices followed by the selected farmers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Breeding management practices</th>
<th>Number of farmers following the practice</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proper heat detection procedures and methods</td>
<td>Adopted</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not adopted</td>
<td>114</td>
</tr>
<tr>
<td>2</td>
<td>Insemination of buffaloes at right time</td>
<td>Followed</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not followed</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>Method of Insemination</td>
<td>Artificial Insemination</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Natural service</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>Availability of breeding bull in village</td>
<td>Yes</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Pregnancy diagnosis at the age of 3 months</td>
<td>Adopted</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not adopted</td>
<td>91</td>
</tr>
</tbody>
</table>
Table 3. Feeding management practices followed by the selected farmers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Feeding management practices</th>
<th>Farmers following the practice</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Feeding balanced ration</td>
<td>Yes</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>106</td>
</tr>
<tr>
<td>2</td>
<td>Feeding dry roughages per day</td>
<td>1-10 kgs</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Above 10 kgs</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>Feeding concentrate supplements</td>
<td>Not feeding at all</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up to 1 kg</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 kg</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>Combination of roughages fed</td>
<td>Dry only</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Green only</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both combined</td>
<td>103</td>
</tr>
<tr>
<td>5</td>
<td>Feeding of silage fodder</td>
<td>Yes</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>Clean drinking water access</td>
<td>Yes</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Special treatment given to feed and fodder</td>
<td>Chaffing</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soaking</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea treated paddy straw</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mixture of green+ dry +concentrate</td>
<td>120</td>
</tr>
<tr>
<td>8</td>
<td>Source of fodder</td>
<td>Home grown</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purchased</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collected from the fields</td>
<td>94</td>
</tr>
<tr>
<td>9</td>
<td>Additional allowance of concentrates during advanced pregnancy and lactation</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>117</td>
</tr>
<tr>
<td>10</td>
<td>Feeding mineral supplements</td>
<td>Yes</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>104</td>
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Table 4. Housing management practices followed by the selected farmers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Housing management practices</th>
<th>No. of farmers</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type of housing</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thatched roof shed</td>
<td>72</td>
<td>60.00</td>
</tr>
<tr>
<td></td>
<td>Asbestos roof shed</td>
<td>44</td>
<td>36.66</td>
</tr>
<tr>
<td></td>
<td>Pucca shed</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>No shed</td>
<td>4</td>
<td>3.34</td>
</tr>
<tr>
<td>2</td>
<td>Type of flooring</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kutcha</td>
<td>101</td>
<td>84.17</td>
</tr>
<tr>
<td></td>
<td>Pucca (Concrete)</td>
<td>19</td>
<td>15.83</td>
</tr>
<tr>
<td>3</td>
<td>Cleanliness in the shed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Satisfactory</td>
<td>75</td>
<td>62.50</td>
</tr>
<tr>
<td></td>
<td>Not satisfactory</td>
<td>45</td>
<td>37.50</td>
</tr>
<tr>
<td>4</td>
<td>Adequacy of floor space</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adequate</td>
<td>118</td>
<td>98.24</td>
</tr>
<tr>
<td></td>
<td>Not adequate</td>
<td>2</td>
<td>1.76</td>
</tr>
<tr>
<td>5</td>
<td>Proper ventilation to the animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Available</td>
<td>112</td>
<td>94.00</td>
</tr>
<tr>
<td></td>
<td>Not available</td>
<td>8</td>
<td>6.00</td>
</tr>
<tr>
<td>6</td>
<td>Type of manger</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cement trough</td>
<td>20</td>
<td>17.00</td>
</tr>
<tr>
<td></td>
<td>Local made trough</td>
<td>100</td>
<td>83.00</td>
</tr>
<tr>
<td>7</td>
<td>Drainage system</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proper</td>
<td>113</td>
<td>94.16</td>
</tr>
<tr>
<td></td>
<td>Not proper</td>
<td>7</td>
<td>5.84</td>
</tr>
<tr>
<td>8</td>
<td>Manure disposal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Manure pit</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Bio gas</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Direct application to the fields</td>
<td>120</td>
<td>100.00</td>
</tr>
<tr>
<td>9</td>
<td>Use of disinfectants</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regular</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Occasional</td>
<td>106</td>
<td>88.33</td>
</tr>
<tr>
<td></td>
<td>Rare</td>
<td>14</td>
<td>11.67</td>
</tr>
<tr>
<td>10</td>
<td>Summer management</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wallowing</td>
<td>61</td>
<td>50.84</td>
</tr>
<tr>
<td></td>
<td>Splashing of water</td>
<td>59</td>
<td>49.16</td>
</tr>
<tr>
<td></td>
<td>Sprinkling of water</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>11</td>
<td>Intervals of the cleaning sheds</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Once in a year</td>
<td>13</td>
<td>10.84</td>
</tr>
<tr>
<td></td>
<td>Occasional</td>
<td>107</td>
<td>89.16</td>
</tr>
</tbody>
</table>
Table 5. Milking management practices followed by the selected farmers.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Milking management practices</th>
<th>Number of farmers following the practice</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Let down of milk</td>
<td>Use of calf 120</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use of oxytocin 0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use of a phantom calf 0</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>Number of times animals are milked</td>
<td>Twice 120</td>
<td>100.00</td>
</tr>
<tr>
<td>3</td>
<td>Interval between each milking</td>
<td>Regular 120</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Irregular 0</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>Type of utensils used for milking and storage</td>
<td>Stainless Steel 106</td>
<td>88.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Iron buckets 5</td>
<td>4.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plastic vessels 9</td>
<td>7.50</td>
</tr>
<tr>
<td>5</td>
<td>Full hand milking</td>
<td>Adopted 10</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not adopted 110</td>
<td>92.00</td>
</tr>
<tr>
<td>6</td>
<td>Sanitary practices followed before and after milking</td>
<td>Yes 28</td>
<td>23.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No 92</td>
<td>76.66</td>
</tr>
<tr>
<td>7</td>
<td>Care against mastitis</td>
<td>Followed 6</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not followed 114</td>
<td>95.00</td>
</tr>
<tr>
<td>8</td>
<td>Washing of animals before milking</td>
<td>Followed 19</td>
<td>15.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not followed 101</td>
<td>84.32</td>
</tr>
<tr>
<td>9</td>
<td>Washing of udder after milking</td>
<td>Followed 2</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not followed 118</td>
<td>98.40</td>
</tr>
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REFERENCES


*Continued on page 119*
ABSTRACT

This study was carried out to determine the prevalence of sub-clinical mastitis, its etiological agents and their antibiogram in Murrah buffaloes at an organized farm. A total of 326 quarter milk samples were screened from 82 apparently healthy buffaloes. The percent prevalence of sub-clinical mastitis was found to be lower on the basis of SCC (>5x10^5/ml) alone (23.17) as compared to cultural examination (29.26). However, the quarter-wise percent prevalence on the basis of SCC (11.04) was similar to bacteriological examination (11.65). On the basis of International Dairy Federation criteria, 7.05% of the quarters (SCC above 500,000/ml of milk and culturally positive), 4.60% quarters (SCC below 500,000/ml of milk but culturally positive) and 3.98% (culturally negative and SCC above 500,000/ml) were found to suffer from sub-clinical, latent and non-specific mastitis, respectively. Out of 38 culturally positive quarters, a total of 44 organisms were recovered. Of these, 15.90% were coagulase positive staphylococci and 47.72% were coagulase negative staphylococci followed by *Streptococcus dysgalactiae* 25%, *Streptococcus agalactiae* 9.09% and *Streptococcus uberis* 2.27%. and 13.63% of the quarters revealed mixed infections with *Staphylococcus* spp. + *Streptococcus* spp. Among Staphylococci, *Staphylococcus aureus* and *Staphylococcus haemolyticus* were the main isolates followed by *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hyicus*, *Staphylococcus pasteuri*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Staphylococcus arlettae* and *Staphylococcus gallinarum*. All the strains of staphylococci and streptococci were found sensitive to cloxacillin, ceftriaxone and cefoperazone. *Streptococci* revealed 100 percent sensitivity towards penicillin, enrofl oxacin, ciprofl oxacin, lincomycin and cephalixin.

**Keywords**: sub-clinical mastitis, Murrah buffalo, prevalence, etiology, antibiogram

INTRODUCTION

In India, the buffalo population is approximately 94 million head. Of the total production of milk, about 53 percent comes from buffaloes and 43 percent from cows. Haryana has the world’s best dairy type buffalo, the Murrah, capable of milk yields as high as 35 kg
a day. In a review on present status of mastitis in buffaloes at periurban dairy farms in India, Joshi and Gokhale (2006) stated mastitis was one of the most important factors in dairy development in the tropics. Sub-clinical mastitis has been reported to be more important (5-20% in buffaloes) than clinical mastitis (1-10) because it is 15-40 times more prevalent than the clinical form, it drastically reduces milk yield, and it usually precedes the clinical form and is usually the basis of herd problems when mastitis outbreaks occur. In India, Dua (2001) has reported annual losses due to mastitis to the tune of Rs 60.5321 billion of which, Rs. 43.6532 billion has been attributed to sub-clinical mastitis. Therefore, the present study was planned to determine the prevalence of sub-clinical mastitis in Murrah buffaloes at an organized farm, to determine the type of organisms responsible for its causation and to determine their antimicrobial sensitivity towards antimicrobials in common use and some of the newer antimicrobials.

**MATERIALS AND METHODS**

**Source of milk samples:** A total of 326 quarter buffalo milk samples were collected from 82 apparently healthy buffaloes of Murrah breed located at an organized farm. Animals which had calved recently (less than two weeks) or those in late lactation (more than nine months) were not included in the study.

**Collection of milk samples:** Milk was collected under aseptic conditions. The udders of cows were cleaned thoroughly with a cloth containing dilute potassium permanganate solution. Hands were properly washed with soap and water and teat apices disinfected with 70 percent alcohol. The first few milk strippings were discarded and 15-20 ml milk sample of each quarter was collected separately in a sterilized test tube. These test tubes were marked as right fore (RF), right hind (RH), left fore (LF) and left hind (LH) and the collection was done first from the near side and then from the off side to avoid contamination of teat apices. Each test tube was given the number possessed by the animal.

**Bacteriological examination:** The prevalence of sub-clinical mastitis was determined following International Dairy Federation Criteria based on bacteriological examination of milk and somatic cell count (SCC).

For bacteriological examination, the milk samples were shaken thoroughly and 0.01 ml of the milk sample was streaked on 5% sheep blood agar and MacConkey’s lactose agar plates. The plates were incubated aerobically at 37°C for 24 to 48 h. Sub-cultures of the resulting growth were made on blood agar for purification of isolates and identified on the basis of Gram’s reaction, morphology and colony characteristics. All the isolates were characterized up to species level following standard bacteriological procedures.

**Somatic cell count:** The SCC on milk samples was performed as described by Schalm et al. (1971) and the milk smears were stained with Newman-Lampert stain (methylene blue-1.2 gm, ethyl alcohol (95%) 54 ml, tetra chloro ethane 40 ml, glacial acetic acid 6 ml).

