RESEARCH ARTICLE

Current Status of Reproductive Performance and Problems in Dairy Animals of Gujarat and their Amelioration including Study of Fertility Gene Markers

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Abstract

The surveillance of breedable 4711 zebu cattle, 8416 crossbred cattle and 15688 buffaloes (total 28,815) from 5 different regions of Gujarat revealed reproductive problems in 27.4, 26.6 and 34.5 % animals, respectively. Reproductive performance, reproductive problems, feeding, and housing practices varied among different classes of animals in different regions. Natural service was still the major means of breeding cattle and buffaloes in Saurashtra and Kutch region, while in middle, north and south Gujarat AI has been practiced for 70-90% breeding. Blood glucose, biochemical and minerals profile of infertile animals also varied greatly in different regions, and it was significantly improved following supplementations of chelated ASMM and bypass fat for 30-45 days. Among so called infertile animals (7390), the incidence of anestrus was maximum (23.34%) followed by repeat breeding (14.94%), subestrus (14.69%), uterine infection (12.91%), infantile genitalia/pubertal anestrus (9.64%), pregnancy or estrus (10.63%) and others (13.83%). Supplementation of minerals in combination with bypass fat in anestrus animals resulted in enhanced estrus response and conception rate as compared to supplementation of minerals and bypass fat alone. Biherbal therapy (pulverized Curry leaves and Bael leaves, 1:1) @ 200 g/day for 5 days modulated genital health and improved estrus expression and conception rate in anestrus, repeat breeding and endometritic bovines (n=75). With respect to molecular identification of fertility marker gene tried in more than 550 animals, targeting 6 specific genes, only LHCGR3 showed appreciable difference in gene frequencies between fertile and infertile crossbred HF cattle. 'T' allele was more prevalent in infertile crossbred cattle (50%), and 'C' allele in fertile crossbred cattle (100%). However, no such differences were observed in indigenous cattle and buffalo. It was also validated on 83 crossbred calves below 1 year of age by revealing genotype frequency of CC, TT and CT as 0.70, 0.11 and 0.19, and allele frequency for T and C allele as 0.20 and 0.80, respectively, predicting 80% of tested calves fertile. Thus, SNP 'T' in LHCGR3 may be involved in infertility at least in crossbred cattle.

Keywords: Dairy animals, Fertility gene, Gujarat, Infertility amelioration, Nutritional supplement, Reproductive problems.

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Introduction

For assessing the extent of infertility and its causes and investigating the gene markers for improving fertility in dairy animals, information on current status of reproductive performance, reproductive problems and feeding-housing practices followed by farmers from different regions of the State/country is mandatory (Dhami *et al.,* 2017, 2018). Livestock fertility is known to vary according to genotype, and the agro-climatic conditions, soil type, cropping pattern and management practices followed in different regions. Among the major causes of bovine infertility, nutritional infertility is the major one for resource poor farmers followed by functional, infectious, and pathological ones. Nutritional supplementations, particularly minerals, bypass fat, protein, and ethno-veterinary formulations are getting interest in veterinary field for its beneficial outcomes (Staples *et al.,* 1998; Boland and Lonergan, 2003; Anonymous, 2016-20; Dhami *et al.,* 2019). Fertility, a complex feature, is under the influence of numerous genes. There are large many QTL, markers, and candidate genes that are associated with bovine fertility (Hu *et al.,* 2007; Joshi *et al.,* 2018). Regions associated with fertility

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traits are found in each of the 30 bovine chromosomes, confirming the complexity of these polygenic traits. It is hard to specify the significant role of a single gene in fertility, requiring further molecular level studies to identify some specific molecule or gene for its use in predicting future fertility of an animal (Anonymous, 2016-20, Joshi *et al.,* 2017, 2018). Hence, an attempt was made to investigate these aspects and ameliorate the infertility in dairy animals of Gujarat by nutritional and biherbal supplementation.

Materials and Methods

Mass contact programmes were organized region wise in Gujarat, India during the year 2015-16 and 2016-17 at farmers' doorstep with preformed questionnaires, followed by sexual health camps, in randomly selected villages of Anand, Kheda and Mahisagar districts of middle Gujarat; Navsari and Surat districts of South Gujarat; Mehsana, Sabarkantha and Patan districts of North Gujarat; Junagadh and Bhavnagar district of Saurashtra region and Kutch district. This was done with the help of dairy co-operatives and state Animal Husbandry Department for collection of data on type of animals, land and animal holdings of farmers, productive-reproductive status and reproductive problems of their animals, feeding and housing practices followed etc. Moreover, annual 10 to 12 fertility improvement (FIP) camps were also organized during 2017-2020. The information on 6112 stakeholders/livestock farmers and 28815 animals were obtained. Further, 7390 so called infertile animals were screened per rectally and 5582 animals were identified to be infertile over 5 years period. All infertile animals identified were dewormed using Inj. Ivermectin s/c or Fendikind plus bolus 3.0 g orally and pour on liquid.

Blood (n=1740; annually 300–350 from 2017–20) from representative animals were collected for estimation of blood glucose, plasma progesterone (by RIA), total protein, cholesterol and minerals status without (280) and with (1460) nutrients supplementations. Depending upon the body condition score and reproductive status, the anestrus cattle and buffaloes (n = 1461) were then nutritionally supplemented with either chelated areas specific mineral mixture (ASMM) 2-kg/Mineral boli 15-20/bypass fat 2-kg alone or bypass fat + ASMM 2-kg each along with an advice to improve feeding level and revisits were made after 2.5–3.0 months to check the reproductive status. Selected anestrus and repeat breeder bovines (n=2165) with good to average BCS were taken up for various estrus synchronization protocols, the results of which are furnished separately (Dhami *et al.,* 2020). A biherbal pulverized formulation (Curry leaves and Bael leaves 1:1) was also tested for treatment of anestrus, repeat breeding and endometritis in 75 bovines @ 200 g per day/head for 5 days.

