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Pathophysiological mechanisms of alcoholic myopathy - Lessons from rodent models



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Abstract

Skeletal muscle dysfunction is highly prevalent and is one of the earliest pathological tissue changes among people with at-risk alcohol use. Clinical studies to elucidate pathophysiological mechanisms of alcohol-mediated muscle disease are hampered due to ethical considerations, and confounded by nutritional, lifestyle, and comorbid conditions. Rodent models have been developed to study the impact of at-risk alcohol consumption and alcohol-mediated end organ injury, including skeletal muscle dysfunction. This review discusses results from well-established rodent models of alcohol administration and highlights key pathophysiological mechanisms underlying alcoholic myopathy identified in rodent models. Salient pathways include impaired regenerative capacity, altered anabolic/catabolic balance, impaired mitochondrial bioenergetic function, and skeletal muscle morphological and contractile changes.

Key words: Alcohol, Muscle, Protein synthesis, Stem cell regeneration, Mitochondria

Alcoholic myopathy, or decreased skeletal muscle mass or function, occurs in 40 to 60 percent of people with at-risk alcohol use (Fernandez-Sola *et al.*, 2007; Preedy *et al.*, 2003) and is one of the earliest pathological tissue changes seen with alcohol use (Lang *et al.*, 2005; Martin *et al.*, 1985). Although alcohol-related muscle disease is nearly five times more common than liver cirrhosis (Estruch *et al.*, 1993), mechanistic data are lacking on its contribution to long-term health, aging, as well as its association with injury, metabolic dysregulation, or disuse atrophy. Ethical issues, complexity of alcohol use patterns, and confounding comorbidities make clinical studies difficult and have led to the development of preclinical models, especially rodents, to understand alcohol-mediated pathophysiological mechanisms underlying human disease. There are always concerns and questions regarding the use of animals in biomedical research and especially in substance use research. However, we obtain reliable data, there is great degree of experimental control and replicability, allowing for mechanistic investigation of human drug use and abuse.

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Why study alcohol and end organ injury?

In the 1930s, E. M. Jellinek, a physiologist, described alcoholism (now referred to as alcohol use disorder; AUD) as a disease. His seminal paper provided clarity and removed stigma associated with AUD, and underscored that it was not due to weakness of will or temperament, moral failing, symptom of mental illness, but that it is a physical illness due to a pharmacologically addictive substance that can affect people from all sections of society (Jellinek and McFarland, 1940). However, at-risk alcohol use has adverse effects on many organ systems, including the liver, lungs, central and peripheral nervous system, immune system, and skeletal muscle. With such wide-ranging effects, it is critical to identify mechanisms leading to alcohol-mediated pathology to better manage and treat alcohol-related disease.

Alcohol administration in rodents

Several reliable models of alcohol administration in rodents have been developed and there are benefits and drawbacks of each. Here, we briefly describe some of the most common models. Some of the models developed allow for excessive drinking and alcohol-seeking behavior demonstrating the rewarding effects of alcohol and their contribution to escalating alcohol use; others are more commonly used to study end-organ effects of alcohol.

