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Research Article **Open Access**

A Rare Case of Athelia in A Cross Breed Cow

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Abstract A three-year-old Jersey cross breed cow was referred with the history of recent calving and congenital absence of three teats. Clinical examination revealed that the udder was normal and engorged with milk. The right fore teat was normal in shape without any physical abnormalities. There was absence of left fore, left hind and right hind teats and they were represented by small eruptions. Permanent therapeutic cessation of lactation was achieved successfully by intramammary infusion of each affected quarter with 120 ml of 5 % povidone iodine solution.
Keywords *Athelia; Cross Breed Cow; Cessation of Lactation; Povidone Iodine*

1. Introduction

The udder is very important organ and economic value in dairy cattle. Though highly vulnerable to various disease conditions eg. Mastitis, congenital anomalies in the udder are of rare in occurrence (Dandale et al., 2013). Congenital abnormalities of the mammary system in cows comprise absence of teats, glands, supernumerary teats and imperforate teats. Absence of teat is extremely rare, but isolated cases in which the teats were only represented by slight eminences have been met with (O' Connor, 1980). Athelia was reported in buffaloes by Sailendra and Sandhya (1998) and Vidyasagar (2009) and in a Japanese Black Heifer by Ghanem et al., (2011). In the present paper, a rare case of athelia in a Jersey cross breed cow and its therapeutic management by permanent cessation of lactation was reported.

2. Case History and Observations

A three-year-old Jersey cross breed cow was presented with the history that the animal calved 2 days back and milking was not possible as there was congenital absence of three teats. Anamnesis revealed that the cow was born of artificial insemination and it's birth weight was 25 kg. At the age of 20 months it attained puberty and it was inseminated during third heat. The animal calved a female calf normally without experiencing any difficulty and the calf did not show any congenital abnormalities. Clinical examination of the udder revealed that the udder was normal and engorged with milk. The animal evinced pain on palpation of the udder. The right fore teat was normal in shape without any physical abnormalities. There was absence of left fore, left hind and right hind teats and

they were represented by small eruptions (Figures 1 and 2). Needle aspiration from the eruptions resulted drainage of colostrum. Based on the clinical symptoms the case was confirmed as Athelia in three quarters.

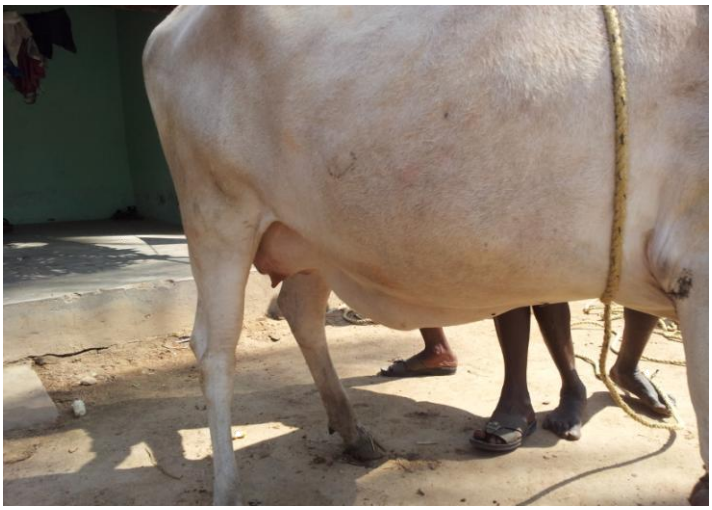

Figure 1: *Absence of three teats in a Jersey cross breed cow*

Figure 2: *Udder showing absence of three teats*

3. Treatment and Discussion

In cows, the udder is a very important organ and of economic value in producing milk for offspring and for other economical purposes. Since surgical correction was not possible, the owner of the present case was advised to cull the cow due to its mammary abnormality. But the owner wanted to maintain the animal for sentimental reasons. Hence it was decided to use povidone iodine for therapeutic cessation of lactation in the three athelia quarters. The colostrum was completely aspirated out from the erupted points of athelia quarters using 18 G needle. The cow was treated with 300 mg of Inj. Flunixin meglumine (Inj. Megludine, Virbac Animal Health, India) intramuscularly in order to minimize udder inflammation and counteract the effects of any aberrant endotoxin or pyrogens introduced during the infusion. Fifteen minutes later, each quarter of athelia was infused with 120 ml of 5% povidone iodine solution (Vetadine solution, Geevet Remedies, India). Treated

mammary quarters were not milked for the rest of the lactation. The degree of mammary quarter inflammation noted following infusion was minimal. It was observed that the povidone iodine eliminated all the treated mammary quarters from lactation permanently. The owner was advised to avoid further breeding of the cow and not to utilize the calf for breeding.

The presence of teats is undoubtedly controlled by genes either single, pair or a few pairs of genes and therefore the athelia condition may be the result of mutation in gene(s) as reported by Verma et al., 1983. Parathyroid hormone-like hormone gene (PTHLH) and the parathyroid hormone/parathyroid hormone like hormone receptor 1 (PTHR1) are functional candidate genes for traits related to mammary gland and teat development (Tetzlaff et al., 2009).

Presently, there are no approved products for therapeutic cessation of lactation. Intramammary infusion of povidone iodine for therapeutic cessation of lactation in cows constitutes an extra label use. Povidone iodine is very effective in completely eliminating all secretion from the treated mammary gland quarters and it appears to be the best choice for therapeutic cessation of lactation (Middleton and Fox, 2001) as also observed in the present case.

4. Conclusion

A rare case of athelia and therapeutic cessation of lactation using povidone iodine solution in a Jersey cross breed cow is reported.

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Research Article **Open Access**

Estrus Response and Conception Rate in Sangamneri and Osmanabadi Goat Does using different Estrus Synchronization Protocols

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Abstract Total 104 parous goats of Sangamneri (62) and Osmanabadi (42) breeds aged 2 - 4 years were used in this study. All goats were randomly distributed in six treatment groups. Group wise applications of hormones used for the estrus synchronization and controlled breeding protocols including both breeds of goat were as under: Group I- Two dosages of Inj. $PGF_2\alpha$; Group II -Intra-vaginal sponges + Inj. eCG i.m. on the day of sponge removal; Group III -Intra-vaginal sponges + Inj. $PGF_2\alpha$ i.m. 24 h prior to sponge removal + Inj. eCG i.m. on the day of sponge removal; Group IV- Intra-vaginal sponges + Inj. GnRH i.m. 24 h prior to sponge removal; Group V -Intra vaginal sponges + Inj. GnRH (i.m.) on the day of sponge removal; Group VI Intra-vaginal sponges + Inj. eCG (i.m.) 24 h before sponge removal. The percentage of goats exhibiting estrus from sponge withdrawal or from the end of treatment for treatment Groups I, II, III, IV and V in Sangamneri goats were 66. 67, 100, 100, 100, 91.67 % and in Osmanabadi goats as 69.23, 100, 100, 100, 57.14 %, respectively. One Sangamneri goat in Group IV exhibited estrus for seven days after sponge removal. Estrus response was 100 % for both Sangamneri and Osmanabadi breeds of goats subjected to estrus synchronization belonging to treatment Groups II, III and IV. The overall percentage of goats exhibiting estrus following different synchronization protocols were 89.28 % and 83.33 %, in Sangamneri and Osmanabadi goats, respectively. The mean time of onset of estrus (h) from progestagen withdrawal for five different treatment groups in Sangamneri goats were 25.20 ± 5.50, 46.91 ± 2.53, 27.33 ± 2.26, 41.33 ± 3.53, and 48.55 ± 4.16 h, respectively and in Osmanabadi goats as 44.4 ± 3.36, 24.0 ± 6.28, 26.75 ± 4.12, 46.84 ± 3.88, and 48.0 ± 9.09 h, respectively. The overall mean interval of onset of estrus in Sangamneri and Osmanabadi goats were 37.20 ± 2.31 h and 37.08 ± 5.72 h, respectively. The mean estrus duration (h) for five different treatment groups following estrus synchronization in Sangamneri goats were 54.60 ± 4.60, 76.36 ± 7.81, 56.67 ± 5.92, 44.00 ± 7.48, and 69.27 ± 8.76 h, respectively and for Osmanabadi goats as 44.00 ± 3.46, 113.14 ± 6.65, 53.50 ± 6.63, 50.86 ± 6.69, and 51.0 ± 15.78 h, respectively. The overall mean duration of estrus in Sangamneri and Osmanabadi goats were recorded as 61.08 ± 3.51 h and 62.34 ± 5.38 h, respectively. There was variation in estrus interval among different treatment groups of both the breeds. The conception rates in Sangamneri goats were 40.00, 72.73, 66.67, 88.89, and 72.72 %,

respectively and in Osmanabadi goats in five treatment groups as 44.44, 57.14, 37.50, 71.43, 100.00 %, respectively. The overall pregnancy rate in Sangamneri goats in this study was 68.00 %, whereas for Osmanabadi goats it was 57.14 %. Out of 34 pregnant Sangamneri goats, 21 goats' kidded and 32 kids were delivered. In Osmanabadi goats 10 goats kidded out of 20 pregnant goats delivering 12 kids. The percentage of single kids, twins and triplet kids born in Sangamneri goats were 34.37, 21.87 and 3.12 percent, respectively whereas, Osmanabadi goats delivered 66.67 % single kids and 16.17 % twins. Overall kidding rate in Sangamneri and Osmanabadi breeds of goats was 63.64 % and 71.43 %, respectively. Multiple kidding rate observed in Sangamneri and Osmanabadi breeds was 47.62 % and 20.00 %, respectively. Overall litter size in Sangamneri and Osmanabadi breeds was found to be 1.52 and 1.20, respectively.

Keywords *Estrus synchronization; Intra-vaginal sponges; Osmanabadi goat; Sangamneri goat*

1. Introduction

Goats are the most conspicuous seasonal breeders among domestic farm animals. Tropical breeds of goats may cycle year around. Sexual receptivity of the female goat is limited to a short period called estrus, the period of desire. The profitability of goat keeping depends on exploitation of fertility potential. This can be achieved by using assisted reproductive technology in goats. The synchronization of estrus using timed hormonal treatments is widely used assisted reproductive technology in goat industry. It reduces the time needed for detection of estrus. Large numbers of goats can be bred in a short period. Outside the normal breeding season, synchronization has additional advantages in herds or animals where heat detection is difficult, goats may be successfully bred without requirement of heat detection. Sangamneri and Osmanabadi are well recognized dual purpose goat breeds from Maharashtra state (India) and are the backbone of economy of small and landless farmers in Maharashtra. Inspite of benefits associated with estrus synchronization; there is no information to study comparative efficacy of different hormonal methods to induce synchronized estrus. Therefore, the present study was undertaken in Sangamneri and Osmanabadi goat does implementing different hormonal methods of synchronization to induce synchronized estrus.

2. Materials and Methods

The study was conducted at All India Coordinated Research Project on Goat Improvement, Department of Animal Science and Dairy Science, Mahatma Phule Agricultural University, Rahuri Dist. Ahmednagar Maharashtra State (India). Total 104 parous goats of Sangamneri (62) and Osmanabadi (42) breeds aged 2-4 years were used in this study. Few Sangamneri and five Osmanabadi breeding bucks were used for breeding purpose during the study. All goats were randomly distributed in six treatment groups.

Group I: Two dosages of Inj. $PGF_2\alpha$ i.m. 10 days apart; Group II: Progestagen-impregnated intra - vaginal sponges kept in vagina for seven days + Inj. eCG i.m. on the day of sponge removal; Group III: Progestagen-impregnated intra-vaginal sponges kept for seven days + Inj. $PGF_2\alpha$ i.m. 24 h prior to sponge removal + Inj. eCG i.m. on the day of sponge removal; Group IV :Progestagen-impregnated intra-vaginal sponges kept for seven days + Inj. GnRH i.m. 24 h prior to sponge removal and breeding during estrus period; Group V : Progestagen-impregnated intra vaginal sponges kept for seven days + Inj. GnRH (i.m.) on the day of sponge removal and breeding during estrus period; Group VI: Progestagen-impregnated intra-vaginal sponges kept for seven days + Inj. eCG (i.m.) 24 h before sponge removal.

The reproductive parameters (as per Karaca *et al.,* 2010) recorded were as follows:

i. Estrus response: Number of goats showing estrus / total number of goats treated in each group X 100; ii. Onset of estrus (h): Interval from sponge removal to time of first estrus identification; iii.

Estrus duration (h): The time between the first and last accepted mount, within the same estrous period.

The goats those exhibited estrus following synchronization were mated at least twice with the respective breed of bucks in all five groups. Sangamneri goats in group VI were inseminated at 48 and 60 hours (fixed time A.I.) following progesterone withdrawal. All goats were scanned for pregnancy 80 days post mating with the aid of a trans-abdominal ultrasonic scanning apparatus. The response of treatment and conception rate was assessed on the basis of pregnancy diagnosis results. The other reproductive parameters recorded were: pregnancy rate, kidding rate, multiple kidding rate and litter size.

3. Results and Discussion

3.1. Estrus detection, behavioral and physical signs of estrus

Estrus detection in all the experimental goats was carried out after sponge removal and / or after end of treatment. In the present study, few goats started exhibiting signs of estrus (commencement of estrus) 12 hours after sponge removal. The prominent symptom of exhibiting estrus in goats treated with different estrus synchronization protocols were the tendency to cluster round the buck at the time of estrus detection, attracted towards male and keeping alliance with males kept in the adjacent pens. Higher incidence of wagging of tail was recorded in goats in estrus. Increased restlessness, frequent urination were other signs of estrus seen in majority of goats during the study. Intermittent bleating was observed in few goats during estrus. The intensity of swelling of vulva in goats was found to be less prominent. The bucks when confronted with synchronized goats, presented sexual arousal, exhibited flehmen reaction and then erection of the penis. The goats during estrus were receptive to mounting, and stood to be mounted. On perusal of literature these estrus symptoms were comparable with the findings of Bhattacharya *et al.* (2000) in Assam local goats and Goel and Agrawal (2002) in Jakhrana goats of Indian origin. Among all the treatment groups in the present study, estrual discharge was observed in only one Osmanabadi goat belonging to treatment Group II (progestagen-impregnated intra vaginal sponge + eCG administered on the day of sponge removal). Three Sangamneri goats in Group II exhibited continuous estrus behavior until fifth to sixth day after sponge removal. One Sangamneri goat in Group IV (Progestagen-impregnated intra-vaginal sponges + GnRH 24 hr before sponge removal) exhibited estrus for seven days after sponge removal.

Exhibition of estrus for prolonged period may be due to prolonged duration of ovulation when eCG is administered at the end of progestogen treatment. Administration of eCG lengthen the estrus duration in goats.

3.2. Estrus response

The overall percentage of goats exhibiting estrus following different synchronization protocols were 89.28 % (range 57.14 to 100 %) and 83.33 % (range 66.67 to 100 %) in Sangamneri and Osmanabadi goats, respectively. Estrus response was 100 % in Sangamneri and Osmanabadi goats subjected to estrus synchronization with Progestagen-impregnated intra-vaginal sponges + eCG on day of sponge removal (Treatment Group II), Progestagen-impregnated intra-vaginal sponges + $PGF_2\alpha$ + eCG (Treatment Group III) and Progestagen-impregnated intra-vaginal sponges + GnRH 24 hr before sponge removal (Treatment Group IV). These three types of estrus synchronization protocols used in these trials were equally efficient and resulted in synchrony of estrus in goats of both the breeds. The overall estrus response in all treatment groups was recorded to be non-significant ($P > 0.05$) between these two breeds.

Ogunbiyi *et al.* (1980) reported similar results of estrous synchronization at first and second injections of prostaglandins (64 % and 84 %). Patil *et al.* (2004) reported only 50 % results of estrus response in Osmanabadi goats. Whereas higher percentage of estrus responses were observed by Medan *et al.,* 2003 (100%), Bitaraf *et al.,* 2007 (97 %), whitely and Jackson, 2011 (87.5 to 100 %) in goats using double injection protocol of PGF_2 α for synchronization of estrus.

Similar results reported by Ozyurtlu *et al.* (2011) (in ewe) and Whitley and Jackson (2011) in goats. Riesenberg *et al.*, 2001 (91.7 %), Motlomelo *et al.*, 2002 (97 %) and Bitaraf *et al.*, 2007 (97 %) reported marginally lower percentages of estrus response in goats when progestagen-impregnated intra - vaginal +. eCG protocol was employed. The mean percentage of goats exhibiting estrus when treated with progestagen + PGF_2α + eCG in the present study corroborate with the findings of earlier workers (Freitas *et al.,* 1996[a]; Leboeuf *et al.,* 2003; Karaca *et al.*, 2010; and Nogueira *et al.,* 2011) and was higher when compared with the findings of Drion *et al.,* 2001 (87 %), Fonseca *et al.*, 2005 (86.8%), Fonseca *et al., (*2008) (88.1%) in goats. Around 80 % estrus behavior was observed in Damascus Baladi goats in studies of Telab and Ashmawy (2007) when GnRH was administered 24 h before sponge removal. Teleb and Ashmawy, (2007) recorded 70 % estrus response in Damascus Baladi goats when progestagen impregnated + GnRH was administered on the day of sponge removal protocol of estrus synchronization was employed.