**Antimicrobial sensitivity testing:**
Different strains of various organisms isolated from udder infections were subjected to in-vitro drug sensitivity testing, using 20 antimicrobials by a disc-diffusion method as suggested by Bauer et al. (1966). The sensitivity was observed on the basis of a zone size interpretation chart provided by the manufacturer. The results were recorded as sensitive, intermediate and resistant.
RESULTS AND DISCUSSION

Results of cultural examination and somatic cell count (SCC) on 326 quarters of 82 buffaloes are presented in Table 1.

Figures in parentheses indicate percentage

The percent prevalence of sub-clinical mastitis was found to be lower on the basis of SCC (>5x10⁵/ml) alone (23.17) as compared to cultural examination (29.26). However, the quarter-wise percent prevalence on the basis of SCC (11.04) was similar to bacteriological examination (11.65).

On the basis of IDF criteria 7.05% of the quarters (SCC above 500,000/ml of milk and culturally positive), 4.60% quarters (SCC below 500,000/ml of milk but culturally positive) and 3.98% (culturally negative and SCC above 500,000/ml) were found to suffer sub-clinical, latent and non-specific mastitis, respectively. Similar prevalence of SCM has been reported by Bansal et al., 1995; In contrast to our study, several workers (Kalorey et al., 1983; Rahman et al., 1983; Tuteja et al., 1999; Maiti et al., 2003 and Chavan et al., 2007) reported high animal-wise and quarter-wise prevalence of SCM. These differences in the prevalence rates of SCM as reported by different workers are perhaps due to difference in managemental and hygienic practices adopted in different dairy herds. The incidence of mastitis varied among farms and the risk increased with increasing parity (Sargeant et al., 1998). Thirunavukarasu and Prabaharan (1998) reported that the incidence of mastitis was significantly associated with animal factors such as breed (Dego and Tareke, 2003), milk yield, stage of lactation (Sharma et al., 2007) and udder morphology, besides farm practices and sanitation. The climatic conditions also affect the prevalence of mastitis (Schultze, 1985). The lower rate of prevalence in the present investigation in comparison to previous studies on the same farm might be attributed to adoption of proper management, hygienic and control measures at the farm.

Tuteja et al. (1999) reported high percent prevalence (26.67) of latent mastitis in comparison to our study. Sereys (1985) and Roder and Gedek (1986) reported that the SCC could be influenced by the type of infecting organisms and season. Thus, a low cell count does not reflect the true bacteriological status of the udder. The significance of latent mastitis cannot be undermined since some of these cases are likely to convert into the sub-clinical form and subsequently into clinical mastitis, particularly under unfavorable environmental conditions. Moreover, latent infection also reflects the possibility of teat canal infections serving as a potential source of infection to the milk secretory tissue. Even mammary parenchyma may be damaged due to liberation of bacterial toxins in the infected teat canal (Nickerson et al., 1986). In comparison to our study, on the same farm Tuteja et al. (1999) observed a high percentage (7.3) of quarters suffering from non-specific mastitis whereas Sindhu et al. (2009) reported a lower percentage (2.19) of quarters having non-specific mastitis. Failure to detect pathogens in such cases might be due to intermittent excretion of the organisms or their disappearance because of spontaneous recovery. Salsberg et al. (1984) observed that somatic cell counts increased more during summer months from June to August in Holstein cows than in cooler months. The possibility of mycoplasmal mastitis cannot be ruled out in such cases, since the organism cannot be cultivated on common bacteriological media.

The relative frequency of various micro-organisms from the apparently healthy milk quarters
including 11 quarters harboring mixed infection is given in Table 2.

Out of 38 culturally positive quarters, a total 44 organisms were recovered. Of these, 15.90% were coagulase positive staphylococci and 47.72% were coagulase negative staphylococci followed by *Streptococcus dysgalactiae* 25%, *Streptococcus agalactiae* 9.09%, and *Streptococcus uberis* 2.27% and 13.63% quarters revealed mixed infections with *Staphylococcus* spp. + *Streptococcus* spp.

All the CPS and CNS were further characterized to species level. Details are given in Table 3. *Staphylococcus aureus* and *Staphylococcus haemolyticus* were the main isolates followed by *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hyicus*, *Staphylococcus pasteuri*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Staphylococcus arlettae* and *Staphylococcus gallinarum*. Results indicated substantial differences in the prevalence of pathogens among different herds. In our study contagious bacteria like staphylococci and *Streptococcus agalactiae* caused most of the infections. Such infections are usually spread from animal to animal at the time of milking. The mastitis situation can be improved by improving milking practices and hygiene. Our findings are in close agreement with those of Bansal et al. (1995) and Petzer et al. (2009) who reported isolation of CNS, *Streptococcus agalactiae* and *Streptococcus uberis*. Rani et al., 2008 reported that the prevalence of mastitis varies with breed, age, lactation and season. Amongst various mastitogenic bacteria isolated, staphylococci were the most prevalent, accounting for 63.62 percent of the infections, followed by streptococci (36.36 percent), respectively. Similar findings were reported in India by Babu et al. (1983), Sharma and Kapur (2000) and Bulla (2002). The high prevalence of staphylococci has been reported by several workers in India (Kalorey et al., 1983; Javed and Siddique, 1999; Tijare et al., 1999; Tuteja, 1999; Kaya et al., 2000; Sharma et al., 2007) and abroad (Hawari and Dabas, 2008; Tenhagen et al., 2009 and Nickerson and Stephen, 2009). The prevalence of a pathogen is influenced by parity, type of sample and season (Sharma et al., 2007; Hagnestan et al., 2009). Distribution of pathogens changes over time; therefore, bacteriological examination at the herd level must be taken regularly to monitor udder health.

Similar to our findings, other workers from India have also reported staphylococci and streptococci to be the main etiological agents of mastitis in different parts of the country (Chavan et al., 2007; Sharma and Sindhu, 2007; Behera et al., 2008; Palanivel et al., 2008; Roychoudhary and Dutta, 2009; Sindhu et al., 2010). Among staphylococci, *Staphylococcus aureus* and *Staphylococcus haemolyticus* were found to be the most prevalent followed by *Staphylococcus epidermidis*. Many workers have found *Staphylococcus aureus* to be more prevalent than *Staphylococcus epidermidis* (Char et al., 1983; Saini et al., 1994; Armenteros et al., 2006; Unnerstad et al., 2009). Contrary to this, several workers (Chavan et al., 2007; Ferguson et al., 2008; Petzer et al., 2009; Tenhagen et al., 2009; Sampimon et al., 2009) reported high prevalence of coagulase negative staphylococci (CNS). These findings show the increasing importance of CNS, which were formally described as a minor pathogen in the case of mastitis. Since, in the veterinary field, our attention has been mainly directed towards coagulase-positive staphylococci, the potential of pathogenicity of CNS remains un-elucidated. Moreover, *Staphylococcus epidermidis* is supposed to be normal flora of teat skin, its higher prevalence, as observed in this study, might
Table 1. Prevalence of sub-clinical mastitis in 326 quarters of 82 buffaloes at an organized farm.

<table>
<thead>
<tr>
<th></th>
<th>Buffaloes culturally positive</th>
<th>Buffaloes showing SCC &gt; 5 lac/ml</th>
<th>Quarters culturally positive</th>
<th>Quarters showing SCC &gt; 5 lac/ml</th>
<th>SCC &gt; 5 lac/ml and culturally positive</th>
<th>SCC &lt; 5 lac/ml and culturally positive</th>
<th>SCC &gt; 5 lac/ml and culturally negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 (29.26)</td>
<td>19 (23.17)</td>
<td>38 (11.65)</td>
<td>36 (11.04)</td>
<td>23 (7.05)</td>
<td>15 (4.60)</td>
<td>13 (3.98)</td>
</tr>
</tbody>
</table>

Table 2. Frequency of isolation of different organisms.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Number (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase positive staphylococci</td>
<td>7 (15.90)</td>
</tr>
<tr>
<td>Coagulase negative staphylococci</td>
<td>21 (47.72)</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>11 (25.00)</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>4 (9.09)</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>1 (2.27)</td>
</tr>
</tbody>
</table>

Table 3. Characterization of staphylococci isolated from buffalo milk.

<table>
<thead>
<tr>
<th>Coagulase test</th>
<th>Sr. No.</th>
<th>Organisms</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase positive staphylococci</td>
<td>1.</td>
<td><em>Staphylococcus aureus</em></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td><em>Staphylococcus hyicus</em></td>
<td>2</td>
</tr>
<tr>
<td>Coagulase negative staphylococci</td>
<td>1.</td>
<td><em>Staphylococcus hyicus</em></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3.</td>
<td><em>Staphylococcus hominis</em> subsp. hominis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4.</td>
<td><em>Staphylococcus pasteuri</em></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.</td>
<td><em>Staphylococcus arlettae</em></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6.</td>
<td><em>Staphylococcus haemolyticus</em></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>7.</td>
<td><em>Staphylococcus saprophyticus</em> subsp. saprophyticus</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8.</td>
<td><em>Staphylococcus gallinarum</em></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9.</td>
<td><em>Staphylococcus simulans</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>TOTAL</td>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>
be a consequence of unhygienic milking practice, due to which the organisms gained access into mammary gland through milkers’ hands, causing an increase in SCC and inflicting pathogenicity in the alveolar tissue. Further studies are required on role of *Staphylococcus haemolyticus* in causing mastitis.

In the current study, amongst streptococcal isolates, *Streptococcus dysgalactiae* were the predominating organisms (25 percent) followed by *Streptococcus agalactiae* (9.09 percent) and *Streptococcus uberis* (2.27 percent). The higher prevalence of *Streptococcus dysgalactiae* than *Streptococcus agalactiae* was reported by Kalra and Dhanda (1964) and Tuteja (1999). In contrast to our study Hameed *et al.*, 2007; Chavan *et al.*, 2007; Getahun *et al.*, 2008; Ferguson *et al.*, 2008, reported higher prevalence of *Streptococcus agalactiae* than *Streptococcus dysgalactiae*, whereas other workers (Javed and Siddique, 1999; Sampimon *et al.*, 2009) found higher prevalence of *Streptococcus uberis* than that recorded in this study. Our findings are in close agreement with Petzer *et al.* (2009) who reported isolation of CNS, *Streptococcus agalactiae* and *Streptococcus uberis*.