- 1) Two-bottle choice method: Animals are allowed an unrestricted choice between alcohol and water for a predetermined amount of time up to 24 hours per day. Animals consume alcohol voluntarily. Work with these models show heterogeneous populations of animals, like humans, some of which consume larger volumes of alcohol than others, and can help elucidate how certain factors, such as previous patterns of alcohol administration (Younis *et al.*, 2019; Nentwig *et al.*, 2019), influence alcohol preference. These studies have also shown that genetic manipulation by inbreeding or selective breeding can produce animals displaying different alcohol preferences (Grahame *et al.*, 1999).
- 2) Operant models: Animals must perform a certain task for alcohol intake. For example, animals are trained to press a lever for alcohol to be delivered through the oral route, or in some cases directly into the brain. These models allow for the assessment of motivation to consume alcohol, such as after traumatic brain injury (Stielper *et al.*, 2019) and to elucidate possible factors underlying motivation for alcohol, such as altered glucocorticoid receptor signaling (Pahng *et al.*, 2019).
- 3) Intragastric administration: Animals are administered alcohol via infusion directly into the stomach through surgically implanted intragastric tubes. This method is used to avoid the influence of taste and has been used to study alcoholic liver disease; e.g., Tsukamoto-French model (Tsukamoto *et al.*, 1990).
- 4) Alcohol-containing liquid diet: After transition from solid to liquid diet, alcohol containing liquid diet is the sole source of nutrition in this model. The alcohol content of the liquid diet is increased over the course of several days at the beginning of the experimental period and subsequently maintained at the target level. The most common diet used is the Lieber-DeCarli diet (Lieber and DeCarli, 1989) and the control diet is isocalorically matched to the alcohol calories. This strategy is widely used to study end-organ injury, including chronic alcohol-induced skeletal muscle pathology (Levitt *et al.*, 2020c; Crowel *et al.*, 2016; Lang, 2018).
- 5) Oral gavage: Animals are orally administered alcohol via gavage. The oral gavage method is commonly used to study acute effects of a single dose of alcohol on muscle pathology or to administer ethanol at binge doses in combination with the Lieber-DeCarli diet; e.g. "binge-on-chronic" or "NIAAA model" (Samuelson *et al.*, 2019; Bertola *et al.*, 2013).
- 6) Systemic injection: This is generally accomplished using an intraperitoneal injection and is commonly used to assess acute effects of alcohol on skeletal muscle (Steiner and Lang, 2015; Steiner *et al.*, 2016).

- 7) Inhalation of alcohol vapor: Animals are placed in chambers and exposed to alcohol vapor for specific periods of time, often referred to as “intermittent ethanol exposure”. This is a common method to make rodents dependent on alcohol (Gilpin *et al.*, 2008; Mouton *et al.*, 2016).
- 8) Genetic models: Mice or rats are bred to create lines of animals that are sensitive or insensitive to alcohol and rat lines have been generated to select for alcohol preference. For example, selectively-bred Sardinian alcohol-preferring (sP) and non-preferring (sNP) rats reliably exhibit strong alcohol preference and motivation (sP) or strong avoidance (sNP) of alcohol (Colombo *et al.*, 2006). Other genetic models of alcohol-related behaviors include inbred strains, recombinant inbred strains, and transgenic/knock-out mice (Mayfield *et al.*, 2016).

The metabolic rate of alcohol varies among species, and doses are optimized to generate blood and brain alcohol levels that produce pharmacological effects. For example, that rate of alcohol clearance is much greater in rats compared to humans and further varies among strains (Erickson, 1984; Holford, 1987). Because of the differences in rates of alcohol metabolism, while the method of alcohol administration may not exactly mimic human consumption, it serves to study the effects of alcohol on end organ injury.

Rodent models commonly used to study alcoholic myopathy are chronic Lieber-DeCarli diet feeding, oral gavage, or systemic injections. In addition, *in vitro* studies using established cell lines (e.g. C2C12 mouse-derived myoblasts or primary myoblasts) are used to mechanistically understand how alcohol affects myoblast proliferation, differentiation, metabolism, and function. Here, we will discuss key findings of alcohol-mediated effects on skeletal muscle reported from studies utilizing rodent models.

Muscle stem cell regenerative capacity

Rodent studies have demonstrated that chronic alcohol feeding increases skeletal muscle gene expression of TNF α and interleukin

(IL)-6, indicating chronic inflammation (Steiner and Lang, 2015a). Our published work showed decreased differentiation potential of skeletal muscle stem cells isolated from chronic binge alcohol-administered macaques. This was associated with reduced expression of myogenic genes and impaired myotube formation, indicative of impaired muscle fiber formation (Simon *et al.*, 2014; Simon *et al.*, 2017). The marked dysregulation of myoblast myogenic and inflammatory gene expression and myotube formation with chronic alcohol administration reflects impaired muscle regenerative capacity and is likely to contribute to decreased muscle mass, especially in response to an injury or disuse atrophy. Alcohol and aging are risk factors for traumatic injury and subsequent immobilization-induced muscle atrophy (Lukaszyk *et al.*, 2016). In fact, about 10-30% of hospitalized older patients have diagnosed AUD (O’Connell *et al.*, 2003). Skeletal muscle recovery may be complicated by alcohol use and altered hormonal status among older individuals (Lukaszyk *et al.*, 2016). To examine this question, we utilized Lieber-DeCarli feeding in rats for 10 weeks culminating in 1 week of unilateral hind limb immobilization followed by 3 or 14 days of remobilization (Levitt *et al.*, 2020c). Our data indicated that alcohol dysregulates the expression of markers of muscle regeneration following unilateral hind limb immobilization. Although alcohol did not significantly exacerbate the immobilization-mediated decrease in muscle weight, it is possible that underlying differences in regeneration may have occurred. Therefore, when immobilization is indicated, caution is warranted about alcohol use during the immobilization and post-immobilization recovery periods.