3.3. Onset of estrus

The overall mean interval to onset of estrus in Sangamneri and Osmanabadi goats were 37.20 ± 2.31 h and 37.08 ± 5.72 h, respectively. In Sangamneri goats, duration of onset of estrus from sponge removal was significantly more ($P< 0.05$) in Groups II, IV and V (41.33 to 46.55 h) than in Groups I and III (25.20 to 27.33 h). In Osmanabadi goats, duration of onset of estrus from sponge removal was significantly more ($P< 0.05$) in Groups Groups I, IV and V (44.00 to 48.00 h) than in Groups II and III (24.00 to 26.75 h). There was significant variation ($P< 0.05$) in the duration of onset of estrus between Sangamneri and Osmanabadi goats in Group II (46.51 vs 24.00 h). Whereas durations of onset of estrus were non-significant ($P > 0.05$) between two breeds in Groups I, III, IV and V.

Patil *et al.* (2004) reported the time required for onset of estrus as 35.20±11.74 h in Osmanabadi goats. Longer interval from end of PGF_2α administration to exhibition of estrus were recorded by Medan *et al.,* 2003 (55.7 ± 3 h) in Shiba goats, Whitley and Jackson, 2011 (60.5 h) in goats. Whereas, Bitaraf *et al.* (2007) recorded 26.0±0.4 h (24-31 h) duration of onset of estrus in synchronized Nadooshani goats treated with double injections of prostaglandins. They observed significant difference for the interval between the end of synchronization protocol and the standing heat amongst treatments. The earlier researchers who reported mean duration of onset of estrus include Motlomelo *et al.* (2002) 30.1 ± 5.5 h, Blaszczyk *et al.* (2004) ranged between 32.9±2.4 and 37.4±3.2 h, Bitaraf *et al.* (2007) 26.2±0.5 h (24-34 h) in estrus synchronized goats. Ozyurtlu *et al.* (2011) reported onset of estrus in synchronized ewes ranged from 41.5±1.81 to 53.0±3.37 h. Riesenberg *et al.* (2001) and Patil *et al.* (2004) recorded very high interval of exhibition of estrus in eCG treated goats ranging from 72 h to 5±7 days in nanny goats and 48 - 120 h in Osmanabadi goats, respectively.

Similar observations when treatment of progestagen + PGF_2α + eCG was employed in goats regarding the time for the interval from sponge removal to onset of estrus in goats have been reported by Freitas *et al.* (1996[a]), Drion *et al.* (2001), Leboeuf *et al.* (2003), Fonseca *et al.* (2008), Karaca *et al.* (2010). Higher range of interval of onset of estrus from the time of sponge withdrawal in goats has been reported by Feritas *et al.* (1996[b]), 32 ± 7.1 h; Kusina *et al.* (*1*999), 11 to 96 h; Lymberopoulos *et al.* (2002), 42 to 44 h; Dogan *et al.* (2005), 12 - 66 h; and Fonseca *et al.* (2005), 49.7±15.7 h in synchronized goats. Compared to the present findings decreased interval of exhibition of estrus was reported by Dogan *et al.* (2004), 15.0 - 15.8 h and Nogueira *et al.* (2011), 13.3 - 13.8 h. Teleb and Ashmawy (2007) reported lower interval of initiation of estrus (30.5 ± 0.9 h) in one group of

Damascus Baladi goats following sponge removal than the interval of onset of estrus in the present study using the protocol of progestagen + GnRH administered 24 h before progesterone removal. Whereas, lower durations of onset of estrus after sponge withdrawal compared to the present findings were observed in earlier studies by Pierson *et al.,* 2003 (25.8±1.22 to 39.3±3.76 h), Teleb and Ashmawy, 2007 (30.5 ± 0.9 h, second group), Karaca *et al.,* 2010 (33.1±2.0 h) when goats were synchronized using the protocol of progestagen + GnRH administered on day of sponge removal.

3.4. Estrus Duration

The overall mean duration of estrus in Sangamneri and Osmanabadi goats were 61.08 ± 3.51 h and 62.34 ± 5.38 h, respectively. There was variation in estrus interval among different treatment groups of both the breeds. Longer estrus durations were recorded in goats treated with progestagen-impregnated intra-vaginal sponges + inj. eCG on the day of sponge removal (Group II) in both Sangamneri and Osmanabdi goats when compared to other synchronization protocols. In Sangamneri goats, duration of estrus was significantly more ($P < 0.05$) in Groups II and V (69.27 to 76.36 h) than Groups I, III and IV (44.00 to 56.36 h). In Osmanabadi goats also estrus durations were significantly different ($P < 0.05$) among the treatment groups (Group II and Groups I, III, IV, V). Between Sangamneri and Osmanabadi breeds, duration of estrus was significantly higher ($P < 0.05$) in Group II (76.36 vs 113.14). Whereas, no significant differences ($P >0.05$) were recorded between two breeds of goats in treatment Groups I, III, IV and V. No significant difference ($P > 0.05$) in duration of estrus was noticed between Sangamneri and Osmanabadi breeds.

Average duration of estrus reported by Patil *et al.* (2004) was 24 to 38 hr in Osmanabadi goats. Akusu *et al.* (1986) reported duration of estrus ranging from 10 to 48 h in West African dwarf goats. The values of estrus duration in both breeds in the present study are much higher than reported by Bitaraf *et al.* (2007) who mentioned overall values for duration of estrus as 22-0±0.3 h (17-25 h) in Nadooshini goats when double injection regimen of $PGF_2\alpha$ was employed for synchronization of estrus. Osmanabadi goats showed significantly longer durations ($P < 0.05$) than Sangamneri goats when exposed to progestagen + eCG treatment. Patil *et al.*, 2004 reported estrus duration of 35 h In Osmanabadi goats. In the present study, longer durations of induced estrus periods in both breeds of goats were observed than the results presented by Motlomelo *et al.*, 2002 (33.3 ± 13.4 h), and Blaszczyk *et al.*, 2004 (27.7±4.2 - 32.0±3.4.I h) when goats were treated with progestagen sponges and eCG. Duration of estrus observed by Ozyurtlu *et al.* (2011) in ewes was in the range of 31.9±1.4 to 33.1±1.6 h. However, the longer durations of estrus interval in the present study in Sangamneri and Osmanabadi goats were recorded when compared with the findings of earlier researchers who treated goats with progestagen + $PGF_2\alpha$ + eCG (Freitas *et al.* 1997 (32 ± 7.1 h), Dogan *et al.,* 2004 (30.5 – 34.0 h), Dogan *et al.,* 2005 (29.7 ± 1.3 h), Fonseca *et al.,* 2005 (28.7 ± 11.5 h), Fonseca *et al.,* 2008 (25 h), and Nogueira *et al.*, 2011 (29.6 - 33.6 h). In the present study, difference in estrus interval was not significant ($P > 0.05$) between Sangamneri and Osmanabadi breeds when progestagen + GnRH administered 24 h before sponge removal protocol for estrus synchronization was employed. Teleb and Ashmawy (2007) reported lower interval of durations of estrus for Damascus Baladi does in goats as 24.6±3.7 h. There was no significant ($P > 0.05$) effect of synchronization methods on estrus duration between these two breeds when goats were synchronized with progestagen + GnRH administered on the day of sponge removal. Relatively lower estrus duration (23.0±3.7 h) was observed by Teleb and Ashmawy (2007) in Damascus Baladi goats than the estrus durations observed in the present study.

3.5. Conception rate

All goats were checked for pregnancy with the aid of a trans-abdominal ultrasonic scanning apparatus with a 3.5-5.0 MHz convex probe on 81st and 88th day in Osmanabadi and Sangamneri goats, respectively after mating. The overall pregnancy rate in Sangamneri goats was 68.00 %, whereas for

Osmanabadi goats were 57.14 %. Non-significant difference was observed (P > 0.05) in overall conception rates between Sangamneri and Osmanabadi breeds of goats.

Whitley and Jackson (2011) reported higher pregnancy rate (75%) in goats when double $PGF_2\alpha$ injection protocol was employed. Patil *et al.* (2004) reported higher pregnancy rate (66.67 %) in Osmanabadi goats. Motlomelo *et al.* (2002) observed 60 % (MAP + eCG group) and 47% (FGA + eCG group) pregnancy rates in Boer and indigenous goats with no significant difference between groups. Whitley and Jackson (2011) recorded 65.8% conception rate in indigenous Damascus does when exposed to progestagen + eCG treatment for estrus synchronization. Dogan *et al.* (2004) recorded 52.63 % and 50.0 % pregnancy rates in Saanen does for MAP+ $PGF_{2\alpha}$ + eCG and FGA+ $PGF_{2\alpha}$ + eCG protocols, respectively which were lower than conception rate in Sanagmneri and higher than conception in Osmanabadi goats observed in the present study. When progestagen + $PGF_{2\alpha}$ + eCG protocol was compared, Dogan *et al.* (2005) reported 70.0 % pregnancy rate in Anatolian black does. Fonseca *et al.* (2008) observed 77.8 %, 44.4 % and 60.0 % pregnancy rates in lactating, non-lactating and nulliparous Alpine goats, respectively. Whereas, Karaca *et al.* (2010) recorded pregnancy rate of 70.8 % in Hair goats. Conception rate of 80 % was achieved by Teleb and Ashmawy (2007) after using treatment with single intra-muscular injection of 4 µg GnRH 24 h before progestagen sponge removal in Damascus Baladi goats. The results of pregnancy rates in the present study were higher when compared to the results obtained by Karaca et *al.* (2010) who reported 58.3 % conception rate in Hair goats when short term progestagen + GnRH treatment protocol was employed.

3.6. Kidding rate

Overall kidding rates in Sangamneri and Osmanabadi breeds were 63.64 and 71.43 %., respectively. The kidding rate was numerically higher in Osmanabadi goats than Sangamneri goats. There was non-significant difference (P > 0·05) in overall kidding rates between Sangamneri and Osmanabadi breeds of goats. The kidding rates in goats in the present study corroborates with previous observations of the kidding rates reported by Freitas *et al.* (1996[a]), Kusina *et al.* (1999), Lymberopoulos *et al.* (2002), Leboeuf *et al.* (2003), Bitaraf *et al.* (2007), Karaca *et al.* (2010) and Whitley and Jackson (2011). The kidding rate recorded in the present study was lower than those recorded by Nogueira *et al. (*2011) (82.2%).

3.7. Multiple kidding rates

Multiple kidding rates observed in Sangamneri and Osmanabadi breeds was 47.62 % and 20.00 %., respectively. The multiple kidding rate was significantly higher (P < 0.05) in Sangamneri breed than Osmanabadi breed of goats. Relatively higher percentage of multiple kidding rate was reported by Karaca *et al.*, 2010 (69.2 %) in Hair breed of goats.

3.8. Litter size

Overall litter size in Sangamneri and Osmanabadi breeds was found to be 1.52 and 1.20, respectively. Overall litter size was significantly higher (P < 0·05) in Sangamneri goats than Osmanabadi goats. These results agree with earlier reports of Bitaraf *et al.* (2007). Whereas, values of litter size in Sangamneri and Osmanabadi goat breeds in the present study were lower than those recorded by Adeoye, 1986 (1.6 ± 0.5, West African dwarf goats), Drion et al., 2001 (1.6 to 1.8, Alpine goats), Lymberopoulos *et al*., 2002 (1.63-2.005, Swiss breeds), Karaca et al., 2010 (1.8, Hair goats) and Nogueira *et al.*, 2011 (1.9, Saanen and Alpine goats). This variation may be due to breed differences of goats.

The Sangamneri goats in Group VI (n=6) were inseminated at 48 and 60 h with fresh diluted Sangamneri buck semen. After fixed time artificial insemination, the overall conception rate, kidding

rate, multiple kidding rate and litter size in Sangamneri goats were found to be 66.67 %, 50.0 %, 50.0 % and 1.50, respectively.

4. Conclusion

Estrus response was 100 % for three treatment protocols in Sangamneri and Osmanabadi goats subjected to estrus synchronization using progestagen-impregnated intra-vaginal sponges + eCG on day of sponge removal; progestagen-impregnated intra-vaginal sponges + $PGF_2\alpha$ + eCG; and progestagen-impregnated intra-vaginal sponges + GnRH 24 h before sponge removal. These three types of estrous synchronization protocols used in these trials were equally efficient and resulted in synchrony of estrus in goats of both breeds. Longer interval to onset of estrus was observed in both breeds of goats when exposed with the treatment of progestagen-impregnated intra-vaginal sponges + GnRH 24 h before sponge removal and progestagen-impregnated intra-vaginal sponge + GnRH on the day of sponge removal. Longer estrus durations were recorded in goats treated with progestagen-impregnated intra-vaginal sponges + inj. eCG on the day of sponge removal in both Sangamneri and Osmanabadi breeds of goats compared with other treatment groups. Administration of progestagen (intra-vaginal sponges) together with eCG lengthened estrous duration in goats. The higher conception rate was recorded in Sangamneri goats (88.89 %) when exposed to synchronization treatment of progestagen-impregnated intra-vaginal sponge + GnRH administered 24 h prior to sponge removal, followed by conception (72.73 %) in goats provided with progestagen-impregnated intra-vaginal sponge + eCG administered on the day of sponge removal and (72.73 %) progestagen-impregnated intra-vaginal sponge + GnRH administered on the day of sponge removal. In Osmanabadi goats, among all treatment groups, highest conception (100 %) was recorded in goats treated with synchronization treatment of progestagen+ GnRH administered on the day of sponge removal, followed by conception (71.43 %) in goats provided with progestagen + GnRH administered 24 h prior to sponge removal. The combination of GnRH with short-term progestagen treatment (intra-vaginal sponges) had a positive effect on the fertility parameters viz conception rate in both breeds of goats.

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Research Article **Open Access**

In vitro Sperm Function Tests and Testicular Biometry for Fertility Prediction in Boar

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Abstract The study was conducted on six porcine males and thirty six semen ejaculates for its fertility assessment. The average length of the left and right testicle of the boars measured by ultrasonography was 9.47 ± 0.73 cm and 9.09 ± 0.65 cm, respectively. The sperm concentration/ml increased significantly as testicular diameter increased in size. The average length of right and left of boar testicle measured by Vernier caliper was 10.4 ± 0.57 cm and average width was 4.3 ± 0.14 cm. The average volume of boar semen was 115.00 ± 11.83 ml with milky colour. Thick consistency was observed in 83.3% semen samples whereas 16.6 % semen samples were having thin consistency. Out of 36 semen ejaculates, 16.6 % semen samples had density of DD where as 83.3 % had a density of DDD. The Mass activity, live percentage, percentage of morphologically abnormal spermatozoa and total sperm concentration in boar semen were 3 ± 0, 75.41 ± 2.07 %, 0 %, and 523 ± 60.07 million/ml, respectively. Mean percentage of hypo osmotic swelling test (HOS-Test) of semen found in the present study was 73.47 ± 2.26. The average time for reduction of resazurin dye from blue to violet was 1.805 ± 0.163 and from violet to pink was 9.944 ± 0.890. None of the sample change colour from pink to white. In the present study 36 ejaculates of boar semen were subjected for TVC of bacteria. In order to differentiate the bacterial species contaminating semen raw semen was placed on different agar plates. The species isolated with higher frequency in boar semen were of Staphylococcus species-83.3% (30 samples) followed by E.coli -63.8% (23 samples). Mean TVC obtained in this study was 45.13×10^3.

Keywords *Porcine; semen; sperm function tests; testicular biometry*

1. Introduction

The challenges faced by the country in securing food as well as nutritional security to fast growing population need an integrated approach for livestock farming. Among the various livestock species, piggery is the most potential source of meat production and more efficient feed converters after broilers. Apart from meat it also provides bristles and manure. But total pig population of India is only 11134. Therefore it is a need of hour that pig farmers should be trained for scientific rearing of the pigs to retain pig fecundity and production. Pigs farrow two times in a year with minimum eight piglets

at each time. They mostly deliver sixteen piglets/annum. Piglets are saleable at the age of six months as they achieve the weight on an average 50 kg.

To mitigate the deficit between demands and supply of quality pig and pork products, the focus should be made on the basic and applied research output through genetic improvement of indigenous pigs, selective breeding and cross breeding. It is important to promote breeding soundness examination (BSEs) on boars, which can help to identify poor or questionable breeders/males before they affect herd fertility. Improvement of physiological and reproductive efficiency of pig production can be achieved through utilization of fertile boar bearing excellent semen quality. In last decade range of *in vitro* tests have been developed to examine structural characteristics and monitor crucial aspects of sperm function. Keeping in view the present scenario of sperm function tests for fertility prediction in boar, the present study was planned to perform the *in vitro* sperm function tests along with testicular biometry and bacterial total viable count of boar seminal plasma.

2. Materials and Methods

Selection of Animals

Fertile boars of White Yorkshayer breed belonging to Pig Farm, KNP College of Veterinary Science, Shirwal Dist Satara Maharashtra state (India) were selected for present research pursuit.