A good amount of literature is available on the antibiogram of different mastitogens. It is not possible to compare our results with their

**Table 4. Antibiogram of different organisms isolated.**

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Percent sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococci (28)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>72.2</td>
</tr>
<tr>
<td>Penicillin</td>
<td>88.8</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>50</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>83.3</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>88.8</td>
</tr>
<tr>
<td>Neomycin</td>
<td>66.6</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>100</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>94.4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>83.3</td>
</tr>
<tr>
<td>Amikacin</td>
<td>50</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>94.4</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>100</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>100</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>83.3</td>
</tr>
<tr>
<td>Colistin</td>
<td>44.4</td>
</tr>
<tr>
<td>Co-Triamoxazole</td>
<td>94.4</td>
</tr>
<tr>
<td>Nitrofurantion</td>
<td>94.4</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>94.4</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>94.4</td>
</tr>
</tbody>
</table>
findings. While considering overall sensitivity, all the strains of staphylococci and streptococci were found sensitive to cloxacinil, ceftriaxone and cefoperazone. Streptococci revealed 100 percent sensitivity towards penicillin, enrofloxacine, ciprofloxacine, lincomycine and cephalxin. Similar to our study, Ranjan et al. 2010 also found high sensitivity towards Enrofloxacine (91.67%) whereas they observed lower sensitivity towards Ceftriaxone (84.10%). It was interesting to note that staphylococci isolates revealed 100 percent sensitivity towards tetracycline. Studies conducted by several workers (Sharma et al., 2007; Chavan et al., 2007; Roychoudhury and Dutta, 2009; Sharma et al., 2010) have showed increased resistance towards different traditional and newly introduced antibiotics. In contrast to these studies, the antibiogram obtained in the current study indicated high sensitivity towards newer and older antibiotics, showing rational use of these antibiotics at farms under study. Antibiotic resistance patterns vary among different farms, regions, states and countries depending upon the type of organisms and use of antibiotics in a particular area; therefore, antimicrobial sensitivity is suggested before institution of treatment. The information obtained by this study will also be of useful to the dairy industry and individual farmers. It will be helpful in prioritizing mastitis control efforts.

ACKNOWLEDGEMENT

The technical help rendered by Sh. Bhupinder Singh and Sh. Randhir Singh of the Veterinary College Central Laboratory, COVS, LLRUVAS, is gratefully acknowledged.

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ABSTRACT

This experiment was carried out to study the effect of yeast culture in the diet on rumen microbial population in buffalo bulls. In a cross over design, six graded Murrah buffalo bulls (254.4±7.98 kg) were randomly divided into two groups of three animals each. The animals in both the groups were offered 1.5 kg concentrate mixture and had access to Hybrid Napier fodder ad libitum. In the treatment group, the concentrate mixture was supplemented with yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) at the rate of 0.5 g/animal/day. The results indicated that supplementation of the diet with yeast culture increased significantly the mean protozoal count (P<0.05) and the total bacterial count (P<0.01) in SRL of graded Murrah buffalo bulls as compared with the control group.

Keywords: yeast culture, protozoal count, bacterial count, buffalo bulls

INTRODUCTION

For many years, ruminant nutritionists and microbiologists have been interested in manipulating the microbial ecosystem of the rumen to improve production efficiency of domestic ruminants. Yeast culture has displayed positive impact on the growth and viability of rumen microflora and the fermenting process in the rumen. But, the results have been inconsistent due to confounding effects of the ration composition, and variations in the strain of the yeast culture and their administration protocol. Addition of Saccharomyces cerevisiae live yeast cultures to ruminant diets has improved fibre digestibility and stimulated cellulolytic bacteria (Dawson et al., 1990), increased protozoal count (Singh et al., 2008) while in other studies no changes in ruminal protozoa (Corona et al., 1999) were observed by the addition of yeast culture to the diet. Therefore, the present experiment was carried out to study the effect of yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) on the rumen microbial population in buffalo bulls.
MATERIALS AND METHODS

In a crossover design, six graded Murrah buffaloes (254.4±7.98 kg), each fitted with a permanent rumen cannula, were randomly divided into two groups (Control and Treatment) of three animals each. Animals in both the groups were offered 1.5 kg concentrate mixture and had access to Hybrid Napier (CO-1 variety) fodder ad libitum to meet the nutrient requirements (ICAR, 1998). In the treatment group, the concentrate mixture was supplemented with yeast culture (Levucell SC 20; Saccharomyces cerevisiae CNCM I-1077) at the rate of 0.5 g/animal/day. The experiment lasted for 60 days and the crossover was made after 30 days of feeding. Rumen liquor was collected from the bulls at 0, 2, 4 and 6 h post feeding and strained through four layers of muslin cloth and was referred to as strained rumen liquor (SRL). The protozoal count in SRL was determined as per Kamra et al. (1991), while the total bacterial count was determined as per Gall et al. (1949).

Statistical analysis of the data was carried out as per the procedures suggested by Snedecor and Cochran (1976).

RESULTS AND DISCUSSION

The DMI expressed as kg / 100 kg b.wt was 3.29 kg and 3.44 kg in control and treatment groups, respectively. Supplementation of the diet with yeast culture increased the DMI (kg / 100 kg b. wt.) but the difference is not significant (P>0.05).

Protozoal Count: The mean total protozoal count (x10^4 / ml of SRL) in the yeast-culture supplemented (YS) group (21.50±1.48) was higher (P<0.05) than in the control (15.42±1.36). Many studies have been conducted to study the effect of yeast cultures on protozoa populations. In some cases (Newbold et al., 1995; Yoon and Stern, 1996) the yeast culture did not modify total protozoa count while in other studies (Plata et al., 1994; Kumar et al., 1994; Singh et al., 2008), the total protozoal count increased.

Time of sampling had significant (P<0.01) effect on the protozoal and bacterial counts in SRL.

Table 1. Effect of dietary supplementation of yeast culture on rumen microbial population in buffalo bulls.

<table>
<thead>
<tr>
<th>Parameter/ Treatment</th>
<th>Time of rumen liquor sampling (h)</th>
<th>Treatment average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Protozoal Count (x10^4 / ml of SRL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.67±1.20</td>
<td>17.00±1.46</td>
</tr>
<tr>
<td>YS</td>
<td>16.33±1.54</td>
<td>23.33±1.89</td>
</tr>
<tr>
<td>Overall*</td>
<td>13.50±1.26</td>
<td>20.17±1.49</td>
</tr>
<tr>
<td><strong>Total Bacterial Count (x10^9 / ml of SRL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.77±0.28</td>
<td>7.96±0.24</td>
</tr>
<tr>
<td>YS</td>
<td>9.13±0.29</td>
<td>14.07±0.42</td>
</tr>
<tr>
<td>Overall*</td>
<td>7.45±0.54</td>
<td>11.02±0.95</td>
</tr>
</tbody>
</table>

a, b, cMeans with different superscripts in a row and column differ significantly.
*(P<0.05) ***(P<0.01)
effect on the total protozoal count. The total protozoal count was the highest (25.83±1.44) at 4 h post-feeding. It increased (P<0.01) from 0 h to 4 h post-feeding, and then declined (Table 1). Feed offer was associated with an abrupt increase in the total protozoal population within the first 4 h of post-feeding, which could be due to migration of protozoa from the reticulo-ruminal wall where they sequester from the rumen medium in response to chemical stimuli originating from the diet. The migration of protozoa into rumen liquor was due to chemotactic movement towards the feed entering the rumen. After the feed was utilized, the protozoa gradually migrated back to the reticulo-ruminal wall after 6 h post-feeding resulting in the observed drop in their numbers in the rumen liquor in both groups. Further, Iqbal et al. (1993) reported that irrespective of diets, the protozoal number increased after feeding and reached a peak at 4 h post feeding, thereafter, the concentration decreased gradually. This increase in protozoal count after feeding may be attributed to availability of substrate for protozoal growth. These results corroborated with those of Singh et al. (2008) in buffalo calves.

**Bacterial Count:** The mean total bacterial count (x10⁹/ml of SRL) in the YS group (14.46±0.44) was higher (P<0.01) than the control (8.67±0.27). The increase in the total bacterial count in the YS group may be attributed to the positive effect of the yeast culture. These results were in agreement with the findings of Rita Sharma et al. (1988), Dolezal et al. (2005) and Kowalik et al. (2008).

Time of sampling had a significant (P<0.01) effect on the total bacterial count. The total bacterial count concentration was the highest (14.78±1.11) at 4 h post-feeding. It increased (P<0.01) from 0 h to 4 h post-feeding, and then declined (Table 1). These results corroborated with those of Singh et al. (2008) in buffalo calves. The probable reason for increased rumen microbial numbers after yeast culture supplementation could be assigned to the capacity of yeast to remove oxygen from the rumen.

Thus, it can be concluded that supplementation of yeast culture (Levucell SC 20) 0.5 g/animal/day in the diet of graded Murrah buffalo bulls increased the microbial population in the rumen.

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*Continued from page 106*


ABSTRACT

The aim of this study was to detect early pregnancy and early embryonic mortality using transrectal ultrasonography in Mehsana buffaloes. The study was carried out on 18 postpartum anestrus Mehsana buffaloes of a dairy farm during breeding season, using different estrus synchronization protocols, followed by fixed time AI. Ultrasound examinations were performed using a real time B-mode ultrasound scanner (ALOKA SSD - 500) equipped with 5 MHz linear array transducer on day 26 and 40 post AI. Ultrasound scanning on day 26 post AI revealed visualization of embryonic vesicle around the conceptus. The embryo proper was observed in nine out of 13 animals (69.23%) on day 26 and all 12 conceived animals (100%) on day 40 post AI. The amniotic and allantoic vesicles were observed as non-echogenic cavities closely surrounding the embryo proper on day 40 post AI. Ovarian corpus luteum and foetal heart beats were observed in conceived buffaloes on both 26 and 40 days post AI. Early embryonic death occurred in one buffalo each between days 26 and 40 and between day 40 and 60 post AI. The overall diagnostic accuracy of ultrasonography for early pregnancy was recorded as 94.44% and 100% on day 26 and day 40, respectively. Plasma progesterone concentrations ranged from 3.00-6.02 ng/ml with an average of 4.42±0.52 ng/ml in pregnant animals and from 0.97-1.91 ng/ml with an average of 1.22±0.06 ng/ml in nonpregnant animals on day 20 post AI. It was concluded that ultrasound scanning is a useful tool in detection of early pregnancy, early non-pregnancy and early embryonic mortality in buffaloes.

Keywords: ultrasonography, early pregnancy diagnosis, Mehsana buffaloes

INTRODUCTION

Livestock production in general and milk production in particular play an important role in our national economy and thus dairying is one of the most important agricultural activities in India. Buffaloes are the major contributor to milk production in our country, especially in Gujarat state. Conditions like early embryonic mortality, early fetal death and unobserved abortions due to the tropical environment lead to an increased...
anestrus period and thereby increase the costly calving interval. To overcome such problems, reproductive ultrasonography may serve as a better tool.

The use of ultrasonography has been increasing as an imaging modality in bovine reproduction. Its use can provide solutions to a number of unanswered questions in dealing with the bovine reproductive cycle and its concurrent disorders, including early pregnancy diagnosis. Ultrasonographic observation of the postpartum changes in female reproductive tract, early pregnancy diagnosis by reproductive ultrasonography and detection of early embryonic mortality are some of the efficient means of improving domestic animal reproduction and production and help in fulfilling the basic necessities of man in the day-to-day world.