Mitochondrial homeostasis and bioenergetics

Alcohol impairs mitochondrial function in tissues including skeletal and cardiac muscle (Guo and Ren, 2010; Kumar *et al.*, 2019; Duplanty *et al.*, 2017; Duplanty *et al.*, 2018), and mitochondrial homeostasis is critical in the maintenance of functional metabolic muscle mass (Romanello and Sandri, 2015). In chronic alcohol fed rats, decreased mitochondrial fusion and connectivity, calcium dysregulation,

and impaired mitochondrial bioenergetics and excitation-contraction coupling have been reported (Eisner *et al.*, 2014; Trounce *et al.*, 1990). Studies also show that chronic alcohol decreases muscle cross sectional area with a decrease in total and free glutathione content, decreased glutathione reductase activity and decreased expression of oxidative stress genes (Otis and Guidot, 2010; Otis *et al.*, 2007). Together, a number of studies have shown alcohol-mediated oxidative damage in muscle as evidenced by an increase in protein carbonyl, cholesterol hydroperoxide, and malondialdehyde content (Otis *et al.*, 2007; Koo-Ng *et al.*, 2000).

Alcohol-mediated impaired bioenergetics are not limited to mitochondria. Chronic alcohol administration results in skeletal muscle glycolytic impairments in rodents. For example, activity of the glycolytic enzymes aldolase, pyruvate kinase, and lactate dehydrogenase were significantly decreased in vastus lateralis after chronic ethanol administration (Trounce *et al.*, 1990), mirroring findings in humans (Trounce *et al.*, 1987). In contrast, opposite effects have been observed in white gastrocnemius muscle in male rats, suggesting a potential fiber type specific effect of ethanol on glycolytic enzyme activities (Vila *et al.*, 2001). Using transcriptomics, decreased expression of genes in the glycolytic pathway were also reported after C2C12 myoblasts were treated with 100 mM ethanol, a supraphysiological dose, for 6 or 24 hours (Kumar *et al.*, 2019). Although these previous findings were at the transcriptomic or enzyme activity levels, we recently confirmed that 3 days of treatment with 50 mM ethanol impairs glycolytic function in live myoblasts *in vitro* (Levitt *et al.*, 2020).

Skeletal muscle protein synthesis

Clinical studies provide evidence that a major mechanism of alcohol-induced myopathy is altered balance of protein synthesis and breakdown (Steiner and Lang, 2015; Reilly *et al.*, 2000; Steiner *et al.*, 2015). To identify alcohol-induced changes in specific proteins in the protein synthesis and degradation pathway, liquid alcohol diets have been used.

The mammalian target of rapamycin (mTOR) pathway plays a central role in protein synthesis and is important for controlling skeletal muscle mass. mTOR activation activates two signaling pathways; S6 kinase 1 (S6K1) phosphorylation leading to activation of the ribosomal protein S6 and phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1) releasing its inhibition of the translation initiation factor eIF-4E. Chronic alcohol consumption decreases phosphorylation of mTOR itself (Lang *et al.*, 2003a), ribosomal protein S6 (rpS6), and phosphorylation of 4E-BP1 in skeletal muscle (Korzick *et al.*, 2013). This is associated with a greater proportion of eIF4E in the inactive eIF4E-4EBP1 complex versus the active eIF4E-eIF4G and thus illustrates one possible mechanism by which alcohol decreases protein synthesis. Insulin and insulin-like growth factor (IGF)-1 are anabolic hormones that activate mTOR signaling. Acute ethanol markedly attenuated the insulin and IGF-1 mediated increases in S6K1 and rpS6 phosphorylation but did not simultaneously impair signaling through 4E-BP1 (Kumar *et al.*, 2002). It should be noted that phosphorylation was measured at only one time point in that study, so it is possible that an acute effect was present at a different time point. However, ethanol attenuated the leucine-stimulated phosphorylation of 4E-BP1 and eIF4G in addition to mTOR, S6K1, and rpS6 (Lang *et al.*, 2003). In addition, rodent models show that alcohol significantly decreases IGF-1 levels in both plasma and muscle, and this decrease is correlated with decreased muscle protein synthesis (Lang *et al.*, 1998). Overall, there is strong evidence that alcohol attenuates basal mTOR signaling after chronic administration, acutely attenuates mTOR signaling in response to anabolic stimulation, and may decrease systemic levels of the anabolic hormone, IGF-1.