Physical Examination of Reproductive Organs

All selected boars (n=6) were subjected to complete physical examination of reproductive organs to determine location, shape, consistency and discomfort on palpation of the testicles, prepuce, penis, scrotum. Physical examination of the reproductive organs was done only once during the first semen collection.

Biometry of Testis

A real time B-mode, portable ultrasonography machine with 5-mHz sector transducer was used for scanning of testis in boars. During scanning the relevant image was frozen and measurements were taken by electric calipers. All the relevant pictures were printed by using electronic printer. The measurements were also taken in standing position by using a digital Vernier Caliper.

Semen Collection

Collection of semen of each boar was done by Gloved Hand Method. Digital pressure was exerted intermittently on the corkscrew end of the penis and semen was collected in collection flask. After the collection semen was filtered by using muslin cloth to remove the gel fraction.

Semen Evaluation

Semen samples were evaluated for various macroscopic, microscopic and sperm function tests. The former consisted of volume, color, consistency and density, while later consisted of mass activity, initial motility, total sperm count, live sperm percentage and abnormal sperm percentage. Additional sperm function tests were performed to evaluate functional ability of sperm, which included Resazurin test, Hypo-osmotic swelling test.

Sperm Function Test

Hypo-Osmotic Swelling Test (HOS test)

Equal volumes of 2.7% aqueous solution of fructose and 1.47% aqueous solution of sodium citrate (0.5 ml each) were mixed and kept in an incubator at 37˚C for 10mins. 0.1 ml of semen was added in

above hypo-osmotic solution and incubated at 37˚C for 30 minutes.10µl of this mixture was taken on glass slide and covered with cover glass. Slide was observed under 40x objective lens to determine the number of spermatozoa showing swollen head and coiled tail indicating sperms with intact plasma membrane.

Resazurin Reduction Test

Resazurin solution was prepared by mixing 50 mg Resazurin (7-hydroxy- 3H, phenoxzin-3, one-10-oxide mixed in 100 ml of 0.9 % normal saline. This resazurin solution was used for further study. 250 µl porcine semen was taken in a test tube and 25µl resazurin solution was added. This solution was layered with small quantity of mineral oil. Test tube was kept in incubator set at 37˚C for colour change. The time required for color change from dark blue to violet, violet to pink, pink to white and was noted in minutes.

3. Results and Discussion

Physical Examination of Reproductive Organs of Boars

On examination it was found that, in all 6 boars, both the testes were symmetrical, firm and slightly resilient. Epididymides on palpation was found to be symmetrical from left to right, head firm and tail were tense. The prepuce and preputial diverticulum were free from signs of irritation or infection. Penis was found to be normal and free from persistent frenulum, lacerations, ulcers and scars. Scrotum was free of scarring, abscesss, thickening, irritation and from evidence of mange. Testes were freely movable within scrotum and no excess fluid was palpable. These observations were in accordance with those of Shipley (1999).

Biometry of Boar Testis

On placing the transducer on the scrotal skin after fixation of the testicle, an image with a relatively homogenous echogenic parenchyma with a centrally located hyperechoic mediastinum testis was seen. The tunica albuginea appeared as a distinct hyperechoic line, which surrounded the testicular parenchyma. The mediastinum testis was used as a reference point for higher accuracy and complete measurement. Clark et al. (2003) reported similar findings in boars. Testicular measurements of boars were taken after freezing the images when the mediastinum testes was identified as having largest diameter, testicular diameter was measured by use of electronic cursor points for all selected boars.

Mean Ultrasonic Measurements of Testis in cm

Left Testicular Diameter- (LTD)	Right Testicular Diameter- (RTD)	Average Diameter (cm)(n=6)
9.475 ± 0.736	9.09 ± 0.65	9.285 ± 0.676

Comparison between Paired Testicular Diameter (PTD) (var 1) and Sperm Concentration per ml(Var1) using Students T test

Correlation Matrix							
-	Var1	Var2	Var3	Var4	Var5	Var6	Var7
var 1	1.000	0.866	0.864	0.879	0.909	0.830	0.872

Students T Test			
Variables Tested	T Value	T Table	Significance at 5%
Var1 -Var2	3.485	2.776	Significant
Var1 -Var3	3.474	2.776	Significant
Var1 -Var4	3.575	2.776	Significant
Var1 -Var5	4.331	2.776	Significant
Var1 -Var6	3.089	2.776	Significant
Var1 -Var7	3.548	2.776	Significant

* (P<0.05)

Significant differences were found between PTD and Sperm concentration/ml (P<0.05). The study indicates that as testicular diameter increased in size, the sperm concentration/ml increased significantly. However, the present observations are higher and significant than those observed by Clark et al. (2003) who found no significant difference between PTD and average total sperm numbers (ATSN),since this study was limited due to the small number of observation in boars this may account for the fact that differences were not detected.

All the experimental boars were subjected to testicular biometry by using Vernier calipers to evaluate length, width once during the study.

Mean Vernier Caliper Measurements of Testis

Parameter,(n=6)	Right	Left	Average
Length (cm)	10.40 ± 0.76	10.68 ± 0.75	10.4 ± 0.57
Width (cm)	4.3 ± 0.21	4.4 ± 0.2	4.3 ± 0.14

Semen Evaluation

Semen samples of 6 boars were evaluated for various macroscopic and microscopic tests. Total 6 ejaculates from each boar (total 36 ejaculates) were collected at weekly interval. Immediately after collection volume, colour, consistency and density were evaluated by visual observations and semen was placed in a water bath at 37˚C, till further evaluation was carried out.

Macroscopic Evaluation of Boar Semen

Mean ± SE values of Macroscopic Semen Evaluation

Parameter	Result
Volume (ml) (n=6)	115.00 ± 11.83

Mean semen value reported by Masenya et al. (2011) in Kolbrek and Large White boar semen was reported as 140.4 ± 48.6 & 177.5 ± 60.4 which is higher than that observed in the present study. This difference may be attributed to hot environmental condition since collection was done in afternoon session. Decreased nutrition and hot environmental conditions also cause decrease in semen volume (Foote, 1978). The difference in collection time volume may be attributed to a decrease in estradiol-17β levels. Estradiol-17β is involved in maintenance of libido and it has been demonstrated that this hormone is responsible for maintaining semen volume in castrated boars. The consistency was recorded as thick and thin with 83.3% showing thick consistency of semen while 16.6% having thin

semen consistency. 16.6% semen samples had density of DD where as 83.3% had a density of DDD. Finding of milky colour of semen is same as described by Shipley (1999) in boars. When the literature was scanned specific references pertaining to consistency and density in porcine were not available for comparison. However present findings corroborate with those reported in dogs. Maximum numbers of semen samples were milky, thick with density DDD.

Microscopic Evaluation of Boar Semen

Parameter (n=36)	Result
Mass activity	3 ± 0
Live sperm %	75.41 ± 2.07
Morphologically abnormal sperm %	0
Sperm concentration (million/ml)	523 ± 60.07

The Mass activity of spermatozoa was 3 ± 0, which is nearby to observation made by Vyt et al. (2004) for boars and Kunbhar et al. (2011) for kundhi buffalo bull. The average percentage of live spermatozoa in the present study was 75.41 ± 2.07 which is lower than observations made by Kumaresan et al. (2009) (90.92 ± 1.56 %) and Masenya et al. (2011) (84.6 ± 6.1 %), and Borg et al. (1993) in boars. Morphologically no abnormality in spermatozoa in any experimental boars was found in the samples during study. In the present study the average sperm concentration in boars found was 523 ± 60.07 million/ml as compared to 727 ± 340.8 million/ml by Masenya et al. (2011) in boars and 133 ± 4, 86 ± 32, 100 ± 22, 117 ± 25 million/ml in Duroc, Landrace, Duroc/Landrace and Yorkshire boars reported by Kommisrud et al. (2002). It can be concluded that conventional microscopic characteristics of semen are good indicators of sperm quality and are simple and rapid semen evaluation tests.

Sperm Function Tests in boars

Plasma Membrane Integrity by Hypo-Osmotic Swelling Test (HOST)

The clinical use of the hypoosmotic swelling test (HOS-Test) to identify spermatozoa with a functional intact membrane has been reported for humans and domestic species, including the boar. This test evaluates response of spermatozoa to hypo-osmotic stress. The basis of this assay is that, when viable sperms are exposed to hypo-osmotic medium, the intact membrane swells and their tail curls and bulges due to influx of fluid. Sperm undergoing coiling reaction are considered to have positive reaction and sperm with damaged membrane will not swell because solution will not enter. Total 36 samples of boars were subjected to hypo-osmotic swelling test.

Mean percentage of Hypoosmotic Swelling Test

	Average (%)
Result (Samples n=36)	73.47±2.26

Samardzija et al. (2008) reported a mean percentage as 16.96 ± 4.27 in boars which is lower than that observed in the present study.

Resazurin Reduction test

Resazurin is a chemical indicator and its reduction by mitochondrial enzymes of metabolically active sperm cells offer an assessment of the reducing capacity of semen, which is manifested by a spectrum of colours. Workers have reported a significant correlation between Resazurin reduction test (RRT) and fertility, as RRT evaluates the metabolic status of active spermatozoa and it is

associated with the concentration of motile sperms (Erb et al., 1952; Dart et al., 1994). RRT has been used successfully in assessing fertility potential in boars (Zrimsek et al., 2004).

Mean Values of Resazurin Reduction Test (Time in minutes)

Semen collected from 6 boars	Blue to violet	Violet to pink
Result	1.805 ± 0.163	9.944 ± 0.890

None of the sample change colour from pink to white; it could be attributed to the lower concentration of spermatozoa in porcine semen as compared to bull and rams. When the literature was scanned specific references pertaining to RRT in porcine were not available for comparison. However present findings corroborate partly with those reported by Erb et al. (1952) for bulls (blue to pink) and Pathak et al. (1989) in cross bred bulls (violet to pink). Present findings are also similar to those reported by Erb et al. (1950) in bulls (Blue to pink), El Battawy (2008) and Dart (1994) in Limousin bulls (blue to pink).

Bacterial Total Viable Count in Boar Semen

Semen collected from 6 boars	Average (10^3 CFU/mL)
Result	45.13

Mean TVC of boar semen obtained in this study was 45.13 × 10^3 which is lower than that reported by Ciornei et al. (2012) in boars. In order to differentiate the bacterial species contaminating semen raw semen was placed on different agar plates. The species isolated with higher frequency in boar semen were of Staphylococcus species-83.3% (30 samples) followed by E.coli -63.8% (23 samples), this findings corroborate with those observed by Ciornei et al. (2012) in boars.

4. Conclusion

Ultrasonographic testicular biometry in porcine can be carried out by excluding epididymis and scrotal skin thickness, which cannot be avoided when testicular biometry is done by using Vernier calipers. Significant differences were found between paired testicular diameter (PTD) and sperm concentration per ml of semen sample. This indicates that as testicular diameter increases in size, the sperm concentration/ml increase significantly. Even collected in the strictest aseptic condition boar semen contains bacteria.

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Review Article | Open Access

Regulatory Framework for Approval of New Animal Drugs in United States

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Abstract Animal healthcare is a booming market catering needs of several companion and production purpose animals. Animal drug approval process in the United States (US) involves submission of an Investigational New Animal Drug Application (INAD), followed by submission of New Animal Drug Application (NADA) to the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM). Although regional variations are present in the regulatory process for veterinary healthcare products across the globe, harmonization initiatives like International Cooperation on Harmonization of Technical Requirements for the Registration of Veterinary Medicinal Products (VICH) are playing a vital role in the veterinary medicinal product registration process by availing detailed guidance documents. The current article focuses on the regulatory requirements for new animal drug registration in the US.
Keywords *US FDA; CVM; new animal drug approval; INAD; NADA*

Introduction

Over the past two decades, global animal healthcare market has captured attention of top global pharmaceutical companies. This is because once a product is approved for human use then it is easier to expand the market share of the product by extending its usage in the animal health segment. Animal healthcare industry is exhibiting an accelerated growth potential. The global animal healthcare market, estimated at $25 billion in 2015, is set to reach $39.7 billion by 2021, with a compound annual growth rate (CAGR) of 8.06% [1]. According to Vetnosis, a leading research and consulting firm specializing in global animal health market, shares of animal healthcare sector are primarily distributed in the West where, 47% of the market is based in the US, 32% in the Europe and 21% in the rest of the world (Figure I) [2]. The most valuable type of product by far is pharmaceuticals (62%), followed by biologicals (26%) and medicinal feed additives (12%) [2].

Rising global population, increased incomes and scarcity of agricultural lands have resulted in swelling demands for protein rich food items originating from livestock animals which include both beef and dairy cattle, pigs, sheep, goats, horses, mules, asses, buffaloes, and camels. With growing

urbanization, there is a rise in popularity of pet ownerships and growing human-animal bond. According to National Pet Owners Survey report, 65% of the US households or about 79.7 million families own a pet [3]. Apart from these, increased risk of developing age-related disorders in pets, increasing prevalence of zoonotic diseases (diseases caused by viruses, bacteria, fungi and parasites), the government initiatives worldwide on animal healthcare along with augmented global trend of large-scale farming practices are resulting into requirement of effective disease prevention and health management strategies. This is leading to constant requirement of good quality drugs, vaccines and nutritional supplements. Animal health companies are well equipped to address all these needs.

Figure I: *Animal healthcare market segments: Geography wise*

The US animal healthcare market, estimated at $4.83 billion in 2016, is set to reach $6.10 billion by 2021, with a CAGR of 5.22% [4]. The animal healthcare products in the US have been categorized into pharmaceuticals, bio-pharmaceuticals and veterinary devices.

The United States Regulatory Bodies for Animal Healthcare Product Approval

As illustrated in Figure II, regulatory agencies for animal health products approval in the US include the US Food and Drug Administration's (US FDA) Center for Veterinary Medicine (CVM), the US Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) and the Environment Protection Agency (EPA). Whilst the US FDA's CVM reviews animal drugs and devices, the USDA does assessment of animal vaccines and biologics applications, whereas the EPA plays a role in regulating topical compounds against fleas, ticks and other parasites.

The CVM ensures that only high quality, safe and efficacious animal drugs reach the animals and consumers of byproducts. It also safeguards the food items derived from treated animals. CVM's responsibilities are handled by six offices, including the Office of New Animal Drug Evaluation (ONADE), which reviews information submitted by sponsors for obtaining manufacturing and marketing approval for animal drugs, and the Office of Surveillance and Compliance (OSC), which conducts post-marketing monitoring of animal drugs.

Animal Drugs

In US, there is no discrete definition for animal drug. The Federal Food, Drug, and Cosmetic Act (FFDCA) defines the term "drugs" as "articles intended for use in the diagnosis, cure, mitigation,

treatment, or prevention of disease in humans or other animals" and "articles (other than food) intended to affect the structure or any function of the body of humans or other animals."

Figure II: *Regulatory bodies for animal healthcare product approval*

In US, the target species for animal drugs are segmented into **major** and **minor** categories. Under major species category, there is a sub-division as companion animals (e.g. dogs, cats, and horses) and food producing animals (e.g. cattle, pigs and chickens). Under minor species, fishes, ferrets, goats as well as all the other animals apart from major species are included. The production animal segment drives almost 59% of the sales with the remainder driven by the companion animal segment [2].

Scientific Assessment of Animal Drugs

Irrespective of variations in regulatory framework across regions, the basic rule of animal drug development includes scientific assessment of animal drug followed by its registration and upon receipt of approval from relevant regulatory bodies, finally marketing of the product.

During scientific assessment, the biopharmaceutical company needs to ensure that adequate information pertaining to safety, efficacy and quality of the test drug has been collected as per the regulatory requirements (Figure III).

Unlike safety assessment of human drugs, veterinary drug safety assessment includes not only patient (animal) safety but also **food consumer safety** (the drugs targeted for food producing animals).

Besides this **handler safety evaluation** is required for assessment of safety of the people those will work with animals or have significant contact with animals. In addition to food consumer safety and handler safety the health authorities also require the submission of an **Environmental Assessment** (EA) or a claim of **Categorical Exclusion** (CE) for environment safety assessment.

For efficacy assessment, effectiveness of the test drug is tested in the target species, in the targeted disease or the targeted condition.

Quality assessment includes testing for ingredients, manufacturing procedures and facilities, purity and stability of the final formulation, maintaining batch-to-batch consistency in the finished products etc.