For fertility marker gene, blood samples from 330 normal cyclic and infertile animals were collected for first 2 years and their DNA extracted was targeted for polymorphism in 6 genes and its different exonic regions (LHR, LHRP6/9, LHRP8/11, LHCGR1, LHCGR3, GH, GnRH, ERα and FSHRP18/13). The fragments of these genes were amplified by gene specific primers and digested with appropriate restriction enzymes. Some SNPs showed monomorphism for genes GnRH, LHR and LHRP6/9, hence were dropped from further study. During subsequent years, 273 known fertile and infertile animals (Gir, Kankrej and CBHF cattle and Surti, Jaffarabadi and Mehsani buffalo) were targeted for SNP genotyping and fertility related genes. DNA extracted from the blood samples by John's method was quantified using Nano spectrophotometer. DNA samples were amplified with gene specific primers (Table 1); samples which were amplified properly were taken for further restriction digestion. PCR-RFLP for LHR, LHRP, GnRH, GH, LHCGR and ERα genes were done using HhaI, Hpy188I, AluI, Hpy166II, BglI and Tsp45I enzymes, respectively. Gene frequencies for major and minor alleles were estimated and compared between fertile and infertile groups, in which LHCGR3 gene was found associated with infertility only in crossbred cattle. Hence, in the last year 83 crossbred (HF x K) calves below 1 year of age were targeted for LHCGR3 gene frequency and was correlated with dams' fertility status.

The data were analyzed using descriptive statistics to arrive at mean ± SEs and per cent frequency of different parameters studied.

Results and Discussion

Surveillance of Reproductive Performance, Problems and Feeding Practices

The detailed observations of pooled data (of 5 regions of

Table 1: Primers used for PCR amplification of genes of different regions of Indian cattle/buffalo

Gene	*Regions*	*Primer Sequence (5'-3')*	*PCR Product Size (bp)*
LHRP	LHRP6/P9	F: TTATTCTGCCATCTTTGCTGAGA	270
		R: AGAAGTCTGCAAAGGAGAGGTTG	
	LHRP8/11	F: CAAACTGACAGTCCCCCGCTTT	300
		R: CCTCCGAGCATGACTGGAATGGC	
LHR	LHR	F: CAAACTGACAGTCCCCCGCTTT	303
		R: CCTCCGAGCATGACTGGAATGGC	
ERα	ERα	F: TTTGGTTAACGAGGTGGAG	248
		R: TGTGACACAGGTGGTTTTTC	
GnRH	GnRHP7/P8	F: AAACTACAACTGAATCAGTC	240
		R: TAGAGAGAAATATCCATATA	
GH	GH	F: TAGGGGAGGGTGGAAAATGGA	404
		R: GACACCTACTCAGACAATGCG	
LHCGR	LHCGR3	F: AAGATGGCACACCATCACCT	389
		R: CAGCTGAGATGGCAAAGA	
	LHCGR1	F: TCTGCCATCTTTGCTGAGAG	550
		R: GCAACACGGCAATGAGAGTA	

Gujarat) on productive-reproductive performance, feeding practices and reproductive problems in dairy animals are furnished in Table 2. The total number of zebu and crossbred cattle and buffaloes surveyed were 4711, 8416 and 15688, out of which 27.4, 26.6 and 34.5 % were having reproductive problems. The distribution of reproductive disorders noted is depicted in Fig. 1.

The reproductive parameters of 28815 breedable bovines studied revealed that the average age at first calving was higher in buffaloes (40.4 ± 1.90 months) and zebu cattle (40.1 ± 2.18 months) than in crossbred cattle (30.5 ± 1.92 months) and it varied with the regions and type of breed/species of animal. The mean numbers of calving were almost similar (2.3-2.9) in all three categories of animals. The mean calving interval (months) and postpartum estrus interval (days) were lower in crossbred cows (13.5 ± 0.49 and 67.4 ± 8.51) than in zebu cattle (16.3 ± 1.07 and 84.1 ± 8.89) and buffalo (17.2 ± 0.89 and 91.5 ± 10.18). A longer calving interval in buffaloes might be due to delayed postpartum breeding apart from summer stress, silent heat problems and low conception rates. The mean duration of estrus was almost similar in zebu cattle and buffalo (20-21 h). However, it was longer in crossbred cows (24–26 h). Charlini and Sinniah (2015) from Sri Lanka, and Singh *et al.* (2016) and Pareek *et al.* (2016) from India reported similar findings in buffaloes and cattle. Parmar *et al.* (2016) made similar observations in cattle and buffaloes in coastal areas of South Gujarat. Patel *et al.* (2014) opined that most dairy farmers (91.30%) bred their animals after 3 to 5 months postpartum and hence had extended calving interval.

Problem of silent heat was common in pluriparous buffaloes (14.27%) and buffalo heifers (23.32 %) which concurred with report of Modi *et al.* (2011) from Mehsana milk shed and of Parmar *et al.* (2016) and Dhami *et al.* (2017) in tribal and arid zones of Gujarat. Metestrus bleeding was recorded mainly in crossbred cattle (3.85%). The estrous cycle length reported was on an average 20-21 days in all classes of animals and concurred with Ingawale and Dhoble (2004) and Dhami *et al.* (2018). The highest incidence of anestrus was observed in buffalo heifers (23.9%) followed by crossbred heifers

Table 2: Productive-reproductive performance, feeding-housing practices in dairy animals