Skeletal muscle protein degradation

Protein degradation in skeletal muscle is directed primarily by two pathways, the ubiquitin proteasome pathway (UPP) and the autophagic-lysosomal system (Steiner and Lang, 2015; Steiner *et al.*, 2015; White *et al.*, 2014). The two ubiquitin ligases or atrogenes,

atrogin-1 and MuRF1 are specific to the muscle and are increased in several catabolic conditions. Both atrogin-1 and MURF1 are increased in acute and chronic alcohol administered rodents (Korzick *et al.*, 2013; Vary *et al.*, 2008; Vargas and Lang, 2008), possibly reflecting increased protein degradation activity. However, chronic alcohol did not further increase atrogin-1 and MURF1 expression or proteasomal activity in aged rats (Korzick *et al.*, 2013). The autophagic-lysosomal system is activated due to cellular stress and mediates degradation of misfolded proteins. Whether alcohol-mediated muscle protein degradation is mediated by autophagy is still not clear. In alcohol-fed mice, there is increased expression of autophagy markers and in vitro treatment of C2C12 myotubes with 100 mM alcohol (a supraphysiological level of alcohol) increased autophagic gene expression within 6h (Thapaliya *et al.*, 2014). However, others have not observed this change in chronic alcohol fed mice (Steiner and Lang, 2015). Similarly, our studies in primary myoblasts derived from chronic alcohol administered macaques do not show changes in autophagic markers compared to myoblasts derived from vehicle administered macaques (Simon *et al.*, 2014). Thus, studies in rodent models suggest that alcohol-mediated decreased protein synthesis and increased protein degradation can potentially contribute to alcoholic myopathy.

Skeletal muscle mass and morphology

Decreased skeletal muscle mass is characteristic of chronic at-risk alcohol use, and structural abnormalities have been observed in muscle biopsy samples from such subjects (Fernandez-Sola *et al.*, 2007). However, studying these phenomena in humans is complicated by differences in nutrition, current and past physical activity, hormonal status, etc. Therefore, rodent models have been employed to examine alcohol-mediated muscle wasting in controlled settings. For example, alcohol-fed rats showed decreased lean body mass compared to pair-fed controls (Korzick *et al.*, 2013). A further benefit of studying such effects in rodents is that many individual rodent muscles have a more homogenous muscle fiber type population (e.g. gastrocnemius is composed

of ~95% type II fibers) than in humans (e.g., gastrocnemius is composed of ~50% type II fibers). The homogenous fiber type composition allows for easier assessment of the effects of alcohol on different fiber types. Chronic alcohol (15% v/v in drinking water) decreased muscle fiber cross-sectional area across all fiber types in the plantaris, which is composed of ~95% type II fibers in rats (Vila *et al.*, 2001). In the same study, muscle fiber cross-sectional area was unaffected in the type I-dominant soleus and red gastrocnemius muscles. We observed decreased quadriceps mass (white and red portions together) in the non-immobilized hind limb of alcohol-fed rats compared to their pair-fed counterparts (Levitt *et al.*, 2020c). Without immobilization, type II-dominant plantaris mass and total protein were decreased in rats fed ethanol for 12 weeks (Clary *et al.*, 2011). Ten weeks of alcohol feeding decreased total protein and RNA in type II-rich gastrocnemius and plantaris but not type I-dominant soleus, with concomitant decreases in expression of the structural proteins titin and nebulin in white gastrocnemius (Hunter *et al.*, 2002), which could further contribute to alcoholic myopathy, particularly in type II muscle. Overall, results from rodent studies confirm that alcohol-induced muscle wasting more severely affects type II-dominant muscle and provide a viable model to elucidate underlying mechanisms.