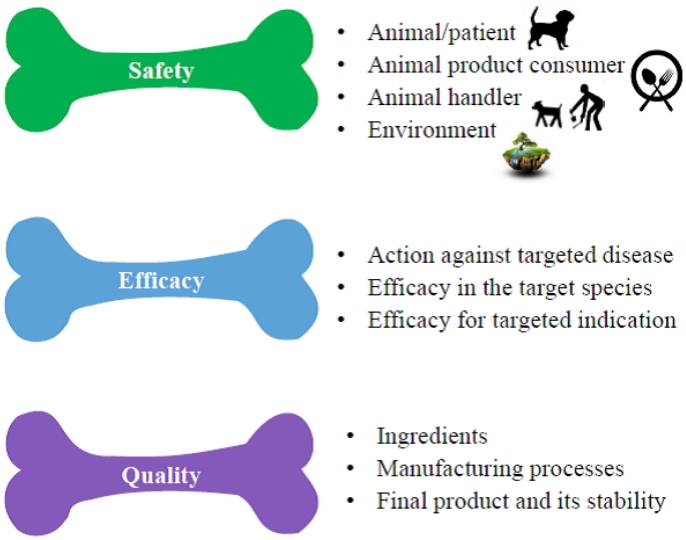

Figure III: *Scientific assessment of animal drugs*

New Animal Drug Registration Procedure

Upon conducting market assessment for unmet animal needs, availability of business opportunities and completion of preliminary screening of new drug in the discovery and development stage, if the biopharmaceutical company decides to go for more expensive registration phase, the company needs to submit a letter to the ONADE of CVM to open an Investigational New Animal Drug Application (INAD) file and to initiate the drug registration process. As a part of the development plan, the ONADE and the drug sponsor need to discuss on the information needed to get the drug approved, including the number and types of studies that may be required and the overall design of each study [5]. A clear flowchart of the new animal drug regulatory approval process is illustrated in Figure IV.

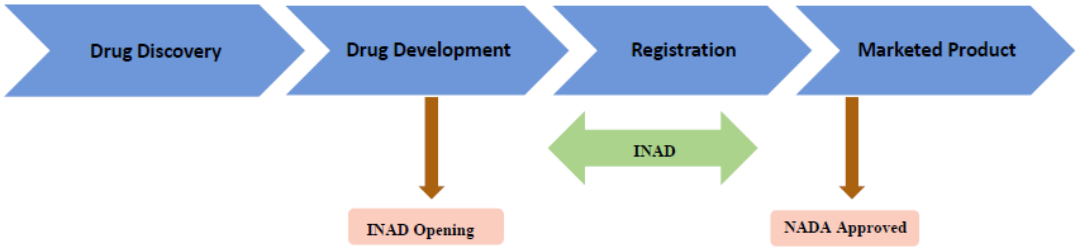

Figure IV: *New animal drug development process*

The INAD file allows shipment of the drug for use in the studies and may authorize the use of edible tissues from animals treated with the investigational animal drug. Upon establishment of the INAD file, the sponsor plans and conducts the studies needed to support approval. Registration requirements for approval are categorized into seven technical sections, out of which five are major *viz.,* target animal safety, effectiveness, human food safety, chemistry, manufacturing, controls, and environmental assessment, whereas two are minor technical sections *viz.,* product labelling and all other information.

Under the ‘target animal safety section’, the sponsor submits data on margin of safety study in the target species, tolerability study, reproductive safety study, animal class safety study and other required studies depending on the drug type.

'Effectiveness section' includes data of dose characterization studies/literature and further the field clinical studies, bio-equivalence studies etc.

'Human food safety section' is compulsory for drugs intended for food producing animals only. If the drug product is to be used in a food-producing animal, residues in food products (such as meat, milk, eggs etc.) derived from that animal must be shown to be safe for human consumption. This section includes the toxicology data to determine acceptable daily intake (ADI), residue chemistry data to set tolerance limit and withdrawal time, and microbial food safety data.

For the quality section of the dossier, plans related to 'chemistry, manufacturing and controls (CMC)', for making the drug should be described. It includes data related to details of ingredients and their sources, manufacturing, packaging and storage details, expiry date for test drugs etc.

'Environment assessment (EA) section' is needed under the National Environmental Policy Act (NEPA), wherein the sponsor needs to generate data related to impact of test drug on environment upon its approval. This section describes how much drug is expected to get into the environment and its potential effects on the environment. Specifically applicable for herd/flock treatments, for individual animal treatment e.g. during treatment of pets i.e. dogs and cats, where chances of the drug entering into the environment are rare, the sponsor can apply for waiver off for conducting EA through categorical exclusion application, which indicates that the drug is unlikely to cause a significant environmental impact.

Minor sections i.e. 'product labelling' encompasses labelling information details for immediate container, package insert, outer packaging, shipping label and client information sheet whereas 'all other information section' includes published scientific literature, foreign experience of test drug, medical experience in people and also studies that were conducted by the drug sponsor but not included in the five major technical sections.

During phased application process, once each section is complete, the sponsor submits data to the CVM for review. CVM ensures that the requirements have been met for a particular section, and issues a "technical section complete" letter. CVM also publishes the Freedom of Information (FOI) Summary, which comprises a summary of the safety and effectiveness studies used to support the CVM's decision of animal drug approval. Upon completion of all the technical sections, the sponsor applies for administrative new animal drug application (NADA). The sponsor submits the copies of the technical section complete letters, detailed information on product labeling, and a completed FDA Form 356V (required only for paper based submissions) for an administrative NADA review. Alternatively, the sponsor may submit a single application.

After receipt of all the information, CVM prepares an approval package for the Center Director's signature. If all requirements have been met, the new animal drug application gets approved. An approved NADA means that the drug is safe and effective for its intended use, and that the methods, facilities, and controls used for the manufacturing, processing, and packaging of the drug are adequate to preserve its identity, strength, quality, and purity. New animal drug approvals are announced by a Federal Register notice and added to the Code of Federal Regulations and the Green Book.

Guidance Documents

CVM Guidance for Industry provides detailed guidelines for planning, conducting and reporting studies with new animal drugs [6]. Moreover, US being the member of International Cooperation on Harmonization of Technical Requirements for the Registration of Veterinary Medicinal Products (VICH); guidance documents issued by VICH are also primarily used during the development of

animal drug candidates [7]. Additionally, applicable human drug guidance from FDA and ICH can also be referred.

Fees

To support FDA CVM's review process for certain animal drugs, the sponsor pays processing fees under the ADUFA (Animal Drug User Fee Act) legislation [8]. The US FDA announces rates and payment procedures for animal drug application review process in the Federal Register for the particular fiscal year.

Conclusion

For the animal health industry, the high cost of drug development and manufacturing and relatively lower returns for certain segments make bringing new drugs to market very challenging.

With the, evolving regulations in the drug development processes, heavy market competition, and rising economic volatility, it is imperative to accelerate the drug approval process and to bring new safe and effective drugs early into the market.

US FDA has established stringent rules and regulations for animal drug approval to ensure animal health and welfare; protect public health as well as environmental safety. The animal drug approval processes in the US are quite sponsor friendly allowing for a phased or complete submission of the data. The US FDA guidelines are evolving and it is important to have thorough understanding of new regulations, policies, and updated rules for timely registration of products.

The drug sponsor needs to have a detailed regulatory strategy and forward planning, better understanding of regulatory requirements, and experienced resources. However this can demand increased efforts and lot of costs to get the drug into market. The sponsors have opportunity to discuss each study with the regulators before its conduct and then the results, thus ensuring right data gets generated for smooth registration of products. It is important to understand the entire ecosystem for the animal and the people directly or indirectly affected from the animals. Hence it is necessary to identify the regulatory requirements before performing any strategic planning for the conduct of studies. There is lot of variability in regulatory requirements for various species. It is important that the trials are carefully planned and executed to generate requisite data. Besides this well written documents with focused discussions around Safety, Efficacy, Quality and Environmental Safety may help to accelerate the product registration.

To overcome the hurdles of drug development process and new product registration to comply with FDA standards, partnering with a strong strategic partner with expertise could be an intelligent option for animal health industry sponsors.

A **strategic** outsourcing partner, offering complete assistance through provision of variety of services under one umbrella, right from project management to clinical data management, biostatistics, medical writing and communications, regulatory affairs, and pharmacovigilance, can prove to be a right and flexible option for partnership to any biopharmaceutical company. A service provider with a wide customer base, huge turnover, years of experience, diversified services portfolio, rich pool of resources, and proposing realistic costs can serve as a valuable tool to empower the biopharmaceutical companies to achieve their goals and help them stay ahead of competition and that to in far more cost effective way.

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Conflict of Interest

The Authors declare that there is no conflict of interest.

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Case Report | Open Access

Case Report: Diagnosis of Cerebello-Pontine Angle Meningioma through Magnetic Resonance Imaging in an Indian Leopard (*Panthera pardus fusca*)

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Abstract A rescued seven-year-old intact female Indian leopard (*Panthera pardus fusca*) with limited previous history and no known clinical parameters was observed showing severe incoordination and altered mentation. Conscious neurological exam was not feasible, but close observation was possible in a confined area. The leopard exhibited ataxia, right-sided head tilt without nystagmus, dysmetria, reduced jaw tone and disorientation. However, appetite, urine and bowel movements were found to be regular. MRI confirmed the presence of a large, well-circumscribed intracranial mass measuring 3.7x2.9x3.4 cm on the right cerebello-pontine angle region and involving parts of the temporal lobe, cerebellum, brain stem and petrous bone, consistent with a meningioma. The right-sided head tilt, ataxia, dysmetria along with Temporalis and Masseter muscle atrophy suggested involvement of cerebellum, cranial nerves fifth (V), eighth (VIII) and possibly seventh (VII).
Keywords *large felid; advanced diagnostic imaging; neurology; Indian wildlife*

1. Introduction

A rescued seven-year-old intact female Indian leopard (*Panthera pardus fusca*) with limited previous history and no known clinical parameters was observed showing severe incoordination and altered mentation. No obvious signs of illness or disease were apparent until two months since the date of capture and the animal appeared alert during this time.

The leopard weighed 50 kg and was anaesthetized using xylazine hydrochloride (Ilium Xylazil; Troy Laboratories Private Ltd, Smithfield, NSW 2164, Australia; 50 mg i.m.) and ketamine hydrochloride (Ilium Ketamil; Troy Laboratories Private Ltd, Smithfield, NSW 2164, Australia; 150 mg i.m.) for basic blood work and physical exam [1]. Following induction, supplemental doses of ketamine (50-75 mg) were used to maintain anesthetic depth as required [1]. Blood parameters were within normal ranges and general physical exam was unremarkable except for mild dehydration (5-6%) and severe unilateral atrophy of Temporalis and Masseter muscles on the right. Anesthetic recovery was uneventful after administration of yohimbine hydrochloride (Reverzine; Bayer Australia Ltd, 875 Pacific Highway, Pymble, NSW 2073, Australia; 5 mg i.v.). Conscious neurological exam was not

feasible, but close observation was possible in a confined area. The leopard exhibited ataxia, right-sided head tilt without nystagmus, dysmetria, reduced jaw tone and disorientation. However, appetite, urine and bowel movements were found to be regular.

Following a month of observation, the leopard was anaesthetized again for magnetic resonance imaging with xylazine (50 mg i.m.) and ketamine (150 mg i.m.). Anesthetic depth was maintained using two supplemental doses of ketamine (50-75 mg i.v.) throughout the procedure. Imaging (MRI; Philips Achiever 1.5T, Philips, Amsterdam, The Netherlands) of the head was performed with the leopard in left lateral recumbence. T1 and T2-weighted images were obtained in sagittal, transverse and dorsal planes. Contrast images were obtained by injecting Gadopentetate dimeglumine in saline (1%) (Magnevist; Bayer Schering Pharma, Berlin, Germany; 10 ml i.v.). Anesthetic revival was uneventful using yohimbine hydrochloride (5 mg i.v.), prior to releasing the leopard back into its enclosure. The following report details the imaging characteristics of a meningioma, as diagnosed using MRI and its associated clinical symptoms. This account appears to be a first in the field of Indian wildlife medicine and provides a comprehensive insight into a wild animal neurological anomaly and its diagnosis using advanced imaging techniques.

2. Diagnosis

MRI confirmed the presence of a large, well-circumscribed intracranial mass measuring 3.7x2.9x3.4 cm on the right cerebello-pontine angle region and involving parts of the temporal lobe, cerebellum, brain stem and petrous bone, consistent with a meningioma. The right-sided head tilt, ataxia, dysmetria along with Temporalis and Masseter muscle atrophy suggested involvement of cerebellum, cranial nerves V, VIII and possibly VII.

On dorsal planes, the lesion seemed hypo-intense on T1 and hyper to iso-intense on T2- weighted images (Figure 1a and 1b). Contrast MRI showed homogenous enhancement of the affected area and highlighted a dural tail (Figure 2).

Transverse planes revealed, a slight deviation of central axis away from midline towards the left, indicating a space-occupying lesion (Figure 3). Additionally, compression of the fourth ventricle and minimal dilatation of inferior horn of the lateral ventricle in the right temporal lobe was observed, illustrating a resistance to the outflow of cerebrospinal fluid. Furthermore, cranial nerves VII and VIII appeared homogenously enhanced with the mass, confirming their incorporation (Figure 4b). However, the extent of their dysfunction could not be ascertained due to lack of possibility for a cranial nerve exam.

Sagittal planes showed involvement of petrous bone and a slight compression of the pons, which resulted in ipsilateral trigeminal neuropathy (mass effect) (Figure 4a). Furthermore, cortical sulci, Sylvian fissures and basal cisterns seemed normal and uninvolved.

The aforementioned pathologies and associated clinical symptoms strongly pointed towards a right cerebello-pontine angle meningioma at the petroclival region of the brain, with involvement of cranial nerves V, VII and VIII.

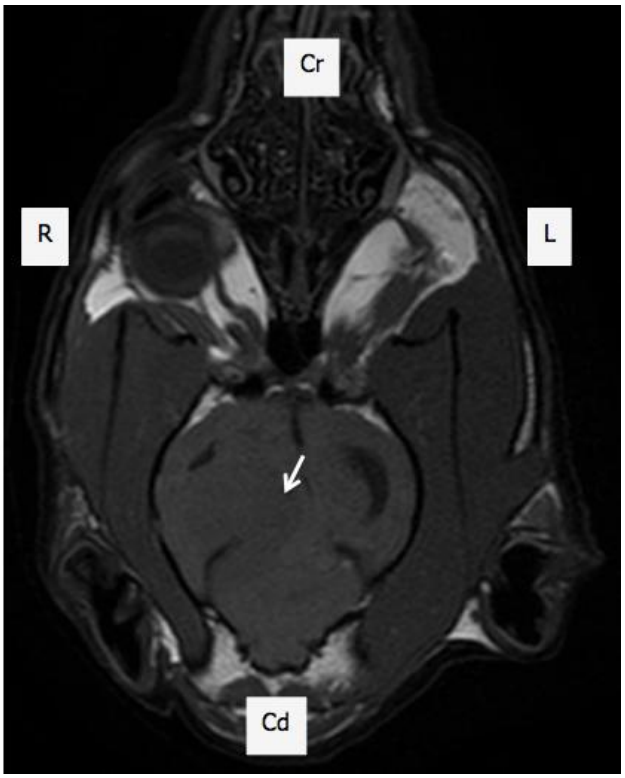

***Figure 1a**:* T1- weighted dorsal plane MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing a well-defined hypo-intense area on the right cerebello-pontine region (white arrow), suspected to be a meningioma. Orientation of dorsal images is denoted by Cr= cranial, Cd= caudal, R=right and L=left

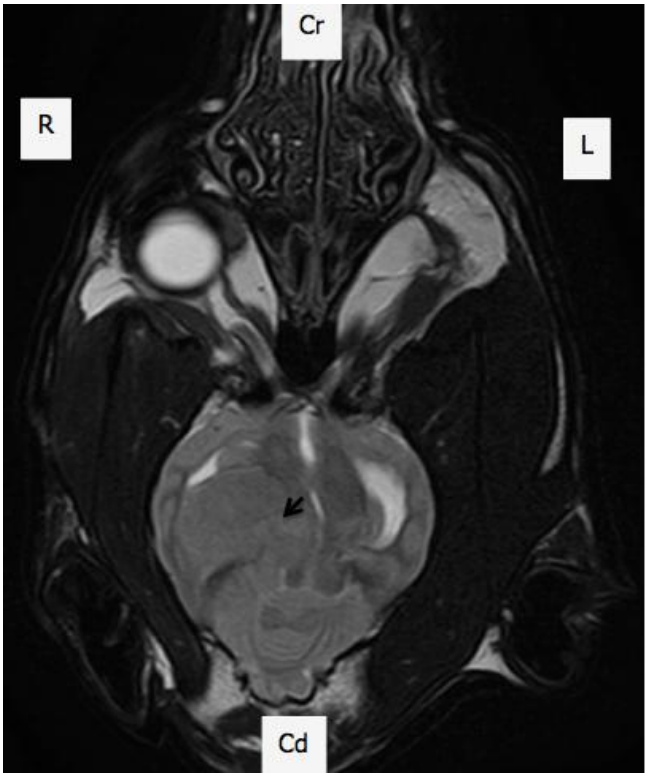

***Figure 1b**:* T2- weighted dorsal plane MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing a well-defined hyper to iso-intense area on the right cerebello-pontine region (black arrow), suspected to be a meningioma. Orientation of dorsal images is denoted by Cr= cranial, Cd= caudal, R=right and L=left