Details of 28815 Animals from 6112 Stakeholders						
	Breedable CB Cattle		*Breedable Zebu Cattle*		*Breedable Buffalo*	
Parameters	*Adult*	*Heifer*	*Adult*	*Heifer*	*Adult*	*Heifer*
Total No. of animals	5743	2673	3275	1436	9786	5902
Age (years)	6.2 ± 0.43	2.3 ± 0.21	7.2 ± 1.22	3.1 ± 0.36	7.6 ± 0.98	3.2 ± 0.38
No. of calving	2.9 ± 0.28	--	2.3 ± 0.55	--	2.8 ± 0.55	--
Age at first calving (months)	30.5 ± 1.92	--	40.1 ± 2.18	--	40.4 ± 1.90	--
Mean calving interval (months)	13.5 ± 0.49	--	16.3 ± 1.07	--	17.2 ± 0.89	--
Postpartum complications (%)	3.7 (222)	--	9.4 (308)	--	7.2 (707)	--
Postpartum estrus interval (d)	67.4 ± 8.51	--	84.1 ± 8.89	--	91.5 ± 10.18	--
Duration of last estrus (hrs)	26.1 ± 1.39	24.1 ± 1.85	21.4 ± 0.59	20.9 ± 0.96	20.3 ± 0.89	19.7 ± 1.09
Metestrus bleeding (%)	4.6 (267)	2.1 (57)	0.9 (30)	0.5 (07)	0.0 (00)	0.0 (00)
Treatment at last estrus (%)	8.3 (477)	4.9 (133)	7.3 (238)	6.6 (95)	8.5 (831)	8.9 (529)
Estrous cycle length (days)	21.3 ± 0.73	21.5 ± 1.06	21.6 ± 1.56	21.0 ± 0.78	20.5 ± 0.96	19.6 ± 1.01
Nature of Reproductive Problem						
Normal cyclic / Early PP (%)	37.96 (2180)	36.80 (986)	29.03 (950)	21.03 (302)	32.37(3168)	31.77(1875)
Pregnant (%)	39.11 (2246)	31.72 (848)	46.84 (1534)	44.08 (633)	35.48(3473)	29.56(1746)
Anoestrus (%)	8.09 (465)	17.02 (455)	9.53(312)	13.72 (197)	12.24(1198)	23.94(1413)
Repeat breeder (%)	12.41 (713)	12.19 (326)	11.02 (361)	15.60 (224)	16.06(1572)	11.61 (685)
Milk yield (liters/day)	10.12 ± 0.88	--	7.47 ± 0.94	--	7.56 ± 0.98	--
Peak milk yield (liters/day)	12.03 ± 1.24	--	9.85 ± 1.19	--	9.43 ± 0.75	--
Lactation length (days)	285.62 ± 7.36	--	272.49 ± 17.82	--	287.56 ± 12.52	--
Feeding & Housing						
Roughage: Green / Dry (kg)	15.26 ± 0.69 / 8.82 ± 0.67		13.46 ± 1.18 / 8.29 ± 0.93		15.75 ± 1.14 / 9.54 ± 0.54	
Concentrate (kg)/ Min. Mix.(g)	3.98 ± 0.50 / 51.28 ± 2.96		1.87 ± 0.61 / 38.51 ± 1.61		3.12 ± 0.78 / 43.27 ± 3.16	
% Animal fed mineral mixture	41.04 (3454)		12.82 (604)		23.08 (3621)	
% Animal fed salt	18.01 (1516)		6.26 (295)		13.14 (2061)	
Housing: Close confinement(%)	70.59 (5941)		37.97 (1789)		57.83 (9073)	

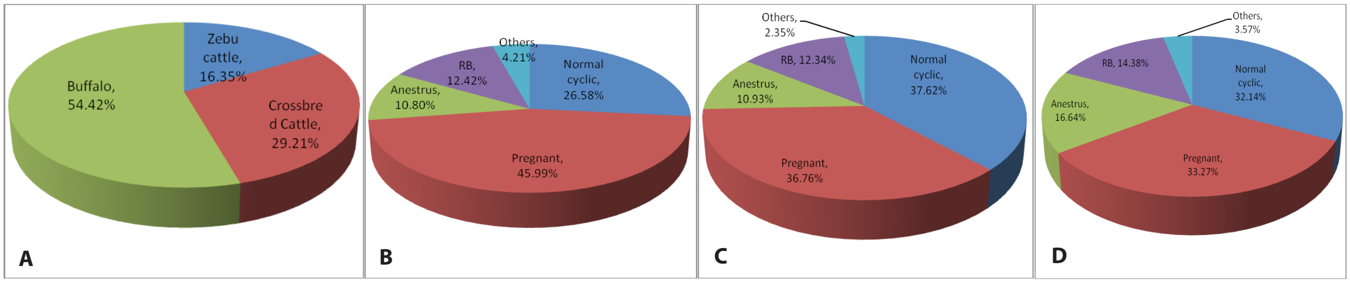

Fig. 1: (A) Frequency of animals surveyed in Gujarat, and the reproductiver status in (B) Zebu cattle (C) CB cattle and (D) Buffaloes surveyed

(17.0%) and the lowest in crossbred cows (8.1%), while repeat breeding problem was more in zebu heifers and buffalo (15-16%) and lower in other classes (11-12%) (Table 2). The incidence of anestrus found in buffaloes agreed with Modi *et al.* (2011), while Kumar *et al.* (2013, 2014) reported higher incidence, and Selvaraju *et al.* (2005) reported lower incidence of anestrus in buffaloes as compared to present finding. The incidence of repeat breeding in buffaloes was higher than that reported by Modi *et al.* (2011), but in crossbreds it agreed with Mohteshamuddin *et al.* (2012). Both these problems varied in different regions and in different breed/species of animals may be due to difference in soil type, quality of feed/ fodder and breeding services available in respective regions. The endometritis, abortion and other reproductive disorders were negligible in the animals surveyed, which concurred with Durgesh *et al.* (2009) and Dhami *et al.* (2017, 2018) in buffaloes of the Malwa region of Madhya Pradesh and tribal and arid regions of Gujarat. In major milk shed areas, 95–99% dairy animals are bred by AI, while in Suarashtra and Kutch region and adjoining parts of North Gujarat, natural service is still the major means of breeding cows and buffaloes as has been reported by Parmar *et al.* (2016) and Dhami *et al.* (2019).

