IDENTIFICATION OF FREEMARTINISM IN ZEBU AND CROSSBRED CATTLE

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ABSTRACT

In the present investigations 12 heterosexual twin born calves of different ages and stages from 'calf to puberty' were examined. The examination involved physical features, reproductive tract and chromosomal complement. At birth, these animals looked similar to newly born calves of respective sex, however, females had more coarse hair on the lower lips of vulva. The clitoris got enlarged at puberty stage and in some cases became tubular structure even visible from distance like glans penis. Rectal palpation of three female co-twins showed underdeveloped infantile reproductive tracts rudimentary ovaries. The chromosomal examination revealed chimaerism as 60,XX/60,XY in all the individuals. However, the proportion of XX complement varied from 41.49 to 76.05 percent and XY varied from 23.94 and 58.51 percent among different animals. In four cases both the co-twins were available while only one member was available in other four cases. Both the co-twins within sets showed similar proportion of XX /XY cells though it varied in different sets. In the present study the frequency of XX /XY was found to be 41.49: 42.42; 61.48: 59.70; 76.05: 75.49 and 46.89: 44.93 percent in the four pairs, which is reasonably same in both the co-twins. Six cases were twin born once to their mothers; however, one cow produced twins thrice. The investigations showed that physical feature help in identification of freemartins, which can be confirmed by chromosome examination at birth and rectal palpation at puberty stage.

Key Words : Cattle, Cytogenetic, Chimaerism, Sex Chromosomes

The frequency of twin births is quite low in cattle and buffaloes (Yadav *et al.*, 1989). The twins can be isosexual (same sex, either males or females) or heterosexual (male and female). In the later category the female calf usually turns out sterile, known as freemartin and is the most frequent congenital chromosomal anomaly in cattle, and occasionally in other species. A freemartin is a female born co-twin with an apparently normal male. The problem of sterility of such females has been known since long, in Roman literature to taurae or female bull (Marcum, 1974). The derivation of freemartin is uncertain, but reportedly antedates the seventeenth century (Forbes, 1946), martin was used in England and Scotland.

In the modern scientific era occurrence of freemartins, in the heterosexual twins of the cattle was first described by Lillie (1916) and Keller and Tandler (1916), which has been further elaborated recently by Capel, and Coveney (2004). Heterosexual cattle twins are well known as blood chimaeras and freemartin reviewed extensively by Marcum (1974). Chimaeras are individuals with two or more cell types in their body, which originated from two different zygotes or developing fetuses. The phenomenon of freemartinism is quite enigmatic where proportion of XY cells varies from 1 to 99% in different twins (Yadav and Balakrishnan, 1986); however, the effect is same level of intersexuality. Reports on freemartinism do come in literature from time to time not only in cattle but also in other livestock species. Crow (1996) described the history, and other anecdotal aspects of freemartinism, which provide useful information on freemartins and freemartinism. In cattle approximately 92% of all females from heterosexual twin or multiple births are freemartins (Marcum, 1974).

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The present report deals with the occurrence of freemartinism, its genesis, biology, incidence, diagnosis, and effects on the male in zebu and crossbred cattle.

Cytogenetical investigations were carried out on 12 individual calves ranging from a few days to three years of age (Table-1). Four of these calves belonged to pure Zebu breed (Sahiwal) and eight were crossbred of Zebu x Taurus (Karan Fries). Each of these calves was born as member in heterosexual twin birth. Among these four cases were of individuals from different twin pairs and eight (four sets) were of both co-twins. Thus there were eight females and four males. The samples were collected from Institute herd (6) and farmer's animals (6). Among the female individuals three had reached puberty stage, and their reproductive tract was examined per rectum. During this investigation one dam was found, which delivered three times heterosexual twins, out of these two individuals were included in this study. The other calves were not available for the study as were sold by the owner earlier.

Blood samples (about 10 ml from each) were collected from jugular vein in a vaccutainer tube containing 143 units of sodium heparin. Metaphase chromosomes were obtained from peripheral lymphocytes using whole blood culture technique (Yadav and Balakrishnan, 1984) with some modifications. The technique comprised addition of 0.4 ml whole blood to a 30 ml culture bottle containing 6 ml Ham F-10 synthetic medium (Sigma), 10µg/ml phytohemagglutinin (Bangalore Genei), and 20 units of penicillin, 20µg streptomycin (Sigma) per ml of medium supplemented with 15% adult cow serum as per standard procedure.