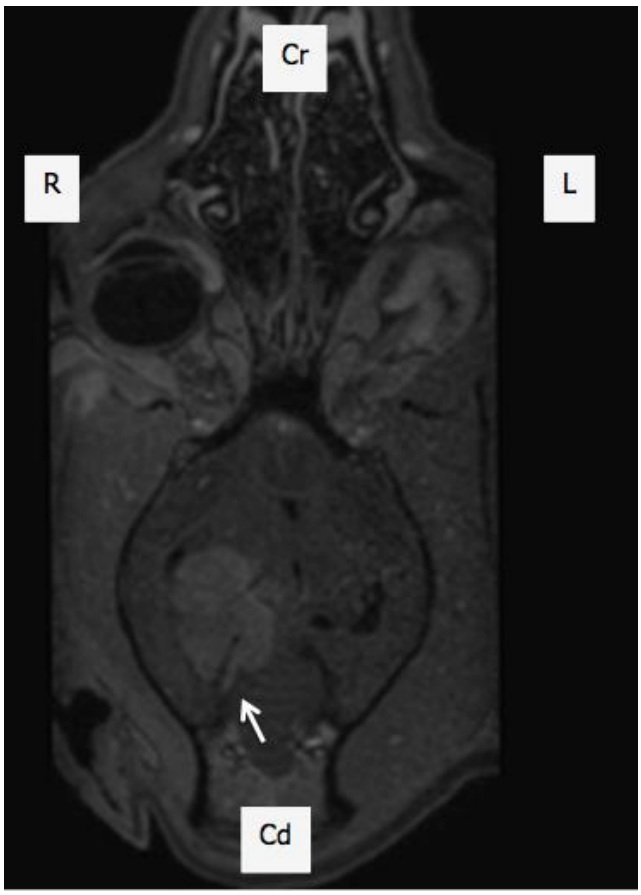

Figure 2: Dorsal contrast MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing homogenous enhancement of an intra-cranial mass with a dural tail at the right tentorial region (white arrow), indicating a possible meningioma. Orientation of dorsal images is denoted by Cr= cranial, Cd= caudal, R=right and L=left

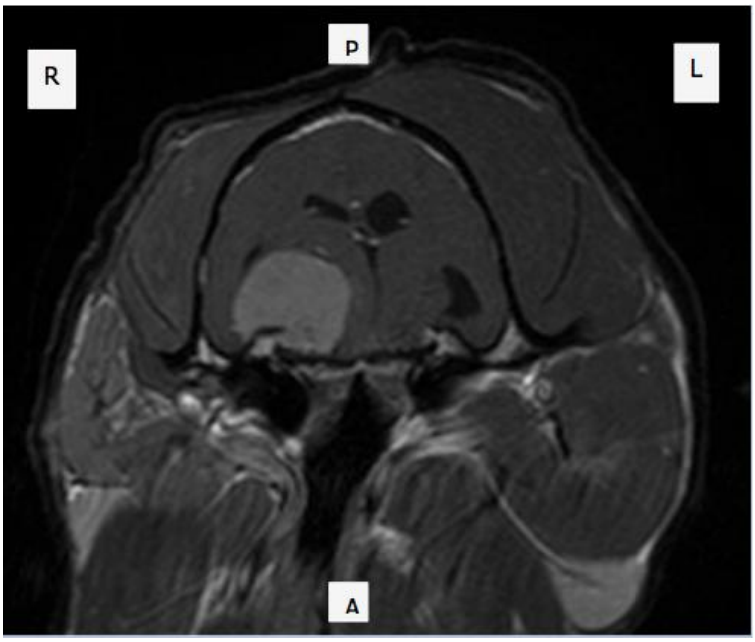

Figure 3*:* Transverse contrast MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing shift in midline axis towards the left and dilation of inferior horn of the lateral ventricle of the right temporal lobe, due to an intra-cranial space-occupying lesion. Orientation of transverse images is denoted by A= anterior, P= posterior, R=right and L=left

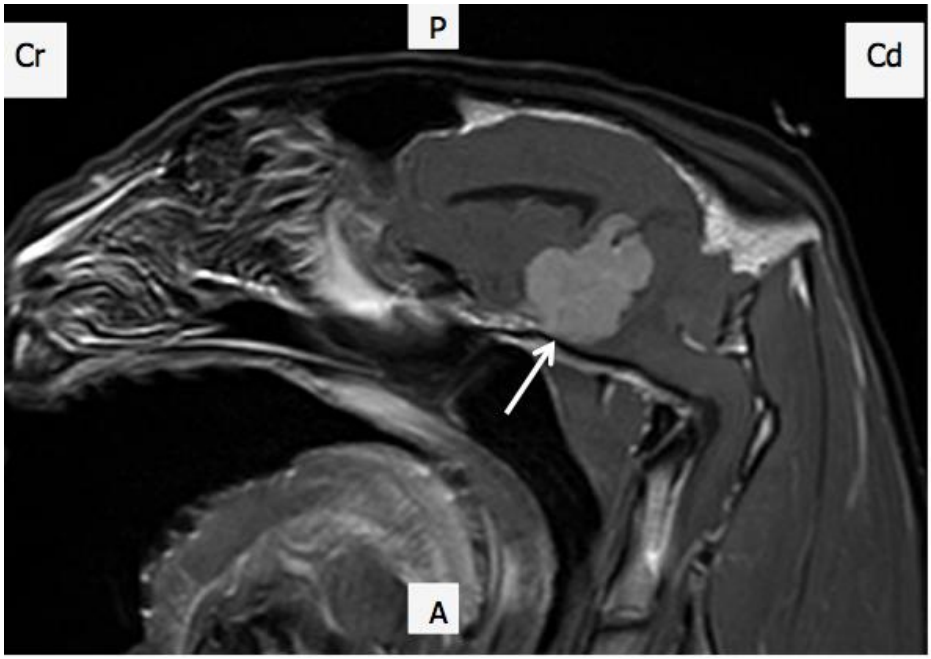

***Figure 4a**:* Sagittal contrast MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing mass effect on the pons with involvement of petrous bone at the petroclival region due to a space-occupying cerebello-pontine angle lesion (white arrow). Orientation of sagittal images is denoted by A= anterior, P= posterior, Cr= cranial and Cd= caudal

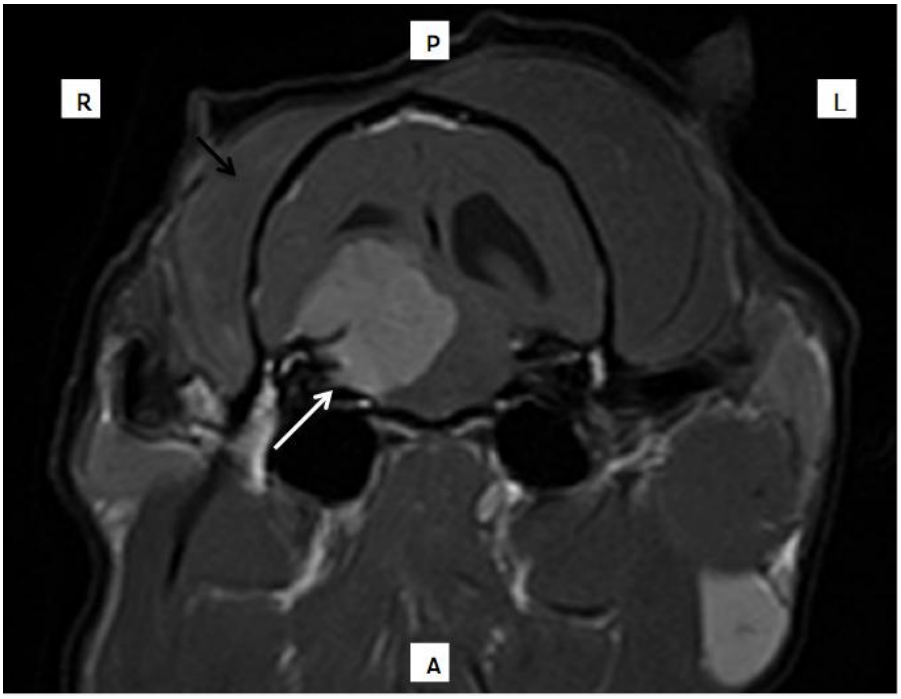

***Figure 4b**:* Transverse contrast MRI of a 7-yr-old female leopard (*Panthera pardus fusca*) showing homogenous enhancement of cranial nerves VII and VIII with the rest of the cerebello-pontine angle mass (white arrow). Severe atrophy of the Temporalis muscle can be appreciated on the right (black arrow), due to mass effect on the pons. Orientation of transverse images is denoted by A= anterior, P= posterior, R=right and L=left.

3. Discussion

Diagnostic imaging descriptions of brain tumors in large wild-caught felids are limited, if any. However, meningiomas are among the most common intracranial neoplasm in domestic cats and sufficient data regarding location, associated symptoms and diagnostic features of these lesions in this species has been documented in literature [4, 9]. Therefore, information for diagnosis of intracranial tumors in wild felids can only be extrapolated from that of small animals.

Meningiomas are slow growing tumors that carry an insidious onset of clinical signs in a patient, with most showing up only one to three months before diagnosis [3]. Moreover, older felines (10-years or above) are commonly affected and a majority of meningiomas occupy supratentorial locations [5]. However in this case, the tumor appeared to affect a relatively younger feline and was found to be infratentorial.

Interpretation of images for this case was largely based on comparison with scientifically accepted descriptions of similar pathologies in small animals, as described. In MRIs, meningiomas in cats are almost always extra-transverse with or without a dural tail and possess marked homogenous contrast enhancement [8]. Lesions are usually reported as being well-margined with decreased signal intensity on T1-weighted and increased signal intensity on T2- weighted images. In most cases, a 'mass effect' can be observed depending on location and peri-tumor edema build up, due to compression of dependent parts; thereby allowing for initial localization of the tumor on physical exam [7]. Similar lesions were appreciated in this case and were sufficient to arrive at a tentative diagnosis at the least. Though imaging may provide sufficient diagnostic insight, confirmation and prognosis of the disease must be sought through histopathological investigation alone [6]. Histologically, meningiomas can be classified into three grades, depending on incidence and severity- Grade I (benign), Grade II (atypical) and Grade III (malignant) [6]. However, owing to the lack of neurosurgical expertise and the endangered status of the animal, neither a biopsy for histopathology nor surgical intervention for cure could be carried out on the patient in concern [2]. Therefore, palliative treatment is being accorded till invasive techniques become feasible in the region.

A handful of reports regarding occurrence of meningiomas in wild and large felids are available, but most of them are isolated incidental postmortem findings that have been described histologically [10]. This clinical case is the first magnetic resonance imaging description of a meningioma in a wild leopard, with an account of associated clinical symptoms, in the Indian subcontinent.

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Research Article

Bromadiolone Induced Oxidative Stress and Cytological Damage in Layer Birds

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Abstract This study was undertaken to evaluate the oxidative stress induced by bromadiolone toxicity in layers. Birds were divided into two groups. Control group (I) received bromadiolone free feed and group (II) was exposed to bromadiolone (5 mg/kg feed) for seven weeks. Blood samples and organs were collected at the end of the experiment. Bromadiolone exposure resulted in significant rise in lipid peroxidation (LPO) levels in erythrocyte and liver. In addition decreased (P<0.05) level of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities were observed in erythrocyte and liver. Histopathological results revealed mild to severe type of necrosis and degenerative changes in heart and liver of bromadiolone intoxicated animals. In addition histopathological findings of tissues ovary, liver, heart and pancreas also showed generalized haemorrhage. It is therefore concluded that feeding of bromadiolone admixed feed lead to depletion of Vitamin K causing multifocal haemorrhages and oxidative stress and cellular damage in different tissues.
Keywords *Histopathology; oxidative stress; layer birds; bromadiolone*

1. Introduction

Bromadiolone, a second generation rodenticide is used as a preferred rodenticide in the urban and farm rodent control and acts by disrupting the normal blood clotting mechanism (Kocher and Parsad, 2003). As whole grain baits are highly attractive and palatable, therefore are readily consumed by animals and birds. Non-target species are potentially at risk from direct consumption of bait and also through eating poisoned rodents (Berny et al., 1997). Recently, ten cases of rodenticide poisoning in dogs were observed in Texas Vet Medical Diagnostic Laboratory and two neonatal puppies (Munday and Thompson, 2003). Eason and Spurr (1995) reported brodifacoum rodenticide toxicity in 200, one week old chicks, in which hundred percent mortality was observed. The source of rodenticide was found to be wood straw mats in the chick boxes. Toxicity data for a variety of animals suggests that anticoagulant rodenticides are no doubt a potential hazard to many wild mammals and birds (Berny et al., 1997; Stone et al., 1999). Thus, the present study was contemplated to study the effect of Bromadiolone induced Oxidative Stress and Cytological Damage in layer birds.

2. Materials and Methods

2.1. Chemicals

Bromadiolone (3 – [3 – [4' – bromophenyl – 4 – yl) – 3 hydroxy – 1 – phenyl propyl] – 4 – hydroxyl coumarin) and all other chemicals were of the analytical grade and were purchased from Merck.

2.2. Animals and experimental design

Twenty egg laying birds, 30 week old were maintained in the poultry sheds of Department of Livestock Production and Management, GADVASU, Ludhiana where proper hygienic conditions were maintained in the cages. These birds were provided with fresh water and feed ad libitum. The birds were randomly divided into two groups of ten birds, each. Control group received bromadiolone free feed and experimental group (II) was exposed to bromadiolone (5 mg/kg feed) for seven weeks. The conduct of experiment was done as per approval of IAEC and as per accordance with the guide lines for animal experimentation.

2.3. Sample collection

At the end of seventh week, blood samples were collected from 5 birds in each group by cardiac puncture for biochemical estimations. The plasma was separated and immediately stored at -5°C till further use. Blood was collected by cardiac puncture before sacrifice and tissue samples were collected for further analysis. The Liver, heart, pancreas and ovaries were removed and in a part were fixed in 10% buffered formalin for histopathological examination (Luna, 1968). These were processed, microtomed at 5 µ and stained with hematoxylin and eosin (H and E) stain. Half portion of the liver from each bird was processed immediately for biochemical estimation.

2.4. Biochemical assay

Lipid peroxide level in 10% RBC hemolysate was determined as per Placer et al. (1966) and was expressed as nmol malondialdehyde (MDA)/mg of hemoglobin (Hb) using 1.56×10^5 as extinction coefficient (Utley et al., 1967). For the preparation of 10% RBC hemolysate, blood samples were centrifuged at 2000 rpm for 10 min and supernatant plasma were separated out. The sedimented cells were washed with sterile 0.85% NaCl solution three times. Washed erythrocytes were hemolyzed with ninefold volume of distilled water to prepare 10% RBC hemolysate. Hemoglobin in the hemolysate was estimated by the cyano-methaemoglobin method (Van Kampen and Ziglstra, 1961). Lipid peroxides in 10% crude tissue homogenate of liver from birds was estimated following Okhawa et al. (1979) and was expressed in nmol of MDA/ mg of protein. Tissues were immediately perfused with cooled buffer that contained 0.9% KCl, pH 7.2. They were homogenized in 9 volume of 1.15% KCl, 125 mM sucrose, pH 7.2. The homogenates were employed for the assays. The protein in 10% tissue homogenate was measured by the method of Lowry et al. (1951). Superoxide dismutase activity in 10% supernatant tissues and RBC hemolysate was estimated as per Menami, M., Yoshikawa, H. (1979). The catalase activity in tissues and RBC hemolysate was estimated as per Cohen et al. (1970).

2.5. Statistical analysis

Data are expressed as means±SEM and were analyzed statistically using analysis of variance to compare the means of different treatment groups with that of the negative and positive control groups.

3. Results

3.1. Effect on LPO

The role of lipid peroxidation was assessed by studying the level of formation of malondialdehyde, an indicator of lipid peroxidation (Table 1). Bromadiolone exposure for 7 weeks resulted in significantly (P<0.05) increased LPO levels in erythrocytes and liver tissues as compared to control birds.

Table 1: *Effect of bromadiolone on malondialdehyde (MDA) concentrations (erythrocyte and liver), superoxide dismutase (SOD), glutathion and Catalase in layers*

Enzymes	Control	Bromadiolone
MDA nM/gm Hb	7.2 ± 0.3^{b}	16.7 ± 3.5^{a}
MDA nmol of MDA/ gm protein	18.7 ± 1.24^{b}	31.4 ± 1.3^{a}
SOD(U/gm tissue	803.5 ± 0.43^{c}	362.2 ± 2.6^{a}
GSH(mM /gm tissue)	6.8 ± 0.4^{b}	2.5 ± 1.2^{a}
Catalase(U/gm Tissue)	116.5 ± 0.4^{b}	72.6 ± 1.8^{a}

Values are represented as mean ± standard error. The different superscript letters mean a significant difference at (P > 0.05) between different groups in the same row.

3.2. Effect on nonenzymatic antioxidant

Result presented in Table 1 represented the reduced glutathione (GSH) levels of studied tissue in experimental animals. Administration of bromadiolone caused significant decrease (P<0.05) in the GSH level in erythrocyte and liver tissues.

3.3. Effect on antioxidant enzymes

Table 1 showed decreased SOD and CAT activities in bromadiolone exposed birds. Significantly (P<0.05) decreased activities of SOD and CAT enzymes were reported in erythrocyte and liver tissue.