Daily milk yield and peak yield were higher in CBHF cattle than zebu cattle and buffaloes, however lactation length in zebu cattle was shorter than CBs and buffaloes which concurred with reports of Rakshe (2003) and Patel *et al.* (2014). The shorter lactation length in zebu cattle observed was due to their inherent dextirity and resource poor owners. The average green and dry fodder fed to the cows and buffaloes were 15 to 17 kg and 8 to 9 kg per day, respectively. The amount of concentrate feed supplied was more in lactating crossbred cattle 4 kg and least in zebu cattle 2 kg per day. Very few farmers fed concentrate to their unproductive animals and heifers. These findings on feeding were in accordance with Rao *et al.* (2015), who stated that farmers in Gujarat fed compounded concentrate of co-operative dairies mainly to their lactating and advanced pregnant animals. Approximately 41 % crossbred cows and 13-23% zebu cows and buffaloes were supplemented with mineral mixture, and salt supplement was in less than 18% animals only with misconception that these are already added in compounded concentrates. These observations concurred well with those of Sabapara *et al.* (2010) from

Table 3: Incidence of major reproductive problems in so called infertile dairy bovines

		Overall	
Sl. No.	*Infertility conditions*	*No.*	*Per cent*
1	Anestrus	1725	23.34
2	Subestrus	1086	14.69
3	Repeat breeding	1104	14.94
4	Uterine infection	954	12.91
5	Infantile genitalia*	713	09.64
6	Estrus/AI	145	01.96
7	Pregnant	641	08.67
8	Others	1022	13.83
Total cases		7390	100.0

* Pubertal anestrus

South Gujarat. Further, 37.8% zebu cows were kept in close confinement. However, buffaloes and crossbred cows kept in close confinement were higher, 57.8 and 70.6 %, respectively. Around 58 to 80 % owners tended to provide drinking water to their animals *ad lib/* 3 times per day in different regions of Gujarat. Similar observations on housing and watering were also made by Sabapara *et al.* (2010), Patel *et al.* (2013), Rao *et al.* (2015) and Dhami *et al.* (2018). Kharadi *et al.* (2006) also reported that majority of farmers (56 %) in Charotar region of middle Gujarat provided close house during day and night to their animals.

Prevalence of Infertility and its Amelioration:

The findings on gynaeco-clinical examinations of so called infertile cattle and buffaloes presented by the farmers in different SHC camps, and their response to nutritional management are presented in Tables 3 and 4, respectively.

Among 7390 so called infertile animals examined, the incidence of anestrus (23.34%) was maximum followed by repeat breeders (14.94%), subestrus (14.69%), uterine infection (12.91%) and infantile genitalia/pubertal anestrus (9.64%). Moreover, 8.67 and 1.96% animals were found to be pregnant and in estrus, respectively (Table 3). Among heifers >3 years of age, infantile genitalia and poor BCS due to malnutrition were the main causes of post-pubertal anestrum. The findings clearly revealed the trend of major causes of infertility in dairy animals under field condition in the area surveyed and concurred well with the previous

Table 4: Estrus response and conception rate within 90 days of supplementation of ASMM, Mineral boli and bypass fat in anestrus cattle and buffaloes

	Cattle			*Buffalo*		
Supplement	*No Supplied*	*No (%) responded*	*No (%) Conceived*	*No Supplied*	*No (%) responded*	*No (%) Conceived*
ASMM	225	94 (41.8)	37 (39.4)	297	110 (37.0)	37 (33.6)
Bypass fat	55	27 (49.1)	14 (51.8)	50	22 (44.0)	10 (45.5)
Min+Bypass fat	175	96 (54.9)	45 (46.9)	219	107 (48.9)	55 (51.4)
Mineral boli	187	72 (38.5)	25 (34.7)	253	90 (35.6)	31 (34.4)
Overall	642	289 (45.0)	121 (41.9)	819	329 (40.2)	133 (40.4)

Figures in parentheses indicate per cent values.

reports from the same areas (Butani *et al.,* 2008; Hadiya *et al.,* 2014; Dhami *et al.,* 2017, 2019).

Various workers have studied the effect of mineral supplementation on estrus induction and conception in dairy animals. Supplementation of ASMM, mineral boli, bypass fat alone or in combination in anestrus animals resulted in 36-55% estrus response and 34-52% conception rate, both being better with mineral plus bypass fat supplementation followed by bypass fat alone than ASMM or mineral boli (Table 4). The overall estrus induction response in anestrus cattle and buffaloes observed under present study was 45.0 and 40.2%, and CRs 41.9 and 40.4%, respectively. Among the non-estrus detected/non-bred and non-conceived animals, majority (65-80%) were having functional ovarian structure when examined 2.5-3.0 months later following one month of mineral and/or bypass fat supplementation compared to non-supplemented group. These results proved the nutritional cause of infertility and beneficial role of minerals and bypass fat supplementation in improving reproductive performance in such animals and concurred with the previous observations from the same and other stations (Anonymous, 2016-20; Dhami *et al.,* 2019).