Skeletal muscle function

Although skeletal muscle mass and function are generally related, a host of factors (bioenergetic, neurological *etc.*) confound this non-linear relationship. Even without sufficient time to decrease muscle mass, acute binge alcohol administration in humans after exercise-induced muscle damage can exacerbate the exercise-induced decrease in force production in men (Barnes *et al.*, 2010), although this effect does not appear to translate to women (Levitt *et al.*, 2017), and is not apparent when participants are accustomed to the exercise (Levitt *et al.*, 2020b). For ethical purposes, the doses of alcohol used in human studies of acute binge drinking produce blood alcohol concentrations of only ~0.08-0.12 g/dL. This may be why an additional stimulus (e.g. exercise-induced muscle damage) is needed to uncover effects.

However, human consumption of ethanol can result in blood alcohol concentrations that far exceed those in these studies and may produce acute and cumulative effects on muscle function as muscle weakness is observed in approximately half of patients with AUD (Preedy *et al.*, 2001). Rodent models have been used to examine acute and chronic effects of alcohol on muscle contractile function. In one such study, extensor digitorum longus (EDL) contractile properties post-fatigue were impaired after chronic ethanol feeding but not 2 hours after acute ethanol intoxication @ 3 g/kg (Crowell *et al.*, 2019). Previous work examining the acute effects of alcohol on muscle contractile function in rat EDL *in vitro* observed decreased twitch tension at higher (0.4 and 2.5 g% ethanol) but not lower (0.1 and 0.2 g% ethanol) ethanol doses (Taylor *et al.*, 1992). These findings suggest dose dependent effects of alcohol on muscle function. Overall, more work is needed to elucidate effects of alcohol on skeletal muscle contractile function, and rodent models are well-suited for such studies.

Conclusion

Alcoholic myopathy is more prevalent than well-known organ injury like alcoholic hepatitis. Clinical studies in human subjects with AUD are challenging for many reasons including the presence of confounding lifestyle factors, comorbid conditions, and varying patterns and severity of alcohol consumption. Rodent models of alcohol administration have allowed for controlled studies to standardize administration across subjects and minimize confounding factors. The specific experimental question and study design parameters must be considered when selecting an alcohol administration protocol. The most used experimental paradigms to study alcohol-mediated muscle pathology are alcohol-containing liquid diets (chronic model) and intraperitoneal injections or oral gavage (acute models). Using these rodent models, many factors underlying alcoholic myopathy, including impaired regenerative capacity, altered anabolic/catabolic balance, impaired bioenergetic function, and skeletal muscle morphological and contractile changes have been described. Thus, mechanistic studies in

rodent models have helped identify key alcohol-mediated pathways that are dysregulated and, in the future, may provide therapeutic targets to ameliorate alcoholic myopathy.

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Conventional and molecular diagnosis of Campylobacteriosis associated with bovine abortion

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Abstract

Campylobacteriosis is responsible for genital tract infections of beef and dairy herds, causing a significant economic loss in livestock sector. Campylobacter foetus species is one of the important pathogens because of its potential impact in Veterinary and Human health. This study was designed to determine the regional incidence of C. foetus infection in Kerala, India by isolation, detection of C. foetus in clinical samples by Polymerase Chain Reaction (PCR), real time PCR (qPCR), and Enzyme Linked Immunosorbent Assay (ELISA) for the detection of C. foetus antibodies in sera of bovines with the history of abortion/infertility. Clinical samples (aborted materials (50), serum (50), Cervico-Vaginal Mucus (CVM) (30) and semen samples (30)) from a total of 160 cattle and buffaloes with the history of abortion and infertility were collected. Aborted materials including placenta, foetal membranes, liver, lungs and stomach contents of the aborted foetus, semen and CVM samples were processed and subjected to isolation and identification of Campylobacter foetus subsp. foetus (Cff) and Campylobacter foetus subsp. venerealis (Cfv) and molecular confirmation by PCR and qPCR respectively. Serum samples from aborted dams were tested using indirect ELISA. All the suspected clinical samples were found negative for Cff and Cfv on both culturing and PCR. All the serum samples tested were negative by ELISA as well. Conclusively the study indicated the infection of C. foetus spp. responsible for abortion in bovine are rare in the location where the study was conducted, which might be due to insignificant endemic levels. As per the breeding policy, only artificial insemination is practiced in Kerala in bovines, which is often considered as a simple control method for Bovine Genital Campylobacteriosis (BGC) and might be one of the factors that prevented extensive spread of C. foetus spp. infection.