Ultrasound pregnancy diagnosis is a reliable method of determining the presence of a conceptus and viability of an embryo, which is essential to increase the profitability of the animal (Tiwari et al., 2002). The present study was conducted to detect early pregnancy and early embryonic mortality by transrectal ultrasonography in oestrus induced postpartum anestrus Mehsana buffaloes.

**MATERIALS AND METHODS**

A total of 18 postpartum anestrus Mehsana buffaloes of a private dairy farm at Vadodara, Gujarat were subjected to different estrus synchronization protocols and fixed time AI with good quality frozen semen. The buffaloes not returning to estrus were subjected to ultrasonographic scanning for early pregnancy diagnosis on days 26 and 40 post AI and finally pregnancy was confirmed by rectal examination on day 60 post AI.

The ultrasound examinations were performed using a transrectal B-mode ultrasound scanner (ALOKA SSD - 500, SN - M10408. Japan) equipped with a 5 MHz linear array transducer designed for intra-rectal placement. The scanning of uterine horns was carried out on their dorsal and lateral surfaces. Ovaries were also scanned for the presence of a corpus luteum and/or any other palpable structure. The images displayed were thoroughly examined and frozen on the screen and the hard copies (sonograms) were taken using a video graphic thermal printer (Sony, UP-895 MDW, Japan).

Blood samples were collected twice from all the buffaloes for estimation of plasma levels of progesterone, i.e. on the day of AI and on day 20 post AI. Plasma progesterone concentrations were estimated by employing the standard radio-immuno-assay (RIA) technique of Kubasic et al. (1984). Labeled antigen (I^{125}), antibody coated tubes and standards were procured from Immunotech - SAS, Marseille Cedex, 9, France. The sensitivity of assay was 0.1 ng/ml. Intra-assay coefficient of variation was 5.4 percent and inter-assay variation was 9.1 percent. Cross reactivity of the antibody with progesterone, 17α-dihydroprogesterone and 20α-hydroxyprogesterone was 100, 0.13 and 0.96 percent, respectively. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated based on findings of ultrasound scanning on different days (day 26 and 40 post AI) and those of plasma progesterone concentrations on day 20 post AI in relation to findings of pregnancy diagnosis by rectal palpation on day 60 post AI.
RESULTS AND DISCUSSION

The results of ultrasound scanning and progesterone assay were correlated with the findings of palpation per rectum on day 60 post AI with respect to accuracy and reliability of these two tests. Although the sensitivity on days 26 and 40 and the specificity on day 40 of ultrasound scanning was 100 percent, the specificity was lower on day 26 being 80 percent. On day 26 one animal was incorrectly diagnosed pregnant. However, the progesterone assay revealed two animals having been diagnosed incorrectly pregnant. The sensitivity, specificity, positive predictive value, negative predictive value and overall diagnostic accuracy of progesterone assay were comparatively lower as compared to ultrasound scanning results on days 26 and 40 (Table 1).

Developmental profile of embryonic vesicle and embryo

The scanning of recently bred buffaloes through B-mode transrectal ultrasonography on day 26 allowed the visualization of non-echogenic embryonic vesicle around the conceptus (Figure 1). The embryo proper was observed on day 26 post AI, however, it was observed in only nine out of 13 (69.23%) buffaloes. Thereafter, the embryo proper was observed in all 12 conceiving animals (100%) on day 40 post AI (Figure 2). The amniotic and allantoic vesicles were observed as non-echogenic cavities closely surrounding the embryo proper on day 40 post AI. These cavities were surrounded by large anechoic area i.e. embryonic fluid. The scanning of ovaries revealed the presence of a pregnancy corpus luteum in conceiving buffaloes on both the days i.e. day 26 and 40 post AI. The heartbeats were observed as a pulsatile flickering

Table 1. Accuracy of ultrasonographic and P₄ assay for early pregnancy diagnosis in Mehsana buffaloes on different days after insemination.

<table>
<thead>
<tr>
<th>Diagnostic results/Predictive values</th>
<th>Days of ultrasound scanning</th>
<th>Plasma P₄ assay by RIA†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Diagnosis pregnant correct (a)</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Diagnosis pregnant incorrect (b)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Diagnosis nonpregnant correct (c)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Diagnosis nonpregnant incorrect (d)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity (%) 100 x a/(a + d)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity (%) 100 x c/(c + b)</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 x a/(a + b)</td>
<td>92.85%</td>
<td>100%</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 x c/(c + d)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Overall diagnostic accuracy (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 x (a + c)/(a + b + c + d)</td>
<td>94.44%</td>
<td>100%</td>
</tr>
</tbody>
</table>

(†) RIA performed at day 20 post-breeding.
Figure 1. Sonogram on day 26 post-insemination.
   a) Conceptus and
   b) Fluid filled embryonic vesicle

Figure 2. Sonogram on day 40 post-insemination.
   a) Embryo proper and
   b) Fluid filled embryonic vesicle
at a faster rate in the centre of the embryo proper within the embryonic vesicle on day 26 and 40 post AI. However, heartbeats were observed in only 10 out of 13 animals on day 26 post AI and in all 12 animals on day 40 post AI. The mean heart rate was 174.60±0.81 beats per minute on day 26 post AI, which decreased to 165.28±1.4 on day 40 post AI. These findings were in accordance with the findings of many workers (Rosiles et al., 2005 and Agarwal et al., 2007).

In the present study it was possible to clearly visualize the embryonic vesicle in all animals and embryo proper in nine out of 13 animals on day 26 post AI. Moreover, both the embryonic vesicle and the embryo proper were detected in all pregnant animals on day 40 post AI. Thus, ultrasonography facilitates diagnosis of all nonpregnant animals at an early stage (day 26 post AI) and is really more advantageous than pregnancy diagnosis by rectal palpation method, wherein 100 percent reliable results cannot be obtained at such an early stage of pregnancy. Agarwal et al. (2007); Ali and Fahmy (2008) reported similar findings in buffaloes. The results of the present study showed that transrectal ultrasound scanning of buffaloes on day 26 post AI was less accurate than on day 40 post AI.

The sensitivity of ultrasound scanning was 100 percent on days 26 and 40 post AI (Table 1). The specificity of ultrasound scanning was 80.00 and 100 percent on days 26 and 40 post AI, respectively. The positive predictive value (PPV) of the ultrasound scanning was 92.85 and 100 percent on day 26 and 40 post AI, respectively. The negative predictive value (NPV) of the ultrasound scanning was 100 percent on both the days i.e. day 26 and 40 post AI. The overall diagnostic accuracy in the present study was recorded as 94.44 and 100 percent on days 26 and 40, respectively. Similar findings were recorded by Bhosreker and Hangarge (2000); Rosiles et al. (2005). They observed 97.90 and 100 percent accuracy in detecting pregnancy through ultrasonography, respectively. The possible explanation for higher overall diagnostic accuracy in the present study might be because ultrasound scanning was carried out on days 26 and 40 post AI with proper restraining of the animals.

Early embryonic mortality and/or early fetal death

In one animal, ultrasound scanning revealed the presence of a conceptus inside the fluid filled embryonic vesicle on day 26 post AI. However, ultrasonography on day 40 post AI revealed the same animal as nonpregnant as evident by the absence of a conceptus and resorption of embryonic fluid; suggestive of early embryonic death between days 26 and 40 post AI. In one buffalo, ultrasound scanning revealed presence of a conceptus and an embryonic vesicle on both the occasions, i.e. days 26 and 40 post AI. Later on, palpation per rectum at day 60 post AI revealed the animal as nonpregnant, suggestive of early embryonic or fetal death between days 40 and 60 post AI. Similar findings were recorded by Campanile et al. (2005); Vecchio et al. (2007).

Hormone profile

In the present study, plasma progesterone concentrations ranged from 3.00-6.02 ng/ml with an average of 4.42±0.52 ng/ml in pregnant animals on day 20 post AI. In nonpregnant animals plasma progesterone concentrations ranged from 0.97-1.91 ng/ml with an average of 1.22±0.06 ng/ml on day 20 post AI. These findings were in close accordance with the findings of Muhammad et al. (2000).

The findings of the present study indicate that ultrasound scanning of freshly bred animals is helpful in detection of early pregnancy, early
nonpregnancy and early embryonic deaths (EED) in buffaloes. The detection of early pregnancy in buffaloes may be accomplished on or after day 26 post AI through ultrasound scanning with positive predictive value and accuracy over 92 percent and the results are instantly available.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Ravi Shetty, owner of Geeta Dairy Farm, Vadodara for providing buffaloes for this research purpose and for his kind cooperation.

REFERENCES


ABSTRACT

Protease inhibitor (PI) genes belong to the superfamily of serine protease inhibitors that includes C1 esterase, antithrombin, and α1-antichymotrypsin, among others. The primary role of PI is to protect tissues against proteolytic digestion by neutrophil elastase. PI is also found to be associated with increased milk and fat yields, increased productive life and decreased somatic cell score. The present study was carried out to reveal PCR-RFLP polymorphism of protease inhibitor exon 2 fragment in Mehsana buffaloes. The buffaloes were registered under the progeny testing programme of the Dudhsagar Research and Development Association, Mehsana, Gujarat state (India). The fragment of 448 bp of PI gene (exon 2) locus was amplified by PCR and subsequently digested with restriction enzyme SfaN1. It revealed monomorphic patterns. Further representative samples were cloned and sequenced. On comparison with published cattle sequences, the nucleotide sequence variation between cattle and buffalo was present at ten nucleotide positions, i.e., 110, 141, 146, 170, 236, 257, 277, 306, 350, and 396.

Keywords: Mehsana buffaloes, Bubalus bubalis, PCR-RFLP, PI gene, protease inhibitors, gene polymorphism

INTRODUCTION

The present study aimed at exploring polymorphism in the PI gene by PCR-RFLP sequencing and its association with production traits in the Mehsana buffalo. Very scanty information is available on PI gene polymorphism in Indian buffaloes (Bubalus bubalis). It has been reported that the PI gene is a candidate gene for production traits in dairy cattle (Khatib et al., 2005). The candidate gene approach consists of the study of different genes potentially involved in physiological processes (e.g., milk protein synthesis and milk fat synthesis) and the identification for each gene of the allele responsible for the desired phenotype.

The protease inhibitor (PI) genes belong to the superfamily of serine protease inhibitors that includes C1 esterase, antithrombin and α1-antichymotrypsin among others (Barra et al., 1987; Anderson and Kingston, 1983; Blank and Brantly, 1994). Khatib et al. (2005) reported five SNP in coding regions of the protease inhibitor (PI) gene by direct sequencing of reverse transcription-polymerase chain reaction products from a wide range of cattle tissues. A total of six different intragenic haplotypes were identified in the North American Holstein population, and these were examined for associations with milk production traits in 24 half-sib families comprising 1007 sons.
utilizing a granddaughter design. One common haplotype was associated with increased milk and fat yields, increased productive life and decreased somatic cell score. Another common haplotype was associated with decreased productive life and increased somatic cell score. One rare haplotype was associated with decreased milk, fat, and protein yields and increased milk protein percentage; another rare haplotype was associated with decreased milk yield, increased protein percentage, and decreased productive life.