The deficiency of phosphorus and/or improper calcium and phosphorus ratio cause anestrus condition in dairy animals or may arrest the phenomenon of fertilization resulting in the repeat breeding. In the present study, satisfactory estrus induction and conception rate were observed which may be due to combined effect of various macro-micro minerals and bypass fat which have positive effects on steroidogenesis, follicular growth and symptoms of ovulatory estrus (Singh *et al.,* 2011; Anonymous, 2016-20). Srivastav (2008) reported that supplementation of 30-40 g commercial mineral mixture daily in concentrate for 20 days induced ovulatory estrus in 93.93 % of anestrus crossbred heifers with first service conception rate of 32.14 %. Devasenat *et al.* (2010) recorded 59 to 62 % estrus response among anestrus heifers and cows following mineral supplementation, while Puvarajan and Vijayarajan (2013) reported estrus induction in 92.16 % (153/166) of pubertal crossbred heifers over three months period following one month of ASMM supplementation, and the first service conception rate recorded by them was 28.18 % (42/149).

Blood Plasma Profile

The blood plasma profiling of representative infertile animals from different regions revealed that the plasma progesterone profile varied from basal (<0.1 ng/ml) in anestrus animals to as high as 15 ng/ml in some crossbred cows with ovarian disorders and persistent CL. Blood glucose varied from 24 to 64 mg/dl, it was lower in heifers and anestrus animals, and higher in normal cyclic/fertile buffaloes and crossbred cows. The plasma protein values ranged from 5.5 to 9.5 g/dl in different categories of animals, lower in buffaloes and infertile heifers than cows. Plasma total cholesterol in cows was 1.5-2.0 folds higher than in buffaloes and it did not reveal clear trend with respect to fertility status of animals. The calcium: phosphorus ratio varied from 1:1 to 1.8:1, and plasma zinc and iron levels were quite different in animals of different regions of the state indicating its role in causing infertility in dairy animals. The plasma levels of calcium, phosphorus and micro-minerals zinc, iron and copper increased significantly following ASMM supplementation (Dhami *et al.,* 2019). Overall, there was improved status of energy, protein and minerals with elevated plasma progesterone around 45-90 days post-supplementation. However, the results varied greatly according to agro-climatic zones, soil types and cropping pattern in different regions of Gujarat.

Plasma or serum proteins bear a close relationship with the tissue proteins and development of endocrine glands, and its deficiency retards the development of reproductive organs leading to failure or delay in onset of postpartum estrus (Roberts, 1986). Cholesterol is the most important sterol essential for life and a precursor for steroidogenesis in the body (Mc Donald, 1980). Kumar *et al.* (2009) and Butani (2013) reported significantly lower serum total protein and cholesterol in repeat breeders than in anestrus and subestrus cows and buffaloes, while serum calcium was significantly ($p<0.01$) lower and phosphorus higher in both anestrus and repeat breeding cows and buffaloes than in subestrus and normal cyclic ones. It was demonstrated that cows with higher milk yield and sub-optimal nutrient intake suffer from greater body weight losses and reduced fertility (Santos *et al.,* 2009). Feeding of supplemental fat to dairy cows improves fertility owing to availability of energy and specific fatty acids that alter oocyte competence and embryo quality (Cerri *et al.,* 2009).

Table 5: Effect of biherbal formulation on clinical recovery and conception rate in repeat breeder, anestrus and endometritic bovines.

			%Conception rate		
Clinical Status	*No. of cases*	*No. (%) responded*	*1st cycle*	*2nd cycle*	*Overall*
Repeat breeders	35	35 (100.0)	48.6 (17/35)	38.9 (7/18)	68.6 (24/35)
Endometritis	28	24 (85.7)	48.8 (11/24)	50.0 (6/12)	60.7 (17/28)
Anestrus	12	09 (75.0)	44.4 (04/09)	50.0 (2/4)	50.0 (06/12)
Overall	75	68 (90.7)	47.1 (32/68)	44.1 (15/34)	62.7 (47/75)

Table 6: Frequency of alleles and genotypes for the reproduction specific targeted 5 genes

	Genotypic Frequency		*Gene Frequency*	
Gene	*Fertile*	*Infertile*	*Fertile*	*Infertile*
LHRP6/P9	GG = 0 AA = 1.0 AG = 0	GG = 0 AA = 1.0 AG = 0	G = 0 A = 1.0	G = 0 A = 1.0
LHR	TT = 1.0 CC = 0 CT = 0	TT = 1.0 CC = 0 CT = 0	T = 1.0 C = 0	T = 1.0 C = 0
ERα	GG = 0.90 AA = 0 AG = 0.10	GG = 0.39 AA = 0 AG = 0.61	G = 0.95 A = 0.05	G = 0.70 A = 0.30
GnRHP7/P8	TT = 1.0 CC = 0 CT = 0	TT = 1.0 CC = 0 CT = 0	T = 1.0 C = 0	T = 1.0 C = 0
LHCGR3	CC = 0.75 TT = 0 CT = 0.25	CC = 0.92 TT = 0 CT = 0.08	C = 0.875 T = 0.125	C = 0.96 T = 0.04

According to Akhtar *et al.* (2004) nutritional deficiencies combined with worm infestation play a major role in infertility of crossbred heifers and cows. Anestrus and repeat breeder buffaloes have been reported to respond favourably to daily supplementation of 30 g ionic or chelated mineral mixtures with 50–70% conception rates (Butani, 2013; Joshi *et al.,* 2020).