3.4. Histopathological alterations

Histopathological findings

Bromadiolone feeding resulted in pathological alterations in various organs. In Liver, vascular compromise viz., congestion to hemorrhage, besides varying fatty changes of mild to severe intensity (Figure 1) were discernible. In ovary, marked hemorrhages were observed, replacing the ovarian stroma (Figure 2). In myocardium of heart, there was evidence of congestion, hemorrhage and edema in myofilness of birds which received bromadiolone (Figure 3).

4. Discussion

Present results showed that bromadiolone causes lipid peroxidation in liver. Increased lipid peroxidation is thought to be consequences of oxidative stress which occurs when the dynamic balance between peroxidant and antioxidant mechanism is impaired (Flora et al., 2008). A marginal decrease in GSH correlated well with an increase in LPO in the respective tissues. Adequate levels of the cellular GSH pool required not only for maintaining the cellular redox status by keeping sulfhydryl groups of cytosolic proteins in their reduced form but also because numerous toxic or potentially toxic compounds are either taken up by or removed from the cells by GSH-mediated pathways

(Chouchane and Snow, 2001). A decrease in cellular GSH concentration has been inversely correlated with lipid peroxidation in the liver (Maiti and Chatterji, 2000, 2001).

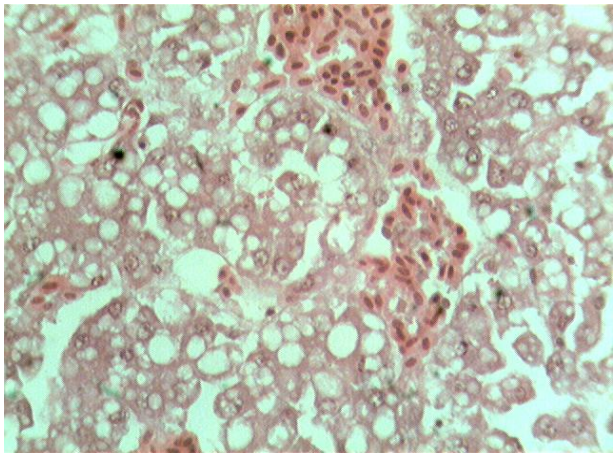

Figure 1: *Liver: Section of liver, with evidence of fatty change in liver. H & E x 150*

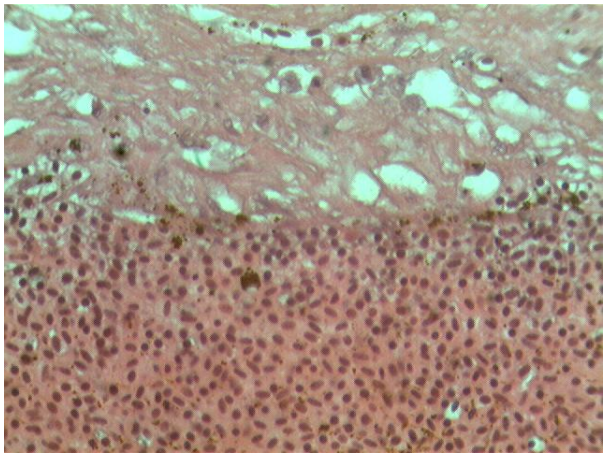

Figure 2: *Ovary: Marked hemorrhages are seen in ovarian stroma. H & E x 150*

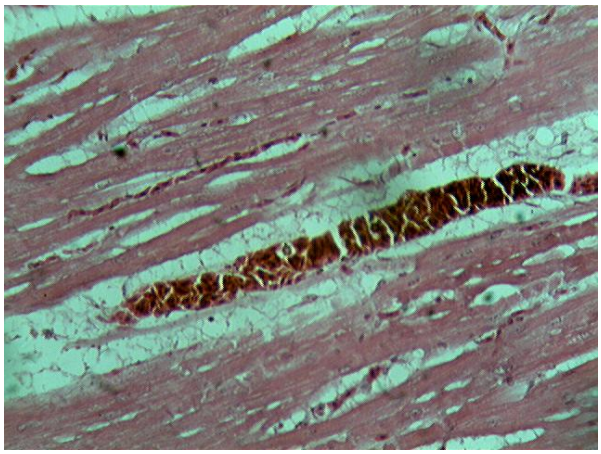

Figure 3: *Heart: Exhibits congestion and edema in myocardium. H & E x 150*

Antioxidant enzymes are considered to be the first line of cellular defense against oxidative damage. SOD is an antioxidant metalloenzyme that reduces superoxide radicals to water and molecular oxygen (McCord, 1976). CAT is a haemoprotein, which reduces hydrogen peroxide to molecular oxygen and water (Gutteridge, 1995). In support to our results observed decreased SOD and CAT enzyme activities in the tissues of experimental birds against bromadiolone intoxication. Santra et al. (2007) conducted a study on arsenicosis and their results are parallel to this study and they concluded the changes in liver cells were due to oxidative stress in mitochondria that plays an important role in the pathogenesis of apoptotic cell injury. A decrease in GSH triggers the activation of neuronal 12-lipoxygenase, which leads to the production of peroxides, and, ultimately, cell death (Schulz et al., 2000). Histopathological observation in birds receiving bromadiolone exhibited extensive cytological alterations as compared to the normal tissue architecture. These gross postmortem lesions of subcutaneous hemorrhage, multifocal hemorrhages in visceral organs and presence of blood clots were evident, which are in accordance with other studies (Kocher et al., 2004).

Bromadiolone is an anticoagulant of high toxic potential for most mammals, as it interferes with the vitamin K dependent clotting factors, when a lethal or sub-lethal dose is ingested (Svendsen et al., 2002). The degree of hazard would be expected to vary by compound ingested, species involved and type of application, as poisoning can occur by direct ingestion of bait (primary poisoning) or via consumption of poisoned rodents causing secondary poisoning (Newton et al., 1990). Based upon the results of this experiment it may be concluded that bromadiolone exposure leads to depletion of antioxidant defense mechanism and varying degree of changes in tissue architecture in layer birds.

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Cloud Publications

Case Report | Open Access

Case Report: Rabies in a Small Indian Civet *Viverricula indica*

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Abstract An adult female Small Indian civet was rescued from a pile of rubble in broad daylight and brought to Pilikula Biological Park's wildlife hospital at Mangalore, India. During initial examination, the animal showed no physical deformities or signs of trauma, but exhibited progressively worsening neurological signs like ataxia, sialorrhea, convulsions and a highly uncoordinated, wobbly gait. Benzodiazepines and antibiotics were administered to manage convulsions and prevent secondary infections respectively, but the animal succumbed four days following admission. Necropsy revealed no gross abnormalities in the organs, but histopathological examination revealed bronchopneumonia, congestion and oedema in the lungs. It also dismissed canine distemper as a differential owing to the lack of intra-cytoplasmic inclusion bodies in the urinary bladder epithelium. However, an impression smear of the brain returned positive for rabies when tested with Fluorescent Antibody Technique (FAT). This is the first report of rabies in a civet from south India. The major implications of this finding are the possibility of rabies transmission to the critically endangered Malabar large-spotted civet *(Viverra civettina)* and the impending risk of zoonosis.
Keywords *Internal medicine; neurology; Viverridae; zoonosis*

1. Introduction

Rabies is a notifiable, zoonotic disease of mammals caused by viruses of the genus *Lyssavirus* of the *Rhabdoviridae* family [1, 3]. It was first discovered 4300 years ago and still continues to be of major public health concern, with an estimated 20,000 human deaths per year in India alone [1, 16]. Several mammalian species like canids, felids, primates, rodents and bovines have been found to suffer from fatal neurological manifestations of the disease; except chiropterans, which are asymptomatic carriers that serve as reservoir hosts for the organism [1, 3]. Though the prevalence of rabies in the aforementioned species has been well-established, only a handful of reports from Africa and Sri Lanka describe rabies in civets (Order: Viverridae) [1, 5, 11]. In southern India, information regarding the incidence of this disease among native wildlife is profoundly scarce and the following case study is probably the first report from this region that demonstrates the presence of rabies in a rescued Small Indian civet (*Viverricula indica)*.

The Small Indian civet is a nocturnal mammal of the Viverridae family [9]. It is widely distributed across the Indian subcontinent, South China, Sri Lanka, Malaysia, Java, Sumatra and surrounding islands [2]. Apart from forests, they are also commonly encountered around human settlements near sub-urban areas and villages, often preying on poultry birds [8]. It has been classified as a 'Schedule II' animal by the Indian Wildlife Protection Act of 1972 and is currently listed by the IUCN under the 'Least Concern' category owing to its abundant and stable population size [4, 8, 9].

2. Case History

An adult female, Small Indian civet was brought to Pilikula Biological Park's Wildlife hospital, Mangalore, Karnataka, India in June, 2014. It was found among a pile of rubble in an open ground during the day, which is unusual for a nocturnal species [9]. The animal appeared to be depressed and in a state of shock, but it was unclear whether this was due to stress of capture or underlying disease. Necessary supportive treatment was accorded and the civet was housed separately for further observation.

3. Clinical Findings

Upon physical examination, the animal appeared to have good body condition with no signs of trauma or dehydration. It weighed 1.6 kg (average for civets is 1.5-4.5kg) and appeared to be eating well [13]. All physiological parameters were within their normal range and the only notable clinical symptom on the first day of observation was an uncoordinated and wobbly gait.

Neurological signs became profound after day three and the animal exhibited a highly uncoordinated gait with ataxia, episodic convulsions and sialorrhea. The observed clinical signs pointed towards diseases affecting the nervous system like rabies, canine distemper, infectious encephalitis or neoplasms of the brain; all of which required further investigation [13]. Meanwhile, the animal was administered Diazepam (CALMPOSE® injection, Ranbaxy Laboratories Limited, India; 10mg/2ml presentation) at 2mg/kg IM (to effect) to manage the convulsions and Enrofloxacin (FORTIVIR® injection, Virbac India; 100mg/ml presentation) at 0.75mg/kg q12h (every 12 hours) to prevent the onset of any secondary infections; considering the animal could be immune-compromised if suffering from a possible viral infection or due to stress of captivity [10]. However, the civet showed no signs of improvement and on day four, it was pronounced dead and subjected to an autopsy.

4. Post Mortem Findings

Canine distemper and rabies featured high on the list of tentative diagnoses, owing to the severity of clinical signs; hence, extreme caution was exercised during post mortem. The civet had moderate to good body condition with adequate sub-cutaneous fat reserves. All organs appeared grossly normal and sections of the vital organs, urinary bladder and intestines were sent to the Department of pathology, Veterinary College, Bangalore of the Karnataka Veterinary, Animal and Fisheries Sciences University, for histopathological examination. An impression smear from different sections of the brain, including the brain stem was also sent to the Institute of Animal Health and Veterinary Biologicals, Southern regional Disease Diagnostic Laboratory at Bangalore to test for rabies.

Histopathology of the aforementioned organs, revealed no pathological abnormalities, except the lungs, which showed congestion, oedema and infiltration by inflammatory cells, suggesting bronchopneumonia. The urinary bladder epithelium also lacked the presence of any intra-cytoplasmic inclusion bodies that could be suggestive of canine distemper, ruling out an important differential [14]. However, an impression smear of the brain tested positive for the presence of rabies virus by Fluorescent Antibody Technique (FAT), thereby confirming the diagnosis of rabies in the Small Indian civet [15].

5. Discussion

This is probably the first report of rabies in a Small Indian civet from southern India. Viverrids, especially civets are highly prone to canine distemper, the neurological form of which can elicit the same clinical symptoms as rabies, making it hard to differentiate between the two; moreover, the latter can be confirmed only after death of the affected animal [12, 15]. Therefore, every precaution must be taken when dealing with such animals to prevent the risk of zoonosis.

Further research is required to identify the strain of rabies virus prevalent in south Indian Viverrids and whether these mammals are subjects of a 'spill-over effect', owing to their regular interaction with domestic dogs near human habitats [16]. If the Small Indian civets are harbouring a sylvatic strain of rabies, they may further endanger the survival of the critically endangered Malabar large-spotted civet *(Viverra civettina)* [7]. Moreover, the transmission of sylvatic rabies to humans through infected civets has previously been reported from Africa and its occurrence in Small Indian civets may present similar zoonotic implications for the Indian sub-continent as well [6].

Acknowledgements

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Case Report

A Rare Case of Lipoma of Vagina along with Perineal Hernia in a Female Dog and Surgical Treatment

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Abstract A 10 year old female dog was presented with history of difficulty in defecation, perineal swelling and swollen mass in the vaginal region. Physical examination showed characteristic signs of hernia on right side of the perineum. Under general anaesthesia surgery was performed and entire growth on the vaginal wall was excised completely. Another incision over the perineal swelling revealed pelvic fat herniation. After reducing the contents, herniorrhaphy was performed by suturing the pelvic diaphragm muscles and skin sutures were applied. Histopathologically, vaginal mass contained numerous polyhydral cells and each cell contains fat globule and nucleus pushed to mature fat cells. These findings confirmed as a case of lipoma. This is a record of successful management of an uncommon case of perineal hernia along with lipoma in a female dog.
Keywords *Female dog; Herniorraphy; Lipoma; Perineal hernia*

1. Introduction

Perineal hernia occurs when there is a breach in continuity of pelvic diaphragm. Pelvic and abdominal contents may protrude between pelvic diaphragm and the rectum. The cause of the muscular deterioration of the pelvic diaphragm may be due to various pathological processes like muscular atrophy, myopathies, hormonal imbalance and prostatic hypertrophy. The swelling is presenting ventrolateral to the anal opening and may be unilateral or bilateral. Generally male dogs are more prone to perineal hernia than the females because female dogs have broader, thicker and stronger levator ani muscle and strong fascial attachments to anal sphincter and rectal wall (Desai, 1982). Perineal hernia in a female dog has been described sporadically in literature (Pettit, 1962; Rochat and Mann, 1998; Niles and Williams, 1990). Perineal hernias in female dogs are often related to trauma (Hedlund, 1994). The following case report describes the clinical presentation and successful management of perineal hernia along with lipoma in a female dog.

2. Case History and Observations

A 10 year old female dog was presented to the College Hospital with history of difficulty in defecation, perineal swelling and swollen mass in the vaginal region (Figure 1). On palpation, vaginal mass was soft in consistency. Routine clinical and radiological examination was carried out for diagnosis. Per rectal examination of pelvic diaphragm reveals weakness of muscles. Clinical and radiographic

examination revealed no disorder related to rectum (Figure 2). Physical examination showed characteristic signs of hernia on right side of the perineum. Hence, it was decided to excise tumor mass and also perform perineal herniorraphy for correction of damaged muscles of pelvic diaphragm.

Figure 1: *Female dog with perineal swelling and swollen mass in the vaginal region*

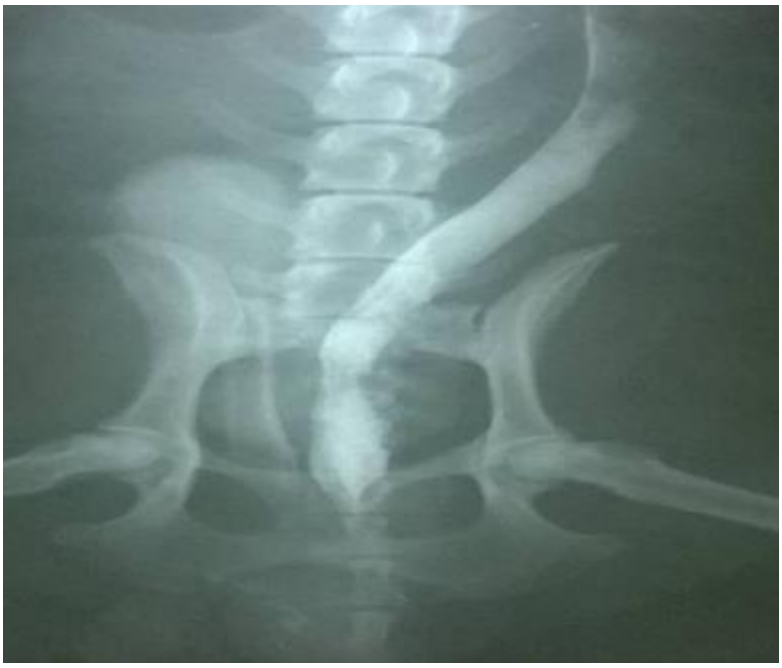

Figure 2: *Radiographic examination revealed no disorder related to rectum*

3. Treatment and Discussion

The dog was prepared for aseptic surgery under Atropine sulphate premedication 0.04 mg/kg body weight given subcutaneously. Then anaesthsia was induced with xylazine hydrochloride intramuscularly 1 mg/kg and maintained with propofol intravenously 6 mg/kg. Animal was restrained

in sternal recumbency (Figure 3). An incision was made over the growth in vagina and entire growth was excised completely. The haemostasis was achieved by ligation (Figure 4). On gross examination the tumour was soft. The cut surface of the tumour was oily and yellowish in colour. After excision of tumor, hernia repair was done by making a slightly curved dorsoventral skin incision extending laterally from the base of the tail to the medial angle of the ischial tuberosity was made. Upon separation of subcutaneous tissue and exploration of area confirmed the diagnosis of perineal hernia, revealing evidence of perineal fat within the herniated mass (Figure 5) and severe atrophy of both coccygeus and levator ani muscle. Then conventional herniorhappy was done by suturing of damaged pelvic diaphragm muscles with PGA No 1 (Figure 6) and skin sutures with Prolene (Figure 7). Postoperatively, ceftriaxone (Intacef, Intas) 20 mg/kg for 5 days and analgesic meloxicam (Melonex, Intas) 0.5 mg/kg for 5 days and alternate day dressing was done. Sutures removed on 10th day postoperatively and animal showed uneventful recovery. Histopathogically vaginal mass showed numerous polyhydral cells and each cell contain fat globule and nucleus pushed to mature fat cells. No mitotic figures were observed. These findings confirmed as a case of lipoma (Figure 8).