MATERIALS AND METHODS

Buffalo population, sampling and DNA extraction

To analyze PI/SfaNI polymorphism, blood samples were collected randomly from 60 unrelated Mehsana buffaloes registered under the progeny testing programme of the Dudhsagar Research and Development Association, Mehsana, Gujarat state. DNA was extracted using a standard protocol by phenol: chloroform extraction procedure (Sambrook et al., 1989).

Molecular genotyping

A primer pair reported by Khatib et al. (2005) F: ATG GCA CTC TCC ATC ACG CG, R: CCA CTA GCT TTG CAC TCT CA was used to amplify the 448 bp region of PI exon 2.

PCR was carried out in a final reaction volume of 25 µl. Amplification cycling conditions for PI involved initial denaturation at 94°C for 10 minutes, followed by 35 cycles at 94°C for 1 minute, 56°C for 45 seconds and 72°C for 1 minute with a final extension at 72°C for 10 minutes) (Figure 1).

For the PCR-RFLP analysis, 10 µl of each PCR amplified product was digested with 5 units of the SfaNI 5’-G C A T C (N)₅^-3’ in a 30 µl total reaction and incubated in a water bath at 37°C for 16 h. The digestion products were separated by electrophoresis on a 2% agarose gel in 0.5 X TBE buffer.

Cloning and sequencing

PCR products from representative sample (448 bp) were purified and cloned in pTZ57R/T vector. Ligated recombinant vector was transformed in competent E. coli (DH5-α) cells. Recombinant plasmids were extracted and used for cycle sequencing and were subjected to automated DNA sequencing.

Sequence data obtained were analyzed in silico by employing software tools, viz. NCBI BLAST, SeqScape and ClustalW, to access the genetic variation.

RESULTS AND DISCUSSION

On screening the PI/SfaNI in the 60 Mahsana buffalo individuals, all the samples showed an identical restriction pattern with RE site at 242 bp and 366 bp on both chromosomes resulting in the appearance of three bands of 242 bp, 124 bp, 82 bp with BB genotype (Figure 2). This result indicates that there was no SfaNI polymorphism at the PI locus in Mehsana buffalo. Allele B for PI is fixed in Mehsana buffaloes. As there is no polymorphism association, studies for production traits were not undertaken.

The RFLP results are not in accordance with Khatib et al. (2005) as there was no SfaNI polymorphism at PI locus in Mehsana buffalo. Khatib et al. (2005) reported five SNP in coding regions of the protease inhibitor (PI) gene. Published reports for PI/ SfaNI polymorphism in buffaloes were not available to compare with till preparation of this manuscript.
Figure 1. PCR product of protease inhibitor exon 2 locus.
Lane 1, 2, 4, 5: 448 bp PI gene product.
Lane 3: 100 bp ladder.

Figure 2. PI exon 2 -SfaNI RFLP: 448 bp PCR fragment in Mehsani buffalo digested by SfaNI and electrophoresed on 2% agarose in 0.5 X TBE at 80 V.
Lanes 1,2: PI /SfaNI digest with three fragments of 242 bp, 124 bp, 82 bp.
Lane 3: PCR product of 448 bp.
Plate 1. Clustal W results of PI exon 2.
Representative samples from Mehsana buffaloes for PI locus were sequenced and forward and reverse sequences were assembled along with reference sequences on SeqScape software. A consensus sequence of 426 bp was obtained. Consensus sequence was then aligned with published in GenBank for PI gene exon-2 in cattle using NCBI BLAST, ClustalW programme revealed strong homology of 94 % to 98 % (Table 1). There was no sequence available in Genbank database for buffaloes until this manuscript was prepared to compare with. The nucleotide sequence variation between cattle and buffalo was present at ten nucleotide positions, i.e., 110, 141, 146, 170, 236, 257, 277, 306, 350, and 396 (Plate 1).

PI (exon2) nucleotide sequences were submitted to NCBI Genbank database accession number GQ385225.

REFERENCES


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Table 1. *Bubalus bubalis* Protease Inhibitor - Blastn in GenBank + EMBL DDBJ.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Accession</th>
<th>Description</th>
<th>Location/Source</th>
<th>Max Indent</th>
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<tbody>
<tr>
<td>1</td>
<td>BT025459.1</td>
<td>Bos taurus serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (SERPINA1), mRNA, complete cds</td>
<td>1-426 (164-589)</td>
<td>97%</td>
</tr>
<tr>
<td>2</td>
<td>BC102730.1</td>
<td>Bos taurus serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1, mRNA (cDNA clone MGC:127781 IMAGE:30957372), complete cds</td>
<td>1-426 (72-497)</td>
<td>97%</td>
</tr>
<tr>
<td>3</td>
<td>X 63129.1</td>
<td>Bos taurus mRNA for alpha-1-antitrypsin</td>
<td>1-426 (55-480)</td>
<td>97%</td>
</tr>
</tbody>
</table>
ABSTRACT

This study aims to compare the Sox-2 gene expression in stem cells derived from various stages of in vitro produced buffalo embryos. Primers were designed based on the Sox-2 sequence (NCBI Ac. No: DQ487021.1) of Chinese swamp buffalo available in Pubmed GenBank by a Web-based primer3 designing programme to obtain a product of 413bp. For zona-analysis and subsequent isolation of ES cells 0.5 percent pronase was used. The DNA sequence of the RT-PCR product submitted to NCBI Pubmed GenBank was given accession number: EU661361. Strong Sox-2 expression was observed in the inner cells obtained from 16-cell stage embryos, morulae and inner cell masses of blastocyst. Out of six trials, in two trials the blastomeres/inner cells of 2-cell, 4-cell and 8-cell stage embryos did not express Sox-2 gene even though they were believed to be totipotent. But in four trials a faint band was observed. The Sox-2 gene expression pattern was low and variable in stem cells derived from early embryos but gradually became more regular, with 100 percent expressing Sox-2 from the 16-cell stage onward. This might be related to the exhaustion of maternally generated Sox-2 transcripts and then its recovery via expression of zygotic transcripts, which takes place in buffalo embryos at the 8-16 cell stage. Epigenetic mechanisms might be the cause of the low levels of Sox-2 gene expression after fertilization. Based on the results it was believed that Sox-2 was co-expressed with Oct-4 in the ES cells and acts synergistically with Oct-4 to activate Oct-Sox enhancers, which regulate the expression of pluripotent stem cell-specific genes, including Nanog, Oct-4 and Sox-2 itself.

Keywords: embryonic stem cells, Sox-2, Gene expression, buffalo, Bubalus bubalis

INTRODUCTION

The pluripotency of ES cells is thought to be maintained by a few key transcription factors, including Oct-4, Sox-2 etc. These transcription factors in ES cells have been extensively characterized in mouse and human, but for those of domestic animals and particularly buffalo (Anand et al., 2008; Kumar et al., 2008) little information is available.

Sox-2 is a member of the Sry (Sex determining region-Y) related transcription factor family. Sox-2 function in ES cells was first identified in relation to Oct-4. Sox-2 and Oct-4 expressions overlap during early embryogenesis, and both are important for the maintenance of the pluripotent state. Sox-2 can be regarded as one of the cofactors.
of Oct-4, since it activates the transcription of target genes, such as Fgf-4, Utf-1, Fbx-15, and Lefty-1 in cooperation with Oct-4. Moreover, Sox-2 expression is regulated by Oct-4 and Sox-2, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal.

**MATERIALS AND METHODS**

Embryos were incubated in solution containing 0.5 percent pronase until the zona was removed. To isolate the inner cell mass (ICM), blastocysts were incubated for 3-4 minutes in solution containing calcium ionophore until the zona and trophectoderm were lysed. Embryos were observed constantly under a zoom stereo microscope until the zona and trophectoderm were lysed. Residual embryos/zonafree embryos were washed with phosphate buffered saline containing 10 percent FBS. Zonafree embryos were incubated in Ca++/ Mg++ free PBS for 10 to 15 minutes at 37°C in CO_{2} incubator. Repeated pipetting through Pasteur pipette disaggregates the zonafree blastomeres/inner cells.

**Sox-2** was detected by reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed based on the sequence (NCBI Ac. No: DQ487021.1) of Chinese swamp buffalo available in Pubmed GenBank by using a Web-based primer 3 designing programme. RT-PCR for Sox-2 was carried out with 100 ng of RNA by using the forward primer (5’ GCCGAGTGGAAACTTTTGTC 3’) and the reverse primer (5’ TGCGAAGCTGTCATAGAGTTG 3’) with the following cycling profile: cDNA synthesis for 15 minutes at 50°C, initial denaturation at 95°C for 2 minutes followed by 36 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, and a final extension for 10 minutes at 72°C. These primers amplified a fragment of 413 bp. The Sox-2 RT-PCR product was analyzed by gel electrophoresis along with a standard 100 bp ladder as marker. Then the gel was visualized under a UV light gel documentation unit.

The cDNA sequence of the Sox-2 RT-PCR product submitted to NCBI Pubmed GenBank was given accession number: EU661361. The sequences were analyzed for phylogenetic conservation and the sequence homology across the species was established. Homologies of the Sox-2 gene (mRNAs) were compared with reported sequences of other species retrieved from annotated databases such as the National Centre of the Biotechnology information (www.ncbi.nlm.nih.gov). The BLASTn search of highly similar sequence homology were explored. The cDNA sequences of the Sox-2 gene were phylogenetically analysed using Lasergene version 4.1 (DNASTAR Package, USA). The Sox-2 gene sequences used for comparison are presented in Table 1.

**RESULTS AND DISCUSSION**

I In two out of six trials the blastomeres/inner cells of 2-cell, 4-cell and 8-cell stage embryos (Pre ZGA) did not express the Sox-2 gene whereas inner cells obtained from 16-cell stage embryos, morulae and inner cell masses of blastocyst consistently expressed the Sox-2 gene. Representative photographs of gel for Sox-2 gene expression are presented in Figure 1.

The percentage of identity among the sequences of the Sox-2 gene from various isolates in comparison with MVC, TANUVAS is shown in Figure 2. The phylogenetic analysis of the Sox-2 gene from various species by clustralW method with
Table 1. Sox-2 gene sequences used for comparison of homology.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No</th>
<th>Percent homology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bubalus bubalis</em></td>
<td>DQ487021.1</td>
<td>99</td>
</tr>
<tr>
<td>(Chinese swamp buffalo)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bos taurus</em></td>
<td>NM 001105463.1</td>
<td>99</td>
</tr>
<tr>
<td><em>Ovis aries</em></td>
<td>X96997.1</td>
<td>98</td>
</tr>
<tr>
<td><em>Canis familiaris</em></td>
<td>XM 545216.2</td>
<td>97</td>
</tr>
<tr>
<td><em>Sus scrofa</em></td>
<td>EU503117.1</td>
<td>96</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>BC013923.2</td>
<td>95</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Z31560.1</td>
<td>95</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>NM 011443.3</td>
<td>93</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>NM 001109181.1</td>
<td>93</td>
</tr>
<tr>
<td><em>Gallus gallus</em></td>
<td>D50603.1</td>
<td>84</td>
</tr>
</tbody>
</table>

Figure 1. Sox-2 gene expression in stem cells derived from different stages of *in-vitro* produced buffalo embryos.