Validation of Herbal Formulation for Ameliorating Infertility

A total of 75 infertile cattle/buffaloes identified under field and farm conditions were subjected to biherbal treatment {pulverized Curry leaves (*Murraya koenigii*) and Bael leaves (*Aegle marmelos*), 1:1} @ 200 g daily PO for 5 days. The biherbal therapy resulted in improved mucus quality at subsequent next estrus in infertile animals. The normal estrus response occurred in 100, 85.7 and 75.0% of repeat breeder, endometritic and anestrus animals. The first service conception rates were fairly good for all three groups. The overall first and second cycle conception rate was 47.1 and 44.1 % respectively and overall two cycles post-treatment conception rate was 62.7%. The conception rate was better in repeat breeder (68.6%) followed by endometitic (60.7%) animals and least in anestrus ones (50.0%) (Table 5). These findings concurred with earlier observations on similar herbal supplements from Gujarat, Odissha, Tamilnadu and Uttar Pradesh (Anonymous, 2016-20),

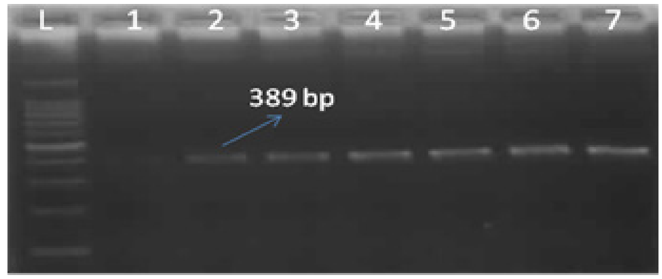

Fig. 2a: PCR amplification of samples in LHCGR3 (389 bp)
Lane 1: 100 bp Ladder, Lane 2-7 PCR products

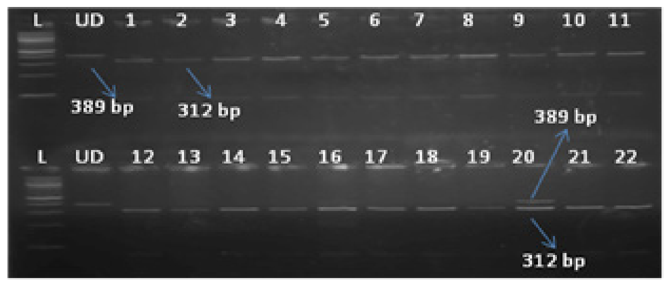

Fig. 2b: Restriction pattern of samples for LHCGR3
Lane 1: 100 bp Ladder, Lane 2: Undigested, Lane 3-13 above: 1-11 samples, below 12-22 samples

Gene Markers for Infertility

The frequency of genotypes and alleles obtained for targeted fertility genes are shown in Table 6. Out of 5 genes investigated with their fragment regions, GnRH, LHR and LHRP6/9 were found monomorphic in both fertile and infertile groups, hence were dropped from further study, while ERα and LHCGR3 were polymorphic and their genotypic and gene frequency varied between normal and infertile animals as shown in Table 6.

Further, based on genotyping of SNPs of 273 animals of indigenous, crossbred cattle and buffalo breeds, the gene frequencies of six selected genes in fertile and infertile groups of animals are shown in Table 7. Out of 5 genes targeted, only LHCGR3 (Leuteinizing hormone/Chorionic gonadotropin receptor) showed appreciable difference in gene frequencies between fertile and infertile crossbred cattle, but not in indigenous cattle or buffalo. The allele frequency of LHCGR between fertile and infertile groups revealed that 'T' allele was more prevalent in infertile crossbred cattle (0.50, *i.e.,* 50%), and 'C' allele in fertile crossbred cattle (1.00, *i.e.,* 100%). However, no such differences were observed in indigenous cattle and buffalo. Thus, it is presumed that SNP 'T' in LHCGR

Table 7: Comparative allelic frequency in indigenous cattle, crossbred cattle and buffaloes

	Allele and Allele Frequency (273 animals)								
	Indigenous Cattle (96)			*Buffalo (111)*			*Crossbred Cattle (66)*		
Gene Targeted	*Allele*	*Normal*	*Infertile*	*Allele*	*Normal*	*Infertile*	*Allele*	*Normal*	*Infertile*
Erα	A-	0.11	0.18	A-	0.13	0.26	A-	0.23	0.30
	G-	0.89	0.82	G-	0.88	0.74	G-	0.78	0.70
GH	C-	0.01	0.02	C-	0.13	0.01	C-	0.20	0.09
	G-	0.99	0.98	G-	0.88	0.99	G-	0.80	0.91
GnRH	C-	0.81	1.00	C-	0.88	1.00	C-	1.00	1.00
	T-	0.19	0.00	T-	0.13	0.00	T-	0.00	0.00
LHR	T-	0.81	0.78	T-	1.00	1.00	T-	0.80	0.47
	C-	0.19	0.22	C-	0.00	0.00	C-	0.20	0.53
LHRP6/9	G-	0.18	0.13	G-	1.00	0.95	G-	0.10	0.32
	A-	0.82	0.88	A-	0.00	0.05	A-	0.90	0.64
LHCGR3	T-	0.28	0.22	T-	0.39	0.33	T-	0.00	0.50
	C-	0.72	0.78	C-	0.61	0.67	C-	1.00	0.50

Table 8: Genotype and gene frequency of crossbred calves (n=83) studied for LHCGR3 gene

Gene	*Genotype*	*Obtained*	*Genotype Freq.*	*Allele*	*No of allele*	*Allele Freq.*
	TT	9	0.11	T-	34	0.20
LHCGR3	CC	58	0.70	C-	132	0.80
	CT	16	0.19	-	-	-

may be involved in infertility at least in crossbred cattle (Joshi *et al.,* 2017, 2018; Anonymous, 2016-20).

It was verified by presence of similar gene frequency in 83 young crossbred calves of fertile and infertile crossbred dams, wherein the restriction site on 389 bp of LHCGR3 gene fragment with C1470T showed fragments of 312 bp and 77 bp (Fig. 2a,b), and the genotype frequency of CC, TT and CT was 0.70, 0.11 and 0.19, respectively, and allele frequency for T and C allele was 0.20 and 0.80, respectively (Table 8), thus predicting 80% of tested calves fertile (Anonymous, 2016–20).