Table-1: Details on animals, status of birth, co-twins and chromosome complements

Sr. No.	Animal No.	Phenotypic sex (F/M)	Metaphases examined	Chromosome sets		Frequency (%)	
				60,XX	60,XY	XX	XY
A. Both female and male co-twins available							
1.	SW1986	F	147	61	86	41.49	58.51
	SW 2038	M	165	70	95	42.42	57.58
2.	KF-012	F	135	83	52	61.48	38.51
	KF-013	M	87	52	35	59.70	40.29
3.	KF-016	F	142	108	34	76.05	23.94
	KF-017	M	151	114	37	75.49	24.51
4.	KF7296	F	177	83	94	46.89	53.10
	KF-7364	M	158	71	87	44.93	55.07
B. Female co-twins only							
5.	SW 1427	F	100	73	27	73.00	27.00
6.	SW1688	F	117	52	65	44.40	55.60
7.	KF-001	F	92	59	33	64.10	35.90
8.	KF-002	F	139	98	41	70.5	29.50

Chromosome preparations were obtained on thoroughly cleaned glass slide with 3-4 drops of cell suspension expelling on it from 3-4 feet height. Thus 5-6 slides were prepared from each animal and air-dried in the room conditions. Slides were stained conventionally in 2% Giemsa dye (Sigma) at pH 6.8 (Sorensen phosphate buffer). In order to make confirmed identification of sex chromosomes C-banding using BSG techniques (Sumner, 1972) was carried out. Chromosomes were examined under a Leica microscope fitted with the Leica automated digital camera. Chromosomes were identified counted at a magnification of 1000x in well-spread metaphases. On an average 100 or more metaphases were examined in each animal with emphasis on well-recognized XX and XY chromosome complements. Selected metaphases were photographed and stored in computer. Karyotypes were also constructed.

Physical examination carried out on all the individuals showed different features. The males showed the breed specific conformity that is Sahiwal or crossbred and irrespective of their age did not differ from single bom calves of parental breeds. The calf stage five female individuals were quite similar to single born calves of parental breed; however had a larger tuft of hair on the vulva (Fig. 1). All the individuals were kept under observations for over two years or till the age of puberty. Along with age features in three heifers gradually changed, clitoris became more prominent (Fig. 2); individuals became more bullish in appearance and looked typical freemartins. Rectal palpation at later age showed underdeveloped infantile reproductive tract and hardly identifiable ovaries. In one case the clitoris became quite large in size and resembles a small tubular structure like glans penis (Fig. 2).

Morphological and numerical evaluation of chromosomes was carried out in 100 or more metaphase plates of each animal (Table-1). All the individuals belonged to cattle and showed 60 chromosomes in their metaphases, which consisted of 58 autosomes and two sex chromosomes (either XX or XY). The observations revealed chimaerism of sex chromosomes as 60,XX/60,XY in all the individuals (Figs. 3-6). However, the proportion of XX complement varied from 41.49 to 76.05 percent and XY varied from 23.94 and 58.51 percent among different animals. Chromosome complement in each cell happened to be normal either 60,XY or 60,XX like normal male or female, however, in the present study both types of cells were present in each individual. The XY- and XX-chromosomes were well identifiable in crossbreds, both being submetacentric (Fig. 3-4). In the same way in Sahiwal X chromosome was identifiable, however, it was difficult to identify Y-chromosome, as it is acrocentric and similar to smaller autosomes. Therefore, C-banding was done, which revealed characteristic features of sex chromosomes (Fig. 5-6). All the autosomes show darkly stained centromere; X- and Y-chromosomes do not take localized dark stain, however, chromatids of Y-take darker colour than that of the autosomes. Thus sex chromosomes in Sahiwal were identified (Fig. 6) and ratio was determined (Table-1).

Among the 12 individuals studied, in four cases (Sr. No. 1-4, Table-1) both the co-twins were available while only one member was available in other four cases (Sr. No. 5-8). Both the co-twins within sets showed similar proportion of XX /XY cells though it varied in different sets. In the present study the observation showed frequency of XX as 41.49: 42.42; 61.48: 59.70; 76.05: 75.49 and 46.89: 44.93 percent in the four pairs (table-1), which is reasonably same in both the co-twins.

In the study six cases (Sr. No. 1-6) were the only twins among other calves born to their mothers, however, two sets of twins were born to one cow (Sr. No. 7-8, Table-1). The dam of these two sets in her first three calvings delivered twins, and is still in her productive life. All the three sets were heterosexual twins. The older have turned out to be freemartins and younger one has chromosome chimaerism, and final features in appearance and reproductive organs or fertility status are to be known at puberty.