- Lane I = Sox-2 amplicon in Two celled embryos
- Lane II = Sox-2 amplicon in Four celled embryos
- Lane III = Sox-2 amplicon in Eight celled embryos
- Lane IV = Sox-2 amplicon in Sixteen celled embryos
- Lane M = 100 bp DNA ladder
- Lane V = Negative Control
- Lane VI = Sox-2 amplicon in Morulae
bootstrap analysis, presented in Figure 2, revealed two major clusters. The Sox-2 gene lineage of Chinese swamp buffalo, cattle, pig, amur tiger, rhesus monkey, Norway rat, dog and lizard were grouped to the first cluster. The lineage of this first cluster again sub-grouped into three clusters of which dog, mouse, human (US, France, Yugoslavia), Norway rat, rhesus monkey, sheep and pig (USA) were grouped to one cluster, Indian water buffalo of TANUVAS, platypus and xenopus were grouped to another cluster and lizard, amur tiger, cattle (Italy), Chinese swamp buffalo and pig (China) were grouped to another cluster. The second cluster revealed a lineage covering the human (Italy), zebra fish and chicken. The bootstrap analysis is shown on every node from common ancestor.

Amino acid analysis of the Sox-2 gene revealed hypervariable regions at the positions 2368-2373 and 2380. The variations were in relation with species specificity.

The HMG domain containing Sox-2 and the POU domain-containing Oct-4 were the transcription factors known to be essential for normal pluripotent cell development and maintenance (Avilion et al., 2003). Their function in pluripotent cells is via a synergistic interaction between the two to drive transcription of target genes. Sox-2 is co-expressed with Oct-4 in the ES cells and acts synergistically with Oct-4 to activate Oct-Sox enhancers, to regulate the expression of pluripotent stem cell-specific genes, including Nanog, Oct-4 and Sox-2 itself as well as Fgf4, Uif1, and Fbx15. Each of these target genes has a composite element containing an octamer and a Sox binding site. Genetic links between the Sox2-Oct4 complex and Sox-2 and Pou5f1 expression, as well as their in vivo binding to these genes in mouse and human ESCs (Chew et al., 2005), suggests that this complex is at the top of the pluripotent cell genetic regulatory network.

Sox-2 mRNA was detected in oocytes as well as in embryos at the different developmental stages analyzed, resembling the profile of Oct-4 (Srinivasa Prasad, 2008). Sox-2 was present as both maternal and embryonic transcript; in particular, a significant increase from the 16-cell stage, concomitant with embryo genome activation, was observed suggesting that Sox-2 expression might be regulated by Oct-4. The results of the present study are in agreement with the findings of Brevini et al. (2008). The Sox-2 gene expression patterns were variable in stem cells derived from early embryos but gradually became more regular, with 100 percent expressing Sox-2 from the 16-cell stage onward. This might be related to the maternal-zygotic transition (activation of the embryonic genome), which takes place in buffalo embryos at the 8-16 cell stage. This might be related to the exhaustion of maternally generated Sox-2 transcripts and then its recovery via expression of zygotic transcripts. An epigenetic mechanism, consisting of DNA methylation and chromatin remodeling, might be the cause of the low levels of Sox-2 gene expression after fertilization as reported by Hattori et al., 2004.

Sox-2 expression in ES cells is regulated by Sox-2 itself and Oct-4, suggesting the possibility that Sox-2 is activated in primitive cells by a positive autoregulatory loop. Therefore, it is speculated that the same positive feedback loop maintained the expression of Sox-2 and Oct-4 together and that the Sox-2 and Oct-4 are regulated coordinately (Boyer et al., 2005).

The essential function of Sox-2 was to stabilize ES cells in a pluripotent state by maintaining the requisite level of Oct-4 expression (Masui et al., 2007). A critical amount of Oct-4 has recently been reported to be crucial for the maintenance
Table 2. Percentage of identity among the sequences of sox-2 gene from various isolates in comparison with Indian water buffalo of TANUVAS.

|          |  1  |   2  |   3  |   4  |   5  |   6  |   7  |   8  |   9  |  10  |  11  |  12  |  13  |  14  |  15  |  16  |  17  |  18  |  19  |  20  |  21  |  22  |  23  |  24  |  25  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|          | 135 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

1. BUBALUS BUBALIS (MC/TANUVAS) seq
2. BUBALUS BUBALIS (CHINA) seq
3. Bos taurus (cattle) seq
4. Bos taurus (cattle) ITALY seq
5. Bos taurus (cattle) ALITALIA seq
6. Bos taurus (cattle) U.S. seq
7. Bos taurus (cattle) U.S. seq
8. Bos taurus (cattle) U.S. seq
9. Bos taurus (cattle) U.S. seq
10. Bos taurus (cattle) U.S. seq
11. Bos taurus (cattle) U.S. seq
12. Bos taurus (cattle) U.S. seq
13. Bos taurus (cattle) U.S. seq
14. Bos taurus (cattle) U.S. seq
15. Bos taurus (cattle) U.S. seq
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18. Bos taurus (cattle) U.S. seq
20. Bos taurus (cattle) U.S. seq
21. Bos taurus (cattle) U.S. seq
22. Bos taurus (cattle) U.S. seq
23. Bos taurus (cattle) U.S. seq
24. Bos taurus (cattle) U.S. seq
25. Bos taurus (cattle) U.S. seq

Data from various isolates are compared with Indian water buffalo (TANUVAS) for the sox-2 gene identity.
Figure 2. Phylogenetic analysis of Sox-2 gene from various species by ClustalW method with bootstrap analysis.

of ES cell self-renewal. A 50 percent decrease in the endogenous Oct-4 levels relative to that of undifferentiated ES cells results in the commitment of ES cells to trophoectoderm lineages, containing both proliferating and endoduplicating giant cells based on the culture conditions. However, an increase beyond the 50 percent threshold level of Oct-4 leads to the concomitant differentiation of ES cells into extra-embryonic endoderm and mesoderm. Interestingly, LIF withdrawal leads to the specification of the same lineages. Less subtle changes in Oct-4 level (both increase and decrease) do not affect ES cell self-renewal. In conclusion, the precise level of Oct-4 protein governs commitment of embryonic cells along three distinct lineages (Niwa et al., 2000; Lanza et al., 2006).

Based on the results it is speculated that the observed lack or low expression of Sox-2 in cells derived from early embryos (pre ZGA) might be the reason for inadequate ability of those cells to retain the property of stemness, to form primary stem cell colonies and subsequently ES-cell lines compared to inner cells derived from morulae and blastocysts (Ito et al., 1996; Hatoya et al., 2006) as Oct-4 is essential for antiapoptosis of stem cells in response to stress effects that might be mediated through the STAT3/Survivin pathway, Sox-2 being responsible for maintaining the requisite level of Oct-4 expression (Masui et al., 2007).
ACKNOWLEDGEMENT

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REFERENCES


*Continued on page 156
The present study was undertaken with the objectives of sequence characterization and identification of polymorphisms in the bubaline CD14 gene. Single strand conformation polymorphism analysis revealed a total of eight different variants viz. CD14-A, CD14-B, CD14-C, CD14-D, CD14-E, CD14-F, CD14-G and CD14-H in exon 2 of the CD14 gene for four breeds of buffalo. Variant CD14-A was observed to be the wild type as it exhibits highest frequencies, whereas CD14-D was observed to be genetically distant from others. Polymorphism or variability may be regarded to be the highest in the Mehsana breed of buffalo, which exhibited six genotypes out of eight. Thus 42 SNPs were identified for the CD14 gene of buffalo. The CD14 gene ranging from the 592nd to the 856th nucleotide of the second exon, which corresponds to the 198th to the 285th codon of the coding sequence was found to be highly polymorphic and may be regarded as the ‘mutational hot spot’, or the hyper variable site, leading to ligand diversity. Comparison of nucleotide sequences of different regions of the buffalo CD14 gene with that of taurine cattle revealed a total of 22 point mutations, of which eleven were non-synonymous codon with eleven amino acid substitutions. The 258 bp fragment of the CD14 gene ranging from the 3rd nucleotide to the 261st nucleotide of the 2nd exon of the CD14 gene revealed identical nucleotide sequences, indicating monomorphism.

Keywords: CD14 gene, buffalo, exon, polymorphism, SSCP

INTRODUCTION

Mastitis is one of the most common diseases affecting dairy cattle and buffaloes causing huge economic losses to dairy farmers. In India, about 3.84% and 24.43% of buffaloes, respectively, are affected with clinical and subclinical mastitis every year (Singh and Singh, 1994). The annual loss due to clinical and subclinical mastitis in buffaloes was estimated to be Rs. 6,962,900,000 and Rs. 17,233,200,000, respectively (Dua, 2001). Incidences of diseases of milch animals is critical to dairy industry, wreaking economic havoc in terms of veterinary and treatment costs, reduced milk production in the subsequent lactation, milk condemnation due to antibiotic residues, early culling, extra labour involvement and deterioration in milk quality. Antibiotic therapy and vaccination have their own limitations. Thus, it seems
understanding and subsequent manipulation of the host immune response is the most precise and effective tool to lower the disease incidences and to nullify the limitations associated with antibiotic treatment or vaccination. Selective breeding of dairy cattle and buffaloes for reduced susceptibility to/increased resistance against mastitis is difficult as it is a polygenic trait with very low heritability. However, indirect selection based on somatic cell count and candidate gene markers can help to increase the efficiency of such breeding programs. A fair amount of genetic research related to udder health has already been performed due to its importance from the economic as well as from the quality control points of view (Ogorevc et al., 2009).