Conclusions

The surveillance study revealed that the reproductive performance, reproductive problems, feeding and housing practices vary greatly among different classes of animals in different regions of Gujarat according to agro-climatic conditions, soil type, cropping patterns and management systems. Among so called infertile animals, the key issues are anestrus, repeat breeding, subestrus, uterine infection and infantile genitalia/pubertal anestrus. Chelated ASMM supplementations improve blood metabolic profile and nutritional status of animals and enhance estrus response and conception rate. Polyherbal therapy for 5 days also modulates genital health and improves estrus expression and conception rate in infertile bovines. Among 6 specific genes targeted as fertility markers, only LHCGR3 showed appreciable difference in gene frequencies between fertile and infertile crossbred HF cattle, 'T' allele being more prevalent in infertile crossbred cattle (50%), and 'C' allele in fertile crossbred cattle (100%). However, no such differences were observed in indigenous cattle and buffalo.

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References

Akhtar, M.H., Samantaray, S., Kumar, A., Roy, G.P., & Singh, A.P. (2004). Effect of deworming and vitamin-mineral supplementation in crossbred heifers. *Intas Polivet, 5*(1), 43-45.

Anonymous (2016-20). *Annual Reports of years 2016 to 2020 of AICRP on nutritional and physiological interventions for enhancing reproductive performance in animals,* ICAR-NIANP, Bangalore, India.

Boland, M.P., & Lonergan, P. (2003). Trace minerals in production and reproduction in dairy cows. *Advances in Dairy Technology, 15*, 319-330.

Butani, J.B. (2013). Supplementation of appropriate mineral mixture to anestrus and repeat breeder buffaloes in tribal areas of Panchmahal district. *MVSc Thesis*, Anand Agricultural University, Anand, Gujarat, India.

Butani, M.G, Kumar Rajesh, Dhami, A.J., Kavani, F.S., & Killedar A. (2008). Incidence of major infertility problems in crossbred cows and buffaloes under field conditions. *Indian Journal of Field Veterinarians*, *4*(2), 1-4.

Cerri, R.L.A., Juchem, S.O., Chebel, R.C., Rutigliano, H.M., Bruno, R.G.S., Galvão, K.N., Thatcher, W.W., & Santos, J.E.P. (2009). Effect of fat source differing in fatty acid profile on metabolic parameters, fertilization and embryo quality in high-producing dairy cows. *Journal of Dairy Science*, 92, 1520-1531.

Charlini C.B., & Sinniah, J. (2015) Performance of Murrah, Surti, Nili-Ravi buffaloes and their crosses in the intermediate zone of Sri Lanka. *Livestock Research for Rural Development,* 27, Webpage http://www.lrrd.org/lrrd27/3/ char27047.html

Devasenat, B., Reddy, I.J., Ramana, J.V., Prasad, P., & Prasad, J. (2010). Effect of supplementation of area specific mineral mixture on reproductive performance of crossbred cattle - A field study. *Indian Journal of Animal Nutrition,* 27(3), 265- 270.

Dhami, A.J., Parmar, S.C., Hadiya, K.K., Patel, J.A., & Parmar, C.P. (2017). Dairy husbandry practices and problems of dairy farmers of tribal and non-tribal areas of Middle Gujarat. *Indian Journal of Dairy Science, 70*(3), 351-356.

Dhami, A.J., Parmar, S.C., & Patel, J.A. (2018). Productive-reproductive performance and problems of dairy animals in arid and semi-arid areas of Kutch and North Gujarat. *International Journal of Livestock Research, 8*(1), 121-128.

Dhami, A.J., Patel, J.A., Hadiya, K.K., Parmar, S.C., & Chaudhari, D.V. (2019). Nutritional infertility and ameliorative measures in dairy animals of middle Gujarat. *The Indian Journal of Veterinary Science & Biotechnology, 14*(3), 5-9.

Dhami, A.J., Patel, J.A., Hadiya, K.K., Chaudhari, D.V., & Panchal, M.T. (2020). Estrus induction and ovulation synchronization in infertile animals: A novel technology towards doubling the dairy farmers' income. A Folder Published by the authors under AICRP, Veterinary College, AAU, Anand, pp. 1-12.

Durgesh, M., Garg, U.K., Shukla, S,, & Jadav, G.P. (2009). Prevalence of different pathological affections of uterus in buffaloes (*Bubaliis bubalis*) in the Malwa region of Madhya Pradesh. *Buffalo Bulletin, 28*, 215-217.

Hadiya, K.K., Patel, J.A., Shah, R.G., Panchal, M.T., & Dhami, A.J. (2014). Monthly pattern of inseminations and reproductive health disorders in dairy bovines of middle Gujarat. *Indian Journal of Field Veterinarians, 9*(3), 28-33.

Hu *et al*. (2007). http://genomes.sapac. edu.au/ bovineqtl/

Ingawale, M.V., & Dhoble, R.L. (2004) Buffalo reproduction in India: An overview. *Buffalo Bulletin, 23,* 4-9

Joshi, P.M., Patel, D.C, Patel, P.D., & Sarvaiya, N.P. (2020). Effect of chelated mineral mixture on blood profile and fertility in anoestrus buffaloes of tribal areas of Dahod district in Gujarat, India. *The Indian Journal of Veterinary Science & Biotechnology, 15*(3), 61-65.

Joshi, R.S., Rank, D.N., Dhami, A.J., & Mankad Pooja (2017). Molecular identification of specific gene marker for infertility in dairy animals. In: *Proceedings of 5th Annual Convention of SVSBT and National Seminar on "Opportunities and Challenges of Translational Research in the Frontier Areas of Animal Biotechnology"* held at OUAT, Bhubaneswar, India, September 22-23, P. 56-57.