Figure 3: *Animal restrained in sternal recumbency*

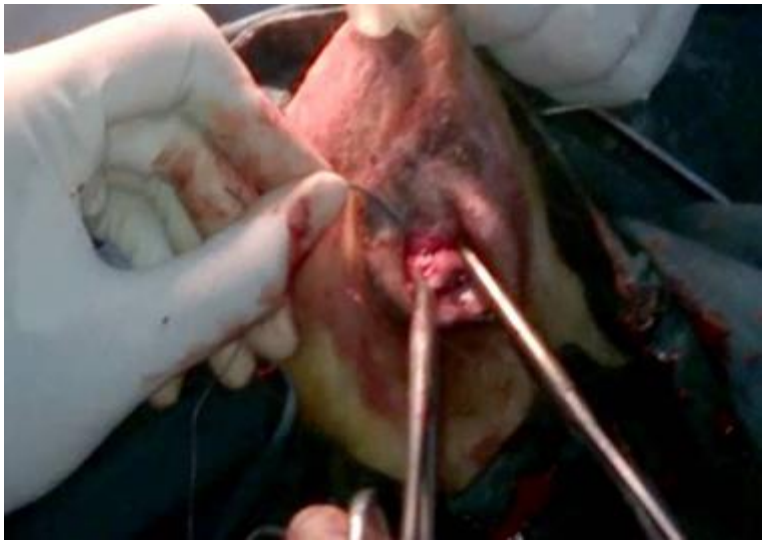

Figure 4: *Haemostasis through ligation*

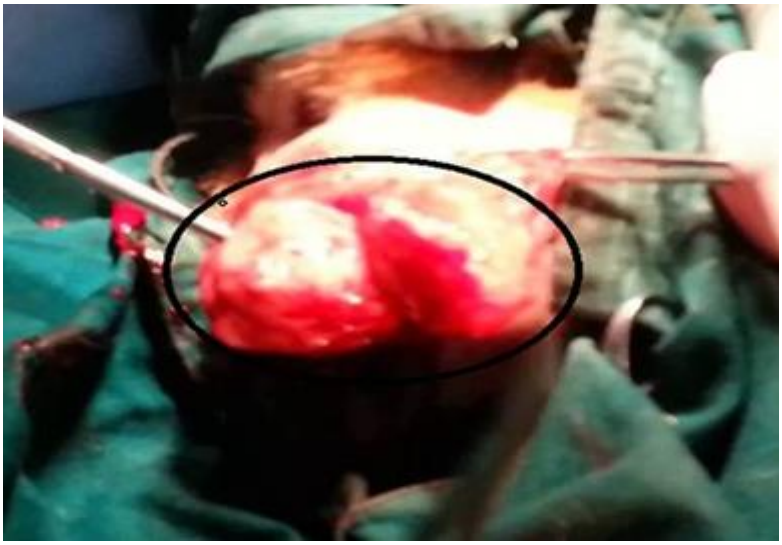

Figure 5: *Perineal fat within the herniated mass*

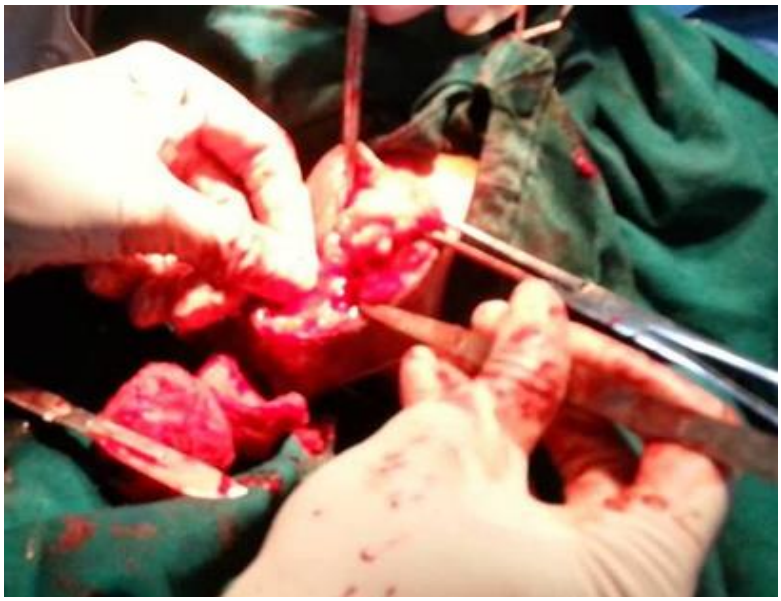

Figure 6: *Conventional herniorrhaphy*

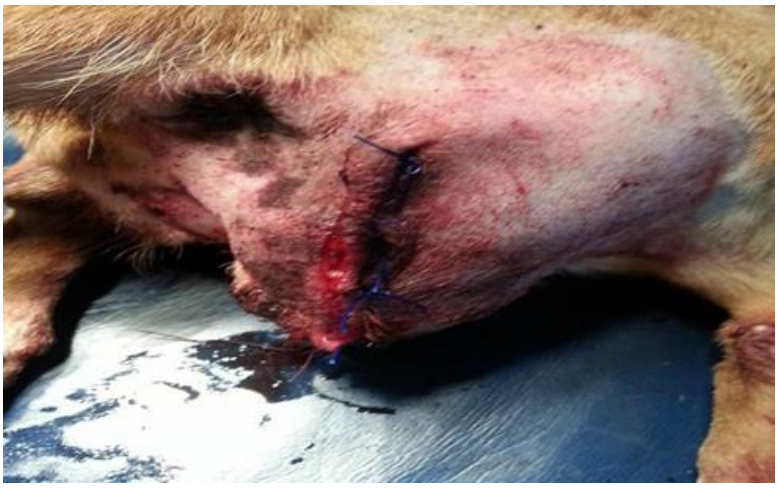

Figure 7: *Animal after surgical repair*

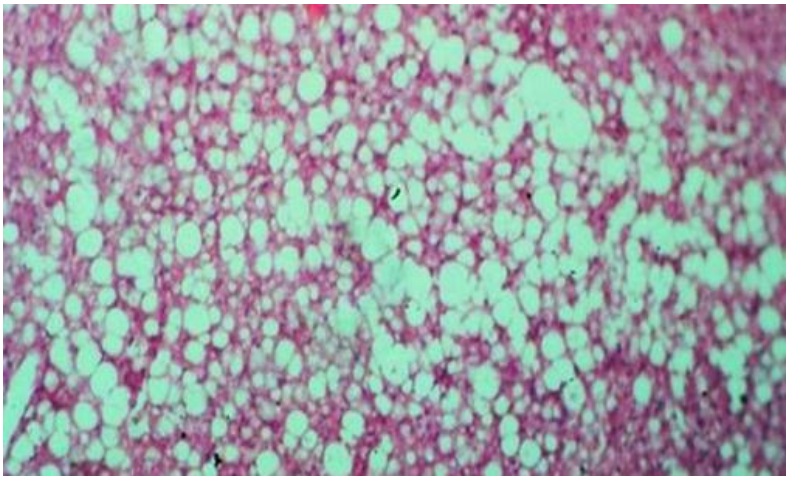

Figure 8: *Histopathogically, the mass showed numerous polyhydral cells and each cell contain fat globule and nucleus pushed to mature fat cells.*

Perineal hernia results from failure of the muscular pelvic diaphragm to support the rectal wall, which stretches and deviates. Pelvic diaphragm is the vertical closure of pelvic canal and it is composed of levator ani muscle medially and coccygeus muscle laterally, and confluence of these structures with external anal spinchter muscle completes the diaphragm. Generally herniation occurs between sacrotuberous ligament and coccygeus muscle in sciatic perineal hernias, ventral to ischiourethralis muscle, between the bulbocavernous and ischicavernosus muscle in ventral perineal hernias, between coccygeus and levtor ani muscle in dorsal perineal hernias and between levator ani, external anal spinchter and internal obturator muscle in caudal perineal hernia (Dorn et al., 1982). Dogs with increased risk of developing perineal hernia are Boston terrier, Collie, Pekingese, Welsch Corgi and Mongrel. Mostly constipation and hormonal imbalance predisposes these animals for occurrence of perineal hernia. The advanced age of the patient and the concurrent inguinal hernias suggest that diminished vitality of the tissues which was an important etiological factor for perineal hernia in a female crossbred terrier (Pettit, 1962). Besalti et al. (2004) reported rectal lipoma as a possible cause perineal hernia in a male dog. But our report showed a rare incidence of vaginal lipoma could be a possible cause of perineal hernia on a bitch. There are many different techniques of perineal hernia repair, of which the two most widely accepted are anatomical reduction technique which was performed in this case and internal obturator trauma. Closure of the hernia defect by transposition of the superficial gluteal muscle (Spreull and Frankland, 1980), semitendinosus muscle (Chambers and Rawlings, 1991) and fascialata (Bongartz et al., 2005) has been reported by various surgeons. Similarly synthetic materials like polypropylene mesh have also been used successfully for repair of perineal hernia in dogs (Szabo et al., 2007). However, in the present case conventional herniorraphy along with excision of vaginal lipoma was performed and animal started normal defecation after 3 days and no recurrence of hernia was reported over a period of 6 months postoperatively.

4. Conclusion

It was concluded that the case report was rare and also the conventional technique can be used successfully for surgical management of clinical cases of perineal hernia in female canine cases.

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Research Article

Occurrence of *Campylobacter* spp., *Salmonella* spp. and *Escherichia coli* in Chicken Carcasses

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Abstract The transmission of Foodborne Diseases (FBD) by bacteria constitute a public health problem in the world. This study aimed to identify the presence of *Campylobacter* spp., *Salmonella* spp. and *E. coli* in broiler chickens' carcasses of Botucatu, SP, Brazil, by means of microbiological and molecular methods. Sixty samples of chilled chickens' carcasses of different brands were randomly collected from supermarkets and meat houses located in both peripheral and central area of the city, from January 2015 to January 2016. Later, *Campylobacter, Salmonella* and *E. coli* was isolated from poultry carcasses by conventional microbiological methods, and confirmed by biochemical and PCR tests. The prevalence was 38.3% of *Campylobacter, Salmonella*, 13.3%, and *E. coli*, 60%.
Keywords *Campylobacter spp.; Chicken carcass; Enteropathogens; Escherichia coli; Foodborne diseases; Salmonella spp.*

1. Introduction

Chicken meat production has expanded significantly in recent years, with Brazil being the second largest producer and the largest exporter worldwide. In addition, poultry consumption has increased globally since considered the cheapest animal protein source. In addition, global poultry consumption has increased considerably since considered as the most economical among animal protein sources. Estimations indicate that over 60% of animal protein consumed comes from chicken meat. Currently, Brazil has a per cápita consumption of 46, 2 (MAPA, 2016).

The contamination of food by pathogens, among them bacteria of the Enterobacteriaceae family, as *Salmonella* spp. and *E. coli*, and the Campylobacteriaceae family, such as *Campylobacter* spp., may cause infection or intoxication in humans. These microorganisms commonly dwell in gastrointestinal tract of animals and humans (Fitch et al., 2005; Nzouankeu et al., 2010). The foodborne disease (FBD) is common in most countries. However, its prevalence has increased exponentially by the massive consumption of poultry and eggs (Wilhelm et al., 2011; Williams et al., 2012).

Campylobacter, *Salmonella* and *E. coli* colonize the gastrointestinal tract of a wide variety of wild and domestic animals. Human consumption of mishandling raw meat is the main propagator of these bacteria (Zhao et al., 2001). This bacterial contamination can occur at various stages along the food

chain, including production, processing and distribution. Several epidemiological studies confirm diseases caused by these pathogens, especially in poultry products (Zhao et al., 2001; Rosynek et al., 2005).

Campylobacteriosis is a disease caused by *Campylobacter* spp. Campylobacter species most common are *C. coli, C. lari*, and *C. jejuni*, being the latter responsible for 90-95% of bacterial gastroenteritis. In humans, gastroenteritis is the most common clinical manifestation. The symptoms are similar to those caused by other enteric pathogens. However, the main complication is the low dose to cause infection. Only the intake of 400 to 500 cells can produces the sickness (Butzler, 2004; Rozynek et al., 2005 Gonçalves et al., 2012). This pathology also appears as a significant cause of Guillain-Barré syndrome, a demyelinating inflammatory polyneuropathy resulting in acute neuromuscular paralysis (Mishu and Blaser, 1993; Silva et al., 2014).

Salmonellosis is caused by *Salmonella* spp. affecting animals and humans. From all serotypes described for *Salmonella enterica*, Enteritidis and Typhimurium are the most frequent cases of human salmonellosis in the world (Galanis et al., 2006; Hendriksen et al., 2009). Clinical manifestations range from mild intestinal signs to septicemia. It is found widely in children, elderly and immunocompetents. Although the diarrhea is the main symptom, the intensity varies among patients. Moreover, abdominal discomfort, cramps, fever, nausea, vomiting and headache are also present (Paixão et al., 2016).

The bacterium *Escherichia coli (E. coli)* is the predominant species in the normal enteric flora of most mammals, and generally as a harmless microorganism. However, some strains are quite pathogenic causing urinary tract infections, septicemia, meningitis and gastroenteritis in humans and animals. The high pathogenicity of *E. coli* is due to several virulence factors. *E. coli* causes enteric and extra-enteric events. Based on pathogenicity, it is classified into different groups: enterotoxigenic (ETEC), enteroinvasivo (EIEC), enteropathogenic (EPEC), enterohemorrágico (EHEC), diffuse adherence (DAEC) and enteroaggregative (EAggEC) (Diaz et al., 2016; Ribeiro et al., 2016).

The prevalence of these bacteria has been reported worldwide. However, official statistics on FBD from developing countries like Brazil are scarce. Studies have primarily described the presence of *Campylobacter* spp., *Salmonella* spp. and E. coli in poultry products (Zhao et al., 2001; Cardinale et al., 2003; Modolo et al., 2005; Ha and Pham 2006; Rall et al., 2009; Carvalho et al., 2010; Silva et al., 2014). Based on these grounds, this research aims to identify the presence of *Campylobacter*, *Salmonella* and *E. coli* in broiler carcasses in Botucatu, SP, Brazil, by microbiological and molecular methods.

2. Materials and Methods

Sixty samples of chilled chicken carcasses of different brands were collected from some retail meat stores and supermarkets located at peripheral and central areas of the city of Botucatu, SP, Brazil, in the period from January 2015 to January 2016. The transport of carcasses to the laboratories was carried out at different times to ensure variety of batches. The samples were kept in containers and transported immediately under refrigeration (4-8°C) in cool boxes to the laboratories of Animal Health Planning and Avian Pathology, both from the Faculty of Veterinary Medicine and Animal Science, Campus Botucatu, UNESP. The project was approved by CEUA with protocol number 35/2015.

For isolation and identification of *Campylobacter* spp. in the laboratory, the packaging of each chicken received a cut at the seal region, and placed for two hours on a sterile beaker to obtain carcass drip. Isolation of the agent was performed by using two procedures: filtration and direct seeding. In the filtration process, 10 ml of liquid sample was placed in each test tube, and centrifuged at 2500 rpm for 5 minutes. The supernatant was filtered with the aid of a cellulose acetate membrane of 0.65 µm in diameter (Sartorius Brasil Ltda). Finally, three drops of the filtrate were plated on agar thioglycolate

(Oxoid Brasil Ltda) containing 20% bovine blood, and incubated at 37°C for 72 hours, under microaerobic (Gerador Microaereofilia CO2 GEN-Oxoid Brasil Ltda).

To direct seeding, an aliquot of previously centrifuged and homogenized sample was seeded on the same agar with the addition of the Buzler selective supplement (bacitracin, novobiocin, cycloheximide, colistin and cefazolin.) (Oxoid Brasil Ltda) and incubated for 48 hours at 42°C, in microaerophilic (Modolo et al., 2005). Typical colonies of *Campylobacter* were separated for their identification through biochemical and molecular tests.

For isolation of *Salmonella* spp., 25 ml of carcasses drip were removed, placed in a sterile plastic bag (WHIRL-PAK) containing 225 ml of peptone water, and then incubated at 37°C, for 24 hours. After this, 1 ml and 0.1 ml were transferred to two test tubes containing 10 ml of selective enrichment media, tetrathionate (Muller-Kauffmann tetrathionate/novobiocin-broth MKTTn) and Rappaport (Rappaport-Vassiliadis medium with soya - RVS broth) (Sigma-Aldrich), respectively.