CD14 is an important molecule for innate immunity. The CD molecule ranges from 1 to 166 with differential structure and functions (Goldsby et al., 2000), of these CD14 is the most important molecule known so far, playing a vital role against several endotoxigenic bacteria. Its pattern recognition receptor binds mainly with LPS (lipopolysaccharide), lipoteichoic acid, arachidonic acid and thus releases various cytokines which acts for the body’s defence. The body’s immunity thus can act against a wide range of pathogens including gram-negative bacteria and gram positive as *Mycobacterium* sp., *Pseudomonas* sp. and *Staphylococcus aureus* etc. CD14 functions both as a cell membrane receptor and a soluble receptor for bacterial LPS. It has been considered as an important molecule for its role in various diseases, like mastitis (Lee et al., 2003), treponemiasis (Schroder et al., 2000) and glomerulonephritis (Yoon et al., 2003). Soluble CD14 enriched bovine colostrums and milk induces B cell growth and differentiation (Fillip et al., 2001). The CD14 gene has been cloned and sequenced in other ruminants (Pal and Chatterjee, 2009a; Pal et al., 2008)

Detection of SNPs is useful for analysis of the evolutionary history of species development, assessment of biodiversity, associative studies between polymorphisms and disease resistance. The variability at the nucleotide level of the CD14 gene leads to the variability in the CD14 encoded molecule, which in turn gives rise to the phenotypic variability in host immune response. Thus, the variants of the CD14 gene and their association with the incidences of disease occurrence may be used as a marker for disease resistance. Association of polymorphic candidate genes with economic traits will help the breeders to search some genetic markers for economic traits. This may be used as an aid to the selection of bulls at an early age and can save huge economic loss for rearing the bulls till maturity. In spite of its tremendous potential to be exploited, very little work has so far been done whereas such type of marker trait association studies have also been documented by a number of workers in various ETLs (Pal et al., 2004; Pal et al., 2005) No reports are available so far for genetic polymorphism of the CD14 gene in any animal at the coding region; however CD14 gene polymorphism study at the promoter region is available in human. In the last decade, many fine mapping experiments have resulted in identification of several QTLs in cattle affecting milk production and udder health emphasizing their potential value in marker assisted selection programs. However, beyond fine mapping, the ultimate target of QTL analysis is the identification of casual gene itself which would facilitate identification of resistance genes and alleles (Reinard and Riollet, 2005). Scanty reports are available so far for genetic polymorphism of the CD14 gene in an animal at the coding region; however CD14 gene
polymorphism study at the promoter region is available in human (Hubacek et al., 1999; Hartel et al., 2004; Guerra et al., 2004).

Buffaloes are economically important animals with higher milk producing ability (54%) with high SNF and fat percentage, better capacity to utilize coarse fodders and their innate resistance to a wide range of diseases (Annon, 2001). Most Indian buffaloes are of the riverine type; there are a few swamp buffaloes in the northeastern region. So far no reports are available regarding molecular characterization of the CD14 gene in buffaloes and very scanty reports are available regarding SNP detection in any farm animal.

Keeping the above facts in view, the present investigation was planned to identify the polymorphism of the CD14 gene of buffalo, analyze the sequences of the identified variants and compare them with those of other species.

**MATERIALS AND METHODS**

**Animals**

Blood samples were collected from a total of 246 unrelated animals belonging to four different breeds of Indian riverine buffaloes (*Bubalus bubalis*) with Murrah (103), Mehsana (69), Surti (36) and Bhadawari (38). Buffaloes were maintained at different government farms in India as well as farmers’ herds, gauushals and NGOs. Samples from Murrah buffalo were collected from farmers’ herds, gauushals and NGOs at Dubrajpur Block, Birbhum district, West Bengal and the Indian Veterinary Research Institute, Izatnagar (U.P.). Samples from Mehsana and Surti buffaloes were collected from Gujrat Agricultural University, S.K. Nagar (Gujrat), Gujrat Agricultural University, Navasari, Anand (Gujrat), and the Government Livestock Farm, Etawah (U.P.) respectively.

Blood was collected from the jugular vein into EDTA containing vacutainer tubes and DNA extraction was performed from whole blood following a standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

**Amplification of the Bubaline CD14 gene**

The 2nd exon of CD14 gene was studied in two fragments. A 258 bp fragment (the 3rd nucleotide to the 261st nucleotide of CD14 cDNA) of the coding sequence of Bubaline CD14 (the 587th to the 854th nucleotide of second exon) was amplified using the forward primer as ATGGTGTCGTGCGCTACCTC and the reverse primer as TATGCTGACACAATCAAGGCT and the fragment obtained was of 258 bp. The reaction mixture used was PCR buffer 1.2X, MgCl₂ 1.5 mM, dNTP 0.5 mM, forward primer and reverse primer 50 ng each, Taq DNA polymerase 1 unit. The reaction condition used was initial denaturation at 94°C for 5 minutes, denaturation at 95°C for 45 seconds, followed by annealing for 61°C for 30 seconds, extension for 72°C for 45 seconds, repeated for 34 cycles followed by final extension for 72°C for 7 minutes.

The second fragment amplified was a 265 bp fragment of the coding sequence of Bubaline CD14 (the 587th to the 854th nucleotide of the second exon). PCR was carried out in a final volume of 25 μl of reaction mixture containing 80-100 ng DNA, PCR buffer 1.2X, MgCl₂ 1.5 mM, dNTP 0.5 mM, forward primer and reverse primer 60 ng each, Taq DNA polymerase 1 unit. The DNA was subjected to amplification with polymerase chain reaction in a thermocycler (PTC-200, MJ Research, USA) using the forward primer as AGCGAACGACAAATTGAGAGACCTTAGTG and the reverse primer as AAGGTCTCTCTCAATT
TGTCGTTCGCTGGGC and the fragment obtained was of 265 bp. The reaction condition used was initial denaturation at 94°C for 5 minutes, denaturation at 95°C for 45 seconds, followed by annealing for 61°C for 30 seconds, extension for 72°C for 45 seconds, repeated for 34 cycles followed by final extension for 72°C for 7 minutes.

Genotyping by PCR-Single stranded conformation polymorphism

SNPs were detected by PCR-SSCP and identified by subsequent sequencing. PCR-SSCP was performed for all the samples. In 0.5 ml PCR tubes, 3 μl of amplified PCR-product was mixed with 9 μl of formamide dye. This 12 μl of PCR-SSCP solution was denatured at 95°C for 5 minutes followed by immediate chilling in ice for 15 minutes. The products were run in 12% polyacrylamide gel at 4°C for 11 h at 200 V in case of the 268 bp fragment and 6 h for the 261 bp fragment. SSCP fragments were visualized by silver staining and documented in the Gel Documentation System. Silver staining was carried out according to the procedure described by Basam et al. (1991) with some modifications. The variants were detected directly by observing the SSCP pattern of each sample in the polyacrylamide gel. PCR products from different variants were selected and sequenced using respective forward and reverse primers to detect variations if any, at the nucleotide level. Then sequences were aligned with those of the reported CD14 sequences of different species using MegAlign Programme of Lasergene Software (DNASTAR, Inc, Madison WI, USA). Multiple sequence alignments were performed with the Megalign program of LASERGENE software. The coding DNA sequences of different exonic regions were conceptually translated to amino acid sequences using the same software.

RESULTS AND DISCUSSION

PCR-SSCP analysis of 258 bp fragment

The 258 bp fragment from the 3rd nucleotide to the 261st nucleotide of the 2nd exon of CD14 gene (Figure 1) revealed identical nucleotide sequences (Gene bank acc no. EU370404), indicating monomorphism of the gene (Figure 2). This indicates the conserved nature of the CD14 gene of buffalo pertaining to this region. It reveals the conserved nature of this gene for the first 258 nucleotides, encoding for 86 amino acids. This region in buffalo may probably be under strong purifying selection which may explain the lack of SNPs in this region. This may be the reason that 1
to 60 nucleotide codes for signal peptide, when no variation is expected. The present finding is similar to CB cattle (Pal and Chatterjee, 2009b), where monomorphism was reported for this nucleotide region except for one nucleotide change.

**PCR-SSCP analysis of 265 bp fragment**

The amplified product of the 265 bp fragment of the CD14 gene was observed by agarose gel electrophoresis (Figure 3). Eight variants designated as CD14-A, CD14-B, CD14-C, CD14-D, CD14-E, CD14-F, CD14-G and CD14-H were detected in 265 bp fragment of CD14 gene of buffalo. The frequencies of the different variants of bubaline CD14 gene are depicted in Table 1. In the Bhadawari breed of buffalo, only three variants were identified as CD14-A, CD14-B and CD14-F (Figure 4) with the respective frequencies being 0.818, 0.045 and 0.136 (Figure 5). In the Mehsana breed, all variants, except CD14-D and CD14-H were identified (Figure 6). The highest frequency was observed for the CD14-A genotype (0.465), whereas CD14-B (0.056) and CD14-G (0.056) were found to be the least frequent genotypes (Figure 7). The Murrah breed of buffalo showed a different picture where the highest frequency was observed for the CD14-D genotype, and the CD14-A (0.071) and CD14-H (0.071) genotypes were the least frequent (Figure 10, Figure 11). Since this is the first report of study for polymorphism of the CD14 gene in farm animals and first time in the coding region in any animal so far, comparison was not possible. However, polymorphism has been identified in the promoter region of the CD14 gene in human being at C (-260)→T (Hubacek et al., 1999), (-159) position (Hartel et al., 2004, Guerra et al., 2004), (-1619), (-550) from the transcription start site of the CD14 gene (Guerra et al., 2004).

CD14-E may be considered as the breed specific marker for the Mehsana breed, whereas CD14-G and CD14-H may be considered as breed specific markers for the Mehsana and the Surti, respectively. In the present study, the Mehsana breed exhibited six genotypes out of eight. Thus polymorphism or variability may be regarded to be the highest in this breed. Simultaneously heterozygosity may also be expected to be high in this breed.

The frequencies of different variants estimated in the population of four different breeds are listed in Table 1 and Figure 12. The most frequent variant (genotype) was identified as CD14-A, with the overall genotypic frequency being 0.47. The least frequent were CD14-G and CD14-H, with very low frequencies (0.018) for both the genotypes. The frequencies for other genotypes were intermediate. Thus CD14-A may be the original wild type allele for the gene. The other variants may therefore be the result of recent mutational events. Among the breeds, Bhadawari had the highest frequency (0.818) for CD14-A and Surti had the lowest frequency for this pattern (0.071) which showed the highest homozygosity in the Bhadawari population and least in the Surti population.

CD14-D was observed to be genetically distant from other variants as evidenced from Figure 13. Since the Surti breed of buffalo was observed to have highest frequency for CD14-D (Figure 11), it is expected that the Surti is genetically distant from the other breeds.
Sequence analysis of PCR-SSCP 265 bp fragments

Six different SSCP variants CD14-A (Gene bank acc no. EU370398), CD14-B (Gene bank acc no. EU370399), CD14-C (Gene bank acc no. EU370400), CD14-D (Gene bank acc no. EU370401), CD14-E (Gene bank acc no. EU370402), CD14-F (Gene bank acc no. EU370403) were sequenced for determining the variation at the nucleotide level. The combined effects of the SNPs were estimated through PCR-SSCP. Moreover, sequence variations were also compared with the cDNA (Gene bank acc no. DQ457089) and genomic DNA of buffalo (Gene bank acc no. DQ444324), we have reported earlier. Since this is the first report of CD14 gene polymorphism in coding region, comparison was not possible. However, polymorphism studies have been conducted at promoter region in human (Hubacek et al., 1999; Guerra et al., 2004; Hartel et al., 2004; Geaghan et al., 2010). The SNPs have been depicted in Figure 14. A very high degree of nucleotide sequence variability was observed for CD14-B (81.3), CD14-C (97.0), CD14-D (86.0), CD14-E (85.8) and CD14-F (85.8) as compared to CD14-A (Table 2). A high degree of nucleotide variation was observed between the alleles/patterns identified within the coding sequence of CD14 gene for the 265 bp fragment ranging from the 587th to the 854th nucleotide the of second exon of buffalo, which corresponds to the 197th to the 285th codon of the coding sequence.