Keywords: Campylobacteriosis, Campylobacter foetus, Bovine abortion, Polymerase Chain Reaction, real time PCR, and Enzyme Linked Immunosorbent Assay.

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Reproductive performance is one of the most important factors influencing profitability in the dairy and beef industries. Though the dairy industry is very important in its contribution to the economics of both developed and the developing countries of the world, it is vulnerable to various factors which adversely affect the cattle production viz., physical, chemical, biological and environmental factors. Infectious biological agents are notable causes of reproductive failures and are being given high priority in the bovine industry (BonDurant, 2007). Infections with these agents are mainly manifested as abortion which is one of the important factors reducing calving rate and consequently causing a significant economic loss to the dairy industry (Mittal *et al.*, 2018).

Campylobacteriosis is a widespread bacterial disease responsible for contagious genital infections and abortion in cattle around the world (Osunla and Okoh, 2017). Bovine Genital Campylobacteriosis caused by *Cfv*, causes significant economic impact in farming industries and requires a careful and thorough diagnosis and treatment for its control. BGC is a notifiable trade disease with countries requiring certification of disease free status of both cattle and semen for import. *Campylobacter foetus* subsp. *foetus* is associated with sporadic cases of abortion in bovines (OIE, 2018).

Bacteriological analyses of these pathogens primarily rely on cultural isolation and phenotypic characterization, which is reported to be the gold standard and confirmatory test for diagnosis of *C. foetus* infection (Brooks *et al.*, 2004). However, isolation of *C. foetus* is labor intensive, accompanied with a high risk for potential and rapid overgrowth of more robust contaminants (Clark and Dufty, 1978). On the other hand, serological tests like ELISA was recognized as an effective screening test for detection of *C. foetus* (Devenish *et al.*, 2005). However, cross-reactive antigens especially within the species pose problems for serological diagnosis (Repiso *et al.*, 2002; More *et al.*, 2017). Hence, to overcome these problems, nucleic acid amplification has been tremendously exploited for the routine and rapid confirmation of these pathogens (Islam *et al.*, 2020). Various methods like PCR and

qPCR have been used for the diagnosis of Campylobacteriosis in human and domestic animals, as the tests are reliable and accurate for the species and subspecies identification of *C. foetus* (Hum *et al.*, 1997; Chaban *et al.*, 2012). A subspecies-specific PCR and qPCR assay for *Cfv* targeting *ISCfe-1* gene, a highly conserved, new insertion element which is present exclusively in *Cfv* strains (Abril *et al.*, 2007), and for *Cff* targeting *SapB2* gene developed by Wang *et al.* (2002) were employed in the present study.

The current communication reports the results of the serological and molecular diagnosis of Campylobacteriosis associated with bovine abortion in organised and unorganised livestock sectors in and around Thrissur district, Kerala.

Materials and methods

Sampling: Aborted materials, serum, CVM and semen were collected from a total of 160 cattle and buffaloes maintained in organized and unorganized farms of Kerala in India. Serum was also recovered for blood samples in separate tubes for ELISA.

Isolation: Stomach contents and pooled sample consisting of liver, lung, kidney, spleen and placenta, CVM and semen were collected aseptically in Cary-Blair medium and inoculated on the same day on Brucella agar base supplemented with five to ten per cent sheep blood and incubated at 37°C for 72 hr under micro-aerophilic conditions. Similarly, reference samples received in the form of swab were subjected for inoculation.

Glycine Tolerance Test: The test was performed as described by OIE (2018). Briefly, a cell-suspension of McFarland no.1 was inoculated onto a Brucella base agar with or without 1% glycine medium and was incubated microaerobically at 37°C for 48 h. The growth in the presence of glycine has been considered to be a presumptive test for *Cff*.