A further incubation was performed for MKTTn at 37 °C for 24 hours, and for RVS at 42°C for 24 hours. Then, by means of a platinum loop, the incubated material was seeded in a selective culture medium Xylose Lysine Desoxycholate (XLD) (Sigma-Aldrich), and in a Brilliant Green Agar (BGA) (Sigma-Aldrich), as described by (ISO 6579-2002).

For isolation of E. coli, 25 ml of carcasses drip were placed in a sterile plastic bag with 225 ml peptone water for subsequent incubation at 37°C for 24 hours. Finally, with the help of platinum loop, the contents were seeded onto MacConkey Agar and Brilliant Green Agar (BGA) (Sigma-Aldrich), and incubated at 37°C for 24 hours. Characterized colonies of *Salmonella* spp. and *Escherichia coli* were separated for identification via biochemical and molecular tests.

Colonies suspected of *Campylobacter* spp. were examined by phase-contrast microscopy (1000X), Carl Zeiss AG, Germany. The diagnosis was made by observing the following morphologies: curved bacillus, and typical movement of spirillum. After the presumptive diagnosis, a suggesting colony were subcultured in a Tarozzi medium, and finally incubated at 37°C for 72 hours, to obtain inoculum with a density adjusted to the scale of the McFarland tube 1 (3x108 CFU/mL). To observe typical features of micro-organism, different biochemical tests were performed such as catalase, growth temperature at 25°C and 43°C, and growth medium at 1% of glycine and 3.5% of NaCl, hydrolysis of hippurate, H2S production with and without cysteine at 0.02%, tolerance to trifeniltetrazóico chloride (TTC), and resistance to nalidixic acid and cephalothin (Veron and Chatelain, 1973; Modolo et al., 2005; ISO 10272-1:2006).

Characterized colonies of *Salmonella* spp. and *E. coli* were placed into agar Triple Sugar Iron (TSI) and agar Lysine Iron Agar (LIA), Sulfide Indole and Motility (SIM), and urea for screening. Colonies compatible with *Salmonella* spp. and *Escherichia coli* were identified by additional biochemical tests (Holt et al., 1994; BAM/FDA, 2014).

Colonies of Campylobacter spp. previously identified by biochemical tests were confirmed by PCR. The isolates were recovered in thioglycolate agar with 20% of blood at 42°C for 48 hours, under microaerophilic. Then, a portion was diluted with 100 µl of ultrapure water, in order to obtain a population of 109 UFC/ml (tube 4 of the McFarland scale). For DNA extraction the kit Gen Elute Bacterial Genomic DNA (Sigma) was used, according to manufacturer instructions.

Typical colonies of *Salmonella* spp. and *Escherichia coli* for each species were inoculated into brain-heart infusion broth (BHI) for 24 hours at 37°C. Subsequently, these colonies were seeded on MacConkey Agar and Brilliant Green Agar (BGA), and incubated at 37°C for 24 hours. The cultures were used for DNA extraction according to the instructions of the kit Gen Elute Bacterial Genomic

DNA (Sigma). The identification of each isolate was confirmed by PCR using primers specific for the genus *Campylobacter, Salmonella* and *Escherichia coli*.

PCR procedures used to identify Campylobacter are previously described by Harmon et al. (1997). The primer pairs used were pg 3: GAACTTGAACCGATTTG and pg 50: ATGGGATTTCGTATTAAC. The reaction had a final volume of 25µL. To complete the reaction volume, 12µL of Go Taq Green Master Mix (Promega), 2µL (20pmol) of each primer, 1µL of DNA (with a concentration of 5nmol/µL), and 8µL water were used. Amplification was performed in a thermocycler by the following program: initial denaturation at 94°C for 4min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1min, and final extension at 72°C for 7min (Silva et al., 2014). The strain of *Campylobacter jejuni* ATCC 33560, courtesy of the Oswaldo Cruz Foundation (FIOCRUZ), served as a positive control. For its recuperation was used the protocol sent by the Microorganism Laboratory Reference. The electrophoresis in agarose gel (1.5%) was useful for the analysis of amplifications.

The identification process of *Salmonella* spp. followed the procedure described by (Rall et al., 2009). The primers used were *invA1*: TCATCGCACCGTCAAAGGAAC and *invA2*: GTGAAATTATCGCCACGTTCGG. PCR reactions had a total volume of 25µL, comprising 12.5µL of Go Taq Green Master Mix (Promega), 1µL (10 pmol) of each primer, 7.5µL of ultrapure and autoclaved water, and 3µL of DNA sample. Amplification was performed in a thermocycler. The cycle parameters were 94°C for 5 min to initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 60°C for 30 secs, amplification at 72°C for 30 secs, ending with a final extension of 72°C for 4 minutes. The positive control was a strain of Salmonella spp. courtesy of Avian Pathology Laboratory of FMVZ-UNESP. The electrophoresis in agarose gel (1.5%) was performed for the analysis of amplifications.

For *Escherichia coli*, reactions were carried out with primers Eco 2083: GCT TTG ACA TGA TGA CAC AG, and Eco 2745: GCA CTT ATC TCT TCC GCA TT. PCR reactions had a total volume of 25µL (5µL (600ng) of extracted DNA), 2.5µL of each primer, 12.5µL of Go Taq Green Master Mix (Promega), and ultrapure water to make up the final volume. Amplification was performed in a thermocycler with an initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 45 secs, annealing of 57°C for 1 min, and amplification at 72°C for 2 min, with a final extension of 72°C for 10 minutes (Rissato et al., 2012).

Statistical analysis was completed using chi-squared test (χ^2 test) and Fisher´s exact test to compare the association between the prevalence of the studied commercial mark of broiler chicken, and the isolation of each bacterium as well as for comparison between the region of collection and isolation. The significance was 5% ($P < 0.05$) (Zar, 1996).

3. Results

Table 1 shows the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli* in isolates of chicken carcasses from stores at peripheral and central locations of Botucatu. Of the 60 samples from analyzed chickens, 23 (38.3%) showed isolation of *Campylobacter*, 8 (13.3%) of *Salmonella* and 36 (60%) of *E. coli.*

Table 1: *Occurrence of Campylobacter spp., Salmonella spp. and E. coli in chilled chickens from the city of Botucatu, SP, Brazil, 2015*

Bacteria	Samples	Prevalence
Campylobacter spp.	23	38.3
Salmonella spp.	8	13.3
E. coli	36	60.0

Total	*67	60

* Simultaneous isolation

Table 2 shows the prevalence of bacterial isolation on carcasses of different poultry brands from the same locations. The statistical test results indicated that there was no significant association (p <0.05) between the commercial marks and the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli*.

Table 2: *Prevalence of Campylobacter spp., Salmonella spp. E. coli in different brands of 181 broiler chickens in Botucatu, SP, Brazil, 2015*

Brand	*Campylobacter* spp.		*Salmonella* spp.		*E. coli*	
	f (nº)	p (%)	f (nº)	p (%)	f (nº)	p (%)
A	4	6.7	1	1.7	3	5.0
B	3	5.0	2	3.3	8	13.3
C	4	6.7	1	1.7	4	6.7
D	5	8.3	0	0.0	8	13.3
E	2	3.3	2	3.3	8	13.3
F	5	8.3	2	3.3	5	8.3
Total	23	38.3	8	13.3	36	60.0
χ^2	8.5145		5.0703		7.9689	
P (Fisher)	0.1519		0.2852		0.1675	

f: frequency; p: prevalence

Table 3: *Proportion of bacterial isolations of Campylobacter spp., Salmonella spp. E. coli in different brands of broiler chickens, Botucatu, SP, Brazil, 2015*

Brand	*Campylobacter* spp.	*Salmonella* spp.	*E. coli*
A	57.1 b	14.3 b	42.9 c
B	23.1 c	15.4 b	61.5 b
C	36.4 c	9.1 c	36.4 c
D	35.7 c	0.0 d	57.1 b
E	22.2 c	22.2 a	88.9 a
F	83.3 a	33.3 a	83.3 a
Total	38.3	13.3	60.0
χ^2	63.966	40.983	36.009
P (Fisher)	<0.001	<0.001	<0.001

Different letters indicate significant differences

The statistical analysis showed a significant difference among the chicken marks referred to the proportions of the three positive bacteria analyzed (Table 3). In this way, brand F showed higher proportion of positivity to *Campylobacter* spp., then brand A. For *Salmonella* spp. and *E. coli*, brands E and F showed higher positivity.

Table 4: *Prevalence of Campylobacter spp., Salmonella spp. E. coli of broiler chickens from different regions of Botucatu, SP, Brazil, 2015*

Region	*Campylobacter* spp.		Salmonella spp.		*E. coli*	
	f (nº)	p (%)	f (nº)	p (%)	f (nº)	p (%)
Central	9	15.0	3	5.0	14	23.3
Peripheral	14	23.3	5	8.3	22	36.7
Total	23	38.3	8	13.3	36	60.0
χ^2	0.5192		0.2098		1.3580	
P (Fisher)	0.1641		0.2713		0.1071	

f: frequency; p: prevalence

Table 4 shows that there was no association between the sample region of origin and the prevalence of *Campylobacter* spp., *Salmonella* spp. and *E. coli*. However, a greater tendency can be noted in the peripheral region when compared to the central region, possibly by inadequate storage conditions, rotation of stocks, and/or hygienic-sanitary control.

Table 5: *Proportion of bacterial isolations of Campylobacter spp., Salmonella spp. E. coli of broiler chickens in different regions of Botucatu, SP, Brazil, 2015*

Region	*Campylobacter* spp.	*Salmonella* spp.	*E. coli*
Central	33.3	11.1	51.9
Peripheral	42.4	15.2	66.7
Total	38.3	13.3	36 (60.0)
χ^2	1.094	0.639	1.847
P (Fisher)	0.3519	0.5455	0.2051

In addition, there was no significant statistical difference ($p < 0.05$) between the samples obtained in the center of the establishments and the periphery in relation to the proportion of positivity of the three bacteria analyzed. However, numerically, the peripheral region was approximately 60% higher in frequency and percentage of positivity than the central region (Table 5).

Table 6: *Prevalence of bacterial isolations of Campylobacter species in broiler chickens Botucatu-SP, Brazil, 2015*

Bacteria	Positive Samples	% Prevalence
Campylobacter jejuni	12	52,17
Campylobacter jejuni\E. coli	9	39,13
Others	2	8,70
Total	23	100

Table 6 shows the results of isolation of *Campylobacter* according to the classification of detected species *C. jejuni* and *C. jejuni/coli* were present in poultry carcasses in 52.17% and 39.13%, respectively.

The positive control of *Campylobacter jejuni* ATCC 33560, through the primer pg3 pg50, which amplifies a conserved region of the flagellin related to both genes *C. jejuni* and *C. coli*, and generates a product of 480 bp. The isolated *Campylobacter* spp. detected from conventional methodology diagnosis, and confirmed by biochemical proofs, resulted also positive for molecular testing (PCR).

The PCR product appears after using the primer pair encoding *inv* A1 and *inv* A2 genes, yielding 284 bp. The presence of *Salmonella* spp. was confirmed by PCR in 7 of samples. However, 8 (13 3%) were positive to the pathogen by traditional methodology. Outcomes related to *Escherichia coli* in chicken carcasses were confirmed by PCR. Primers were used in all reactions for the highly divergent and specific region of the DNA encoding rRNA 16S and 23S of *E. coli*. (Figure not shown). Likewise, based on the antimicrobials tested and on the PCR assays specific, isolates displayed features linked to multidrug resistance and integron class 1 (data not shown).

4. Discussion

The present study demonstrates bacterial prevalence of three major pathogens on chicken carcasses from some supermarkets and retail stores located at peripheral and central areas of the city of Botucatu, SP, Brazil.

Such an occurrence linked to meat consumption varies considerably. For the genus *Campylobacter* spp., it depends on the number of microorganisms originally present in the carcass, hygienic

conditions of slaughterhouses, storage time in shelf and proper conservation (Carvalho and Costa, 1996; Modolo et al., 2005). In addition, some reports indicate the difficulty of isolating *Campylobacter* spp. related to sensitivity to oxygen, desiccation, heat, and pH (Silva et al., 2014). Variations in *Salmonella* spp. depend on the batch origin (primary infection), sanitary conditions of the slaughterhouse, and cross-contamination during the stages of slaughter, transportation and marketing (Olsen et al., 2003; Rall et al., 2009).

These results agree with those obtained in different countries regarding the isolation of the bacteria analyzed. For example, in Senegal, Africa, a research found 56% of prevalence for *Campylobacter* jejuni, and 96% for *Salmonella* spp. (Cardinale et al., 2003). In Washington, USA, authors reported prevalence of *Campylobacter* spp. in 70.7% of broiler chickens, 4.2% for *Salmonella* spp., and 38.7 for E. coli (Zhao et al., 2001). In Vietnam, Asia, 45% of E. coli was identified, followed by 28.3% of *Campylobacter* spp., and 8.3% of *Salmonella* spp. (Ha and Pham, 2006). In Brazil, related studies also revealed such bacteria in chicken carcasses. Detected 47% positive isolates in *Campylobacter* spp., higher values than those indicated in the present work.

The tests performed at 400 chicken carcasses indicated major presence of the bacterium in the carcass sold in the central region than in peripheral region (Modolo et al., 2005). However, Carvalho et al. (2010) found lower percentage of isolation of these bacteria, 14.2%. Silva et al. (2014) determined occurrences in 61% of samples of chicken droppings, 20% in chicken products for consumption, and 3% in human stool, all linked to this microorganism. Carvalho et al. (2010) pointed out the presence of gene complexes of the extensional citoletal toxin (CDT) in 36.4% of the samples. However, Silva et al. (2014) determined 93.5% of such grouped genes for CDT.

In the case of *Salmonella* spp., official organizations such as the European Centre for Disease Prevention and Control (ECDC) and the Center for Disease Control (CDC) have documented a reduced presence of this bacterium when compared with *Campylobacter* spp., mostly due to different regulations implanted worldwide (ANVISA, 2001). However, the prevalence of 13.33% found in this study is still considered high. Moreover, Rall et al. (2009) cited the presence of the pathogen in 8% of the samples in chickens in Botucatu. These values are lower than those expressed in the present research. These authors also indicate that 70% of the samples were out of microbiological parameters.

A similar study on *E. coli* was conducted on chilled chicken carcasses with the brands available in markets and butcher shops of the city of Campo Mourão, Paraná, Brazil. Brand A showed 12.12%, followed by C and D with 6.06% of the positive samples. Brand B showed no positive samples. The results found in poultry carcasses in the city were 24.2%, lower than those reported in this study. (Rissato et al., 2012).

The results presented indicate a public health problem due to the commercial marks used for this research are extensively available for human consumption. Isolates of *Campylobacter* spp. *Salmonella* spp e *E. coli* detected from conventional methodology diagnosis, and confirmed by biochemical proofs, resulted also positive for molecular testing (PCR). *Campylobacter* spp. isolates did not present clear distinction at biochemical tests.

Strains featured both *C. coli*, with a tolerance of 2`3`5 to Triphenyl tetrazolium chloride, and *Campylobacter jejuni*, when hydrolyzed by hippurate. Therefore, they have been named *Campylobacter jejuni/coli*. Similar behavior has been reported by both studies Modolo et al. (1991) in calves and dogs, with and without diarrhea, and Modolo et al. (2005) in broiler carcasses, Botucatu, SP. Véron & Chatelain (1973) indicated problems associated to this bacterium to achieve taxonomic studies on the genus *Campylobacter*. In addition, further investigations highlight difficulties in its classification, probably due to the presence of a common plasmid on such strains (Bradybury, 1983).

Some authors describe unrevealed samples as false negatives, since some medium components of the *Campylobacter* culture like blood or hemoglobin strongly inhibit PCR (Denis et al., 2001). The high protein levels on liver could also explain this interference due to all samples were stored in Tarozzi mediums. Other researchers as Silva et al. (2014) pointed out unsuccessful efforts to obtain *Campylobacter* material for molecular analysis linked to its culturing hardships. Isolates of *Salmonella* spp. and *E. coli* was confirmed through complementary biochemical tests. Unlike *E. coli*, *Salmonella* spp. did not show typical characteristics in all strains.

Thus, molecular diagnosis is important as a viable and reliable alternative to confirm the presence of food bacteria. Speed, specificity and sensitivity are its main advantages (Rall et al., 2009; Rissato et al., 2012; Silva et al., 2014).

Despite previous studies on the assessed bacteria about pathogenic contamination of commercialized broiler chickens, this research demonstrates a significantly remaining prevalence. This must be still considered a very high-risk factor for public health despite Brazilian food health organizations have warned consumers about FBD. Special attention should deserve *Campylobacter* before the absence of specific regulations to monitor and control it. Low infective doses and its relationship with Guillain-Barré syndrome, a disease that leads to muscle paralyses.

In the present study demonstrates the presence of *Campylobacter* spp., *Salmonella* spp., *E. col*i and in different brands of chicken carcasses of meat Botucatu supermarkets and homes, conventional molecular and microbiological methodology. This presents a danger to public health because these bacteria are considered by international organizations as the main causes of foodborne diseases.

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