Twinning occurs in most of the breeds of cattle, however, the incidence varies in different breeds, populations and even in families or dams. Rutledge (1975) in a review of earlier reports on twinning presented that some of cows in different breeds show high fecundity and high number of twins in consecutive calvings. Twinning in cattle ranges from about 1% for beef breeds to about 4% for dairy breeds (Yadav *et al.*, 1989; Komisarek and Dorynek, 2002). The incidence of twin births causes negative effects in milch animals though positive in beef breeds. The loss is not only due to freemartinism, but also management problems connected e.g. with a greater risk of dystocia and retained placenta, it is an undesirable trait in dairy herds (Komisarek and Dorynek, 2002). Silva Del Río *et al.* (2007) reported twinning rate as 4.2%, and it increased with parity [1.2% for nulliparous vs. 5.8% for multiparous cows], and with time (3.4% in 1996 to 4.8% in 2004), with parity by time interaction in America Holsteins. Twinning rate is also slightly influenced by seasonal effects, with a trend toward more multiple births during the spring (Yadav *et al.*, 1989) or autumn months (Gregory *et al.*, 1996).

In twin pregnancies in cattle soon after implantation, placental membrane usually reorient and blood vascular anastomoses occurs between the two foetuses. Thus blood supply becomes common and various constituents keep exchanging between co-twins. This placental fusion occurs before the sexual differentiation of foetuses (Jost *et al.*, 1972). Moreover, the differentiation of gonads starts several days earlier in males than in females. Thus, in the event of twin foetuses of different sex, sex-determining factors from the developing male gonad are transported to the female foetus through the common blood vessels and suppress the development of the reproductive tract of the latter. There have been various theories for the incomplete development of female co-twins (Marcum, 1974); however, the basis is exchange of blood through vascular anastomoses, which carries various hormones. In heterosexual twins blood vascular anastomoses leads to the establishment of sex chromosome chimaerism (60, XX/XY), which is easily detectable in peripheral blood leucocytes (Basrur *et al.*, 1970).

There have various methods for the identification of freemartinism such as physical and rectal examination, chromosome evaluation, hormone profile, DNA profile etc. The animals examined in the present study revealed various features and could be characterized freemartins as in literature.

The observations on twin born calves of the present study were typically similar in their phenotype and chromosomes configuration as per previous reports. The observation of the females born twin to bulls agreed with the results of other reports (Yadav and Balakrishnan, 1985). All XX/XY chimaeric, bovine, heterosexual twin females in the literature have been freemartins. Females born twin to a bull possessing erythrocyte mosaicism would be freemartins whereas those females not expressing mosaicism would be as fertile as if singly born without any suppressing factor from male co-twin.

Physical examination of the cow is a fairly accurate means of determining a freemartin. However, laboratory testing should be conducted to confirm the diagnosis since physical findings may not be clear. Presently the observation of the sex chromosome complement of 100 lymphocytes from a suspected freemartin is usually sufficient for diagnosis. Examination of the sex chromosome complement of the male twin along with the female twin would enable a more positive diagnosis of freemartinism since XX cells in the male twin would indicate the probable chimaeric nature of the female twin. In the genomic ear DNA based testing for freemartinism is likely the most accurate method of diagnosis, yielding 100% accuracy rates. Using a technique known as PCR (polymerase chain reaction), molecular probes are used to specifically detect the presence of a Y chromosome in a suspected freemartin (McNiel *et al.*, 2006). In the present study both the co-twins were available in four sets, and only females in other four cases, however, freemartinism was well identifiable at birth and the same was confirmed at puberty.

The analysis of sex chromosome complement in cultured lymphocytes from bovine heterosexual twins is effective and reliable for early diagnosis of the freemartin. One or more XY chromosomes among cells from the female twin or XX chromosomes among cells from the male twin is sufficient to conclude that the female is a freemartin and will not fit for breeding. Freemartinism cannot be prevented; however, it can be diagnosed in a number of ways ranging from simple examination of the placental membranes, chromosome to PCR evaluation. The breeder can predict the reproductive value of female calf at birth and save the feed and development costs if he is aware of the high probability of freemartinism.

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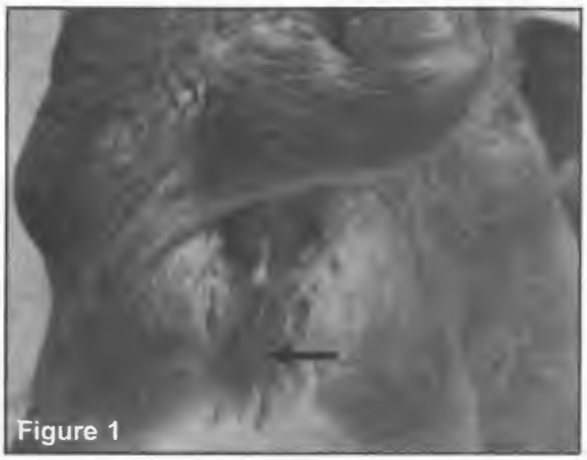