Joshi, R.S., Rank, D.N., Dhami, A.J., & Zala Hinal (2018). Infertility gene markers: molecular identification in dairy animals. In: *Proceedings of 34th ISSAR Convention and "International Symposium on "Productivity Enhancement through Augmenting Reproductive Efficiency of Livestock for Sustainable Rural Economy"* held at Veterinary College, AAU, Anand, India, December 28-30, p. 123.

Kharadi, V.B., Desai, P.M., Sabapara, G.P., Vij, P.K., Singh, P.K., Singh, G, & Ahlawat, S.P.S. (2006) *Buffalo Genetic Resources of India* - Surti. Network Project on Animal Genetic Resources (ICAR), NBAGR, Karnal, India

Kumar, P.R., Shukla, S.N., Shrivastava, O.P., & Purkayastha, R.D. (2013). Incidence of postpartum anestrus among buffaloes in and around Jabalpur. *Veterinary World, 6*, 716–719.

Kumar, P.R., Singh, S.K., Kharche, S.D., Chethan Sharma, G., Behra, B.K., Shukla, S.N., Kumar, H., Agarwal, S.K. (2014) Anestrus in cattle and buffalo: Indian perspective. *Advances in Animal and Veterinary Sciences, 2,* 124-138.

Kumar Rajesh, Butani, M.G., Dhami, A.J., Kavani, F.S., & Shah, R.G. (2009). Effects of different therapies on fertility and serum progesterone, metabolites and minerals profile in repeat breeding crossbred cows. *Indian Journal of Field Veterinarians, 5*(2), 1-8.

Mc Donald, L.E. (1980). *Veterinary Endocrinology and Reproduction*. 3rd edn, Lea and Febiger, Philadelphia, USA.

Modi, L.C., Patel, P.A., Patel, S.P., Patel, G.G., Joshi, A.H., & Suthar, D.N. (2011). Prevalence of reproductive problems in buffalo in Mehsana milk-shed area of Gujarat. *International Journal of Agro-Veterinary Medical Sciences, 5,* 424-428.

Mohteshamuddin, K., Dhabale, R.B., Prakash, N., Tandle, M.K., & Awati, B. (2012). Incidence of repeat breeding in cattle of Bidar taluka of Karnataka, India. *International Journal of Agro-Veterinary Medical Sciences, 6,* 11-13.

Pareek, N.K., Ankuya, K.J., Patel, M.P., Joshi, N.A., Rathod, B.S., Prajapati, K.B., & Patel, J.B. (2016). Factors affecting age at first calving in Kankrej cattle. *Journal of Animal Research, 6,* 127-130.

Parmar, S.C., Dhami, A.J., Parmar, C.P., & Chaudhary, M.M. (2016). Animal husbandry practices and problems of dairy farmers in coastal areas of South Gujarat. *The Indian Journal of Veterinary Science & Biotechnology, 12*(2), 12-17.

Patel, N.B., Kavad, S.D., & Rao, T.K.S. (2014). Eco-friendly livestock management practices followed by tribal households of Narmada valley region of India. *Journal of Applied and Natural Science, 6,* 512-518.

Patel, N.B., Patel, J.B., Rao, T.K.S., Singh, R.R., & Modi, R.J. (2013). Housing management practices of dairy animal in Patan district of North Gujarat reg ion. *Indo-American Journal of Agricultural and Veterinary Sciences, 1,* 31-37.

Puvarajan, B., & Vijayarajan, A. (2013). Effect of area specific mineral supplementation in anoestrous crossbred heifers. *Indian Journal of Field Veterinarians, 8*(4), 43-45.

Rakshe, P.T. (2003). Effect of age at first calving and subsequent period of breeding on the performance of buffaloes from the college of agriculture Pune (MS), India. *Buffalo Bulletin, 22*, 7-11.

Rao, T.K.S., Patel, N.B., & Tyagi, K.K. (2015). Eco-friendly housing and feeding management of dairy animals in tribal area of high rain coastal India. *Research in Environment and Life Science, 8*, 75-80.

Roberts, S.J. (1986). *Veterinary Obstetrics and Genital Diseases*. 3rd edn., Ithaca, New York, USA.

Sabapara, G.P., Desai, P.M., Kharadi, V.B., Saiyed, L.H., & Singh, R.R. (2010). Housing and feeding management practices of dairy animals in the tribal area of South Gujarat. *Indian Journal of Animal Sciences, 80*, 1022-1027.

Santos, J.E.P., Rutigliano, H.M., & Sá Filho, M.F. (2009). Risk factors for resumption of postpartum cyclicity and embryonic survival in lactating dairy cows. Animal Reproduction Science, *110*, 207-221.

Selvaraju, M., Veerapandian, C., Kathiresan, D., & Chandrahasan, C. (2005). Incidence of bovine reproductive disorders. *Indian Veterinary Journal, 82,* 556.

Singh, K.P., Singh, B., Singh, J.P., Singh, S.V., Singh, P., & Singh, H.N. (2011). Mineral and salt supplementation for anoestrous crossbred heifers. *Indian Veterinary Journal,, 88*(4), 31-32.

Singh, B., Pushkraj, S., Dhruvaraj, S., Gautam, D., & Sachin, T. (2016). Study of productive traits in Sahiwal x Holstein Friesian crossbred cows - Frieswal. *Indian Journal of Animal Research, 50*, 425-429.

Snedecor, G.W., & Cochran, W.G. (1994). *Statistical Methods*. 8th edn. Iowa State University Press, Ames, Iowa, USA.

Srivastav, S.K. (2008). Effect of mineral supplementation in oestrus induction and conception in anoestrus crossbred heifers. *Indian Journal of Animal Sciences, 78*, 275.

Staples, C.R., Burke, J.M., & Thatcher, W.W. (1998). Influence of supplemental fats on reproductive tissues and performance of lactating cows. *Journal of Dairy Science, 81*, 856-871.