Reference strains: Two reference isolates (received as swab) were used in this study. *Campylobacter foetus* subsp. *foetus*

and *Campylobacter foetus* subsp. *venerealis* were kindly provided by Institute for Veterinary Bacteriology, University of Bern, Switzerland.

Genomic DNA extraction:

HiPurA™ Multi-Sample DNA Purification Kit, Hi Media, was used for the extraction DNA from clinical samples. DNA concentration of stock solutions was measured using Nano drop (Thermo Scientific). The purity of the extracted DNA was checked by measuring the ratio of absorbance (OD of DNA preparation at 260 and 280 nm). Finally, the extracted DNA was stored at -20° C until use.

Campylobacter foetus subsp. *foetus* identification by PCR and qPCR:

In the present study, genus and subspecies specific PCR and qPCR were standardized using the reference sample DNA extracted from *Cff* (ATCC 27374) and *Cfv* (NCTC 10354).

PCR was carried out using the already published *Campylobacter* genus specific *16S rRNA* gene primers by Linton *et al.* (1996). The sequences of primers were: forward primer 5'- GGATGACACTTTTCGGAGC -3' and reverse primer 5'- CATTGTAGCACGTGTGTC -3'. *Campylobacter foetus* subsp. *foetus* identification was performed using specific primers published by Wang *et al.* (2002). The forward primer was 5'- GCAAATATAAATGTAAGCGGAGAG -3' and reverse primer was 5'- TGCAGCGCCCCACCTAT -3'.

Primers for real-time PCR were designed based on *sapB2* gene sequence for *Cff* (GeneBank Accession no. CP008808.1) using NCBI primer designing software. The forward primer was 5'- TTTAGGAGCCGTATCAGCAA -3' and reverse primer was 5'- TCACCAGCAAGAGCTCCTAT -3'.

Campylobacter foetus subsp. *venerealis* identification by PCR and qPCR:

The PCR and qPCR was conducted using the primers specific for *Cfv ISCfe-1* gene (Abril *et al.*, 2007). The forward primer was 5'- AGGCGAAGAGAATGTTAAATTTGAA -3' and reverse primer was 5'- CCATAAAGCCTAGCTGAAAAAACTG -3'.

PCR was performed in a reaction volume of 12.5 µL containing approximately 100 ng/µL of genomic DNA, 10 pM/µL of forward and reverse primer and 6.25 µL of PCR master mix ((2X, Thermo Scientific).

Identification of the PCR product was done in a submarine agarose gel electrophoresis system using one per cent agarose containing ethidium bromide, using Tris Borate EDTA buffer at a voltage of 50V. The gel was visualized under a UV trans-illuminator and results were documented using a gel documentation system (Biorad).

SYBR green PCR protocol:

Reactions were carried out in Eco™ Real-Time PCR System (Illumina) using 48-well plates, sealed by an adhesive seals after pipetting all reagents into the wells. Reaction mixture and thermal cycling parameters are given in (Tables 2 to 4). SYBR green real-time PCR amplification plots and melt curves were viewed and analysed.

C. foetus antibody ELISA:

Conventional method of ELISA was performed as per the method described by Seyyal *et al.* (2000) with slight modifications. The immunogenicity of whole cell antigen of *C. foetus* was confirmed by AGPT and the protein concentration was standardized by Lowry's method. Hyper immune serum raised against *C. foetus* in New Zealand White male rabbits. Pre inoculation bleeding for preparation of control non-immune sera was done. A typical injection schedule consisted of 0.5 mL antigen (2 x 10⁸ cells per mL) on the 1st day of inoculation. One millilitre antigen (2 x 10⁸ cells per mL) was inoculated on the 4th day, and 2 mL antigen on the 7th, 10th, 14th and 16th days (Seyyal *et al.*, 2000). The rabbits were bled by cardiac puncture one week after the last injection. The sera were separated from blood collected from two New Zealand white male rabbits and stored at -20°C.