Phylogenetic analysis of CD14 gene of riverine buffalo

The percent homology of different regions of the bubaline CD14 gene with that of various species is presented in Table 3. The sequence homology of the 258 bp region was 97.7% with \textit{Bos taurus}, 95% with \textit{Bos indicus}, 96% with \textit{Bos grumniens}, 91.5 with \textit{Capra hircus} and 94.6 with \textit{Ovis aries}. The percent sequence similarity of the bubaline 265 bp region was greater than 90% with ruminants (\textit{Bos taurus}, \textit{Capra hircus} and \textit{Ovis aries}) while it was less than 90% with monogastric species (\textit{Sus scrofa}, \textit{Canis familiaris} and \textit{Equus caballus}). Similarly, the percent homology of bubaline CD14 was more than 90% with ruminants while it was 84% with \textit{Equus caballus}. Comparative analysis of the buffalo CD14 peptide sequence with that of cattle has been depicted in Table 4. This represents the species specificity. Phylogenetic closeness of buffalo with cattle as observed in the present study has also been reported while studying other genes like growth hormone gene (Pal and Chatterjee, 2010). Chicken has been found to be genetically most distant to buffalo.

SNP detection of bubaline CD14 gene

Very high degrees of nucleotide variation with 42 SNPs were identified with 39 non-synonymous changes, leading to 23 amino acid changes. Twenty-nine transversional mutations and 18 transitional mutations were detected (Table 5). At some sites, both transitional and transversional mutations were detected: at 702, 703, 764, 767, 770 nucleotide positions of CD14 gene of buffalo. Non-synonymous substitutions exceeding synonymous substitutions indicates the evolution of this protein through positive selection among domestic animals. SNPs for the CD14 gene of buffalo was reported here for the first time and moreover, this is the first report of SNPs in any farm animal. Combined genotypes of SNPs were analyzed using a PCR-SSCP method. Thus, the 268 bp fragment of the coding sequence of Bubaline CD14 was found to be highly polymorphic and may be regarded as the ‘mutational hot spot’ or the hyper variable site,
leading to ligand diversity. Similar higher degrees of mutations have been observed in some other genes as 25 SNPs in the leptin gene in Korean cattle (Chung et al., 2008), 96 SNPs from TLRs and their associated intracellular signaling molecules (Dhiman et al., 2008) in human.

Most of the SNPs identified were within LRR at amino acid positions 209, 210, 215, 217, 246, 249, 252, 255, 256, 257, 266, 267, 271, 273, 274, 275, 277, 280. It has been reported that LRR in the extracellular domain is responsible for the recognition of pathogens and participates in receptor and ligand interactions (Gordon, 2002). Grooves within the CD14 molecule were responsible for receptor recognition and binding (Kim et al., 2005). Since CD14 molecules can bind to a wide range of substances including lipopolysaccharides from gram-negative bacteria, lipo-arabinomannans of mycobacteria, manuronic acid polymers of Pseudomonas sp. and to peptidoglycans of Staphylococcus aureus, it is expected that there should be sufficient variability in the pathogen recognition and receptor binding site, which is primarily comprised of LRR region of the CD14 molecule (Kim et al., 2005). Thus, the presence of the LRR coding region may be the region for maximum variability to enable the CD14 molecule to bind with a wide range of substances. From the sequence alignment studies, it was observed that the particular leucine moiety was almost unaltered within the leucine rich repeats and the variations were observed for other amino acids. Thus the basic function of leucine rich repeats remains unaltered.

However, in the absence of any available report regarding the polymorphism study of this particular region in livestock species, comparison is not possible. Moreover, similar nucleotide variations were also observed for this particular region of the CD14 gene between different species, which may give some insight.

Further research need be directed to finding out the association of allelic variants with traits related to disease incidences, to establish markers. There is a need to ascertain the differential biological potency of different allelic variations of CD14. On the basis of such functional study, the most potent variant can be expressed in vitro for further therapeutic or immuno competence study. Gene inserts containing the resistant variety of CD14 gene of buffalo may be approached for somatic gene therapy, particularly against mastitis. Transgenic animal production with the buffalo CD14 gene insert may provide scope for future research to develop disease resistant stock.

REFERENCES


Figure 1. Amplification of the 258 bp fragment of the CD14 gene of buffalo.
Lane 1: Amplified product of 258 bp fragment.
Lane M: 100 bp DNA ladder.

Figure 2. PCR-SSCP pattern of the 258 bp fragment of the CD14 gene of buffalo.
Figure 3. Amplification of the 265 bp fragment of the CD14 gene of buffalo.
Lane 1: Amplified product of 265 bp fragment.
Lane M: 100 bp DNA ladder.

Figure 4. PCR-SSCP pattern of the 265 bp fragment in the Bhadawari buffalo CD14 gene in 12% PAGE.
Lane 1-4 and 8: CD14-A.
Lane 5-7: CD14- B.
Lane 9-12: CD14-F pattern.
Figure 5. Frequency distribution for different SSCP variants in the Bhadawari breed of buffalo.

Figure 6. PCR-SSCP pattern of the 268 bp fragment in the Mehsana buffalo.

Lane 1-3: CD14- F.  
Lane 5, 6, 10: CD14- B.  
Lane 12-13: CD14- C.  
Lane 4: CD14- G.  
Lane 8, 9, 11: CD14- A.  
Lane 7: CD14- E.
Figure 7. Frequency distribution for different SSCP variants in the Mehsana breed of buffalo.

Figure 8. PCR-SSCP pattern of the 265bp fragment in the Murrah buffalo.
Lane 5-8: CD14- A.  Lane 1-4: CD14- B.
Lane 12-13: CD14- D.  Lane 9-11: CD14- F.
Figure 9. Frequency distribution for different SSCP variants in the Murrah breed of buffalo.

Figure 10. PCR-SSCP pattern of the 268 bp fragment in the Surti buffalo.
Lane 1-2: CD14- C.  Lane 7-8: CD14- D.
Lane 3-4: CD14- A.  Lane 11-12: CD14- H.
Lane 5-6, 9-10: CD14- B.
Figure 11. Frequency distribution for different SSCP variants in the Surti breed of buffalo.

Figure 12. Frequency distribution for different SSCP variants of buffalo.
Figure 13. Phylogenetic tree constructed for variants of CD14 gene in riverine buffalo.

Figure 14. SNPs for different sscp fragments (265 bp) of CD14 gene in buffalo.
Table 1. Frequencies of the identified variants of the 265 bp fragment of the CD14 gene in buffalo.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td>Bhadawari</td>
<td>0.818</td>
<td>0.045</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.136</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Mehsana</td>
<td>0.465</td>
<td>0.056</td>
<td>0.183</td>
<td>-</td>
<td>0.070</td>
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<td>Murrah</td>
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<td>0.105</td>
<td>-</td>
<td>0.193</td>
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<td>-</td>
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<tr>
<td>Surti</td>
<td>0.071</td>
<td>0.16</td>
<td>0.089</td>
<td>0.607</td>
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<td>-</td>
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<tr>
<td>Overall</td>
<td>0.47</td>
<td>0.09</td>
<td>0.08</td>
<td>0.18</td>
<td>0.022</td>
<td>0.13</td>
<td>0.018</td>
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Table 2. Genetic identity of different variants of CD14-A with respect to others.

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<tr>
<th>SSCP variants</th>
<th>Gene bank Accession no.</th>
<th>Percent identity</th>
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<td>CD14-B</td>
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<td>CD14-C</td>
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<td>CD14-D</td>
<td>EU370401</td>
<td>86.0</td>
</tr>
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<td>CD14-E</td>
<td>EU370402</td>
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</tr>
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<td>CD14-F</td>
<td>EU370403</td>
<td>85.8</td>
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Table 3. Percent sequence homology of amplified region of bubaline CD14 gene with various species.

<table>
<thead>
<tr>
<th>Species</th>
<th>258 bp 3rd nucleotide to 261st nucleotide</th>
<th>265 bp 587th to 854th nucleotide of second exon</th>
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<tbody>
<tr>
<td></td>
<td>Percent homology</td>
<td>Accession</td>
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<tr>
<td>Cattle</td>
<td>97.7</td>
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<td>Goat</td>
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<td>DQ457090</td>
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<td>Sheep</td>
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Table 4. Comparative analysis of the buffalo CD14 peptide sequence with that of cattle.

<table>
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<tr>
<th>Sl. No.</th>
<th>Amino acid sequence position</th>
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<th>Cattle</th>
<th>Nucleotide substitution</th>
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<td>14*</td>
<td>Proline</td>
<td>Serine</td>
<td>C/T</td>
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<tr>
<td>2</td>
<td>62*</td>
<td>Glycine</td>
<td>Alanine</td>
<td>G/C</td>
</tr>
<tr>
<td>3</td>
<td>209**</td>
<td>Serine</td>
<td>Threonine</td>
<td>C/G</td>
</tr>
<tr>
<td>4</td>
<td>235-236**</td>
<td>Serine, Lysine</td>
<td>Threonine, Proline</td>
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<tr>
<td>5</td>
<td>277**</td>
<td>Serine</td>
<td>Arginine</td>
<td>C/G</td>
</tr>
</tbody>
</table>

* indicates aa changes resulting due to the 258 bp fragment (the 3rd - 261st bp of the 2nd exon of the CD14 gene of buffalo).

** indicates aa changes resulting due to the 265 bp fragment (the 587th to 854th nucleotide of the second exon of the CD14 gene of buffalo).
Table 5. Identified SNPs in the CD14 gene of buffalo include 6 variants.

<table>
<thead>
<tr>
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<th>CD14-</th>
<th>CD14-</th>
<th>CD14-</th>
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<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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Synm: Nucleotide substitution leading to synonymous codon change.
Non-synm: Nucleotide substitution leading to non-synonymous codon change.
Vaccine, 26(14): 1731-1736.
in *Bubalus bubalis* and *Capra hircus*. 

Conference on Development of Dairy Cattle. NDRI, Eastern Regional Station, Kalyani, India.


*Continued from page 137*


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- …liquid nitrogen vapour freezing technique (Verma et al., 1975)
- …and buffaloes (Singh et al., 1983; Shah et al., 1987; Misra, 1996; Pant et al., 2002)

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- Books: Author(s) or editor(s). Year. Title. Publisher name, Place of publication. Number of pages.
  Example: Citation in text: Snedecor and Cochram. (1980)

  Example: Citation in text: Sloss and Dufty. (1980)

- Ph. D. Thesis: Author(s) of the chapter. Year. Title of the chapter, pages of the chapter. In author(s) or editor(s). Title of the book. Publisher name, Place of publication.
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