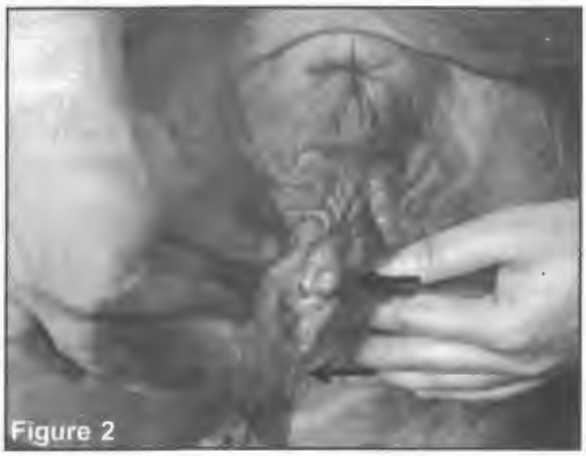

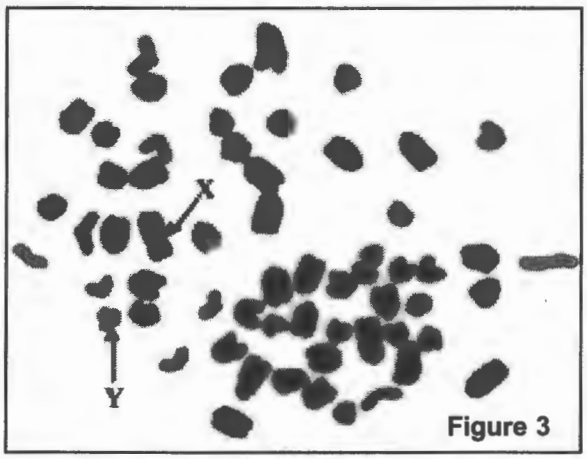

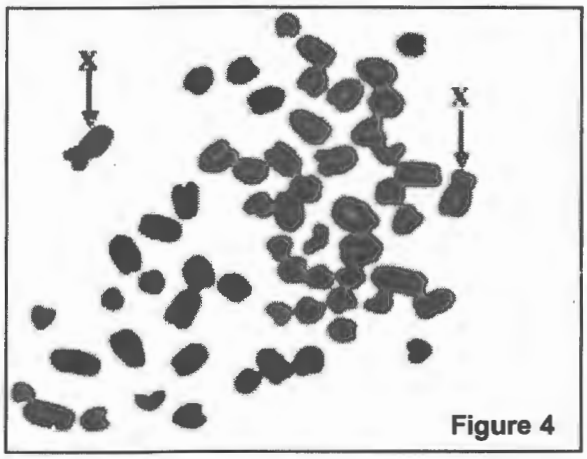

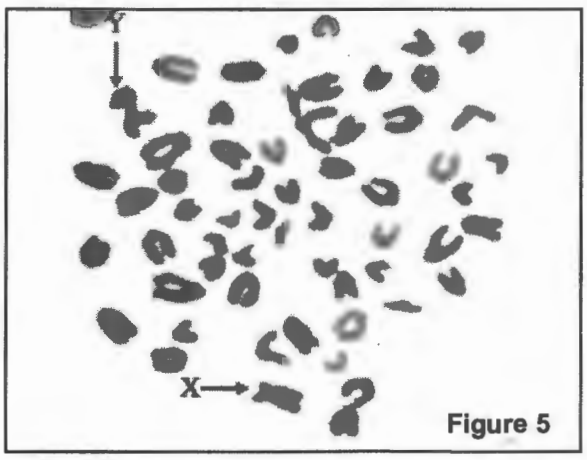

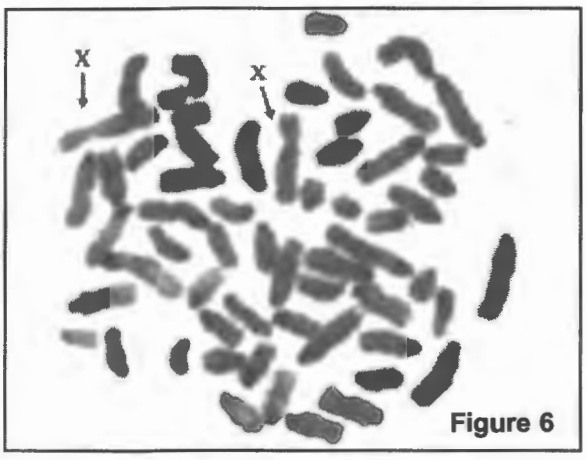

Figures 1-6: Showing vulvar hair tuft and clitoris in freemartins; Karan Fries (Fig 1) and Sahiwal (Fig 2) with quite large clitoris like glans penis. Figs 3-4: Conventionally stained two metaphase plates showing XY (left side) and XX (right side) chromosome complements of Karan Fries. Figs 5-6: C-banded metaphase plates showing by arrows XY (left side) and XX (right side) chromosome complements of Sahiwal freemartin