Checker board analysis was performed for standardizing the concentration of antigen, sera and commercial rabbit anti-bovine IgG peroxidase conjugate. The DAB

Table 1: Amplification cycle for *Campylobacter foetus* subsp. *foetus* and *Campylobacter foetus* subsp. *venerealis*.

Initial denaturation	94°C °C for 2 min.	1 cycle
Denaturation	94°C °C for 30sec.	30 cycles
Annealing	61.4°C °C for 30sec.	
Extension	72°C °C for 30 sec.	
Final extension	72°C °C for 4 min.	1 cycle

Table 2. Real-time PCR Reaction mix for the detection of *Campylobacter foetus* subsp. *foetus* and *Campylobacter foetus* subsp. *venerealis*

Components	Volume
Orion 2X Real time PCR smart mix	6.25 µL
Forward primer (10 pM/µl)	0.5 µL
Reverse primer (10 pM /µl)	0.5 µL
Template DNA	4.0 µL
Nuclease free water	1.25 µL
Total	12.5 µL

Table 3. Amplification cycle for *Campylobacter foetus* subsp. *venerealis* used for real-time PCR

Steps	Temperature	Time	Number of cycles
Initial denaturation	95°C °C	10 min	1
Denaturation	95°C °C	30 sec	40
Annealing/Extension	61.4°C °C	30 sec	40

Table 4. Amplification cycle for *Campylobacter foetus* subsp. *foetus* used for real-time PCR

Steps	Temperature	Time	Number of cycles
Initial denaturation	95°C °C	10 min	1
Denaturation	95°C °C	30 sec	40
Annealing/Extension	60.4°C °C	30 sec	40

(50X) was diluted in distilled water in the ratio of 1:20 and used.

The extracted whole cell antigen was diluted in the coating buffer (0.05 M carbonate - bicarbonate buffer, pH 9.6), and 100 µL was added to each well. The plates were incubated at 4°C overnight, washed three times with ELISA washing solution (Phosphate buffered saline, pH. 7.4, containing 0.5 per cent Tween-20) and dried by shaking (Shaker Incubator at 180 rpm, 55°C for 20 min). One per cent bovine serum albumin in PBS-T was used to block the wells which were then subjected to incubation

for one hour at 37°C and again washed three times with PBS-T. One hundred microlitres each of 50 serum samples of bovine having history of recent abortion (each diluted in the ratio of 1:100) were loaded into the wells in duplicates and incubated for one hour at 37°C and washed as before. One hundred microlitres of 1:10,000 commercial rabbit anti-bovine IgG peroxidase conjugate diluted (1:10,000) was added in each well. After incubation for one hour at 37°C, the plates were washed with ELISA washing solution and followed by loading of wells of each plate with 100 µL of DAB (50X) diluted in distilled water in the ratio of 1:20. The plates

were incubated at room temperature for 10 min, after which the reaction was stopped using 50 μ L of 1.25 M H_2SO_4 . The optical density (OD) was measured on an ELISA reader (Biorad) at 450 nm.

Results and discussion

Even though the isolation of causative agent is considered as the gold standard test for the diagnosis, the difficulties associated with the isolation of *C. foetus* on non-selective media have limited its practical use in the timely diagnosis (Sanhueza *et al.*, 2014). The attempts made for isolation of *C. foetus* spp. in Brucella agar was unsuccessful for all the samples tested under the present study and *Proteus* was the major contaminant grown in the culture during the study. Meanwhile it was reported that reduced viability and fragile nature of the agent in clinical samples could also be affecting the culture results (Clark and Dufty, 1978; Brooks *et al.*, 2004). Furthermore, chances of contamination with ubiquitous, faster growing microorganisms, such as *Pseudomonas* spp. and *Proteus* spp. often contaminate the samples and make it difficult to detect the presence of *C. foetus* (Monke *et al.*, 2002). Many of the contaminating materials like faeces, urine or other contaminants may potentially interfere with the direct culturing (Radstrom *et al.*, 2004). Thus the viability of *C. foetus* outside the host is important which might also get affected by the exposure to atmospheric levels of oxygen that have a toxic effect on the bacteria. These might be the reasons for unsuccessful isolation in the present study. The suppression of growth of fastidious *Campylobacter* by the contaminants like *Proteus* could also be one of the reasons for failure of isolation.

PCR offers a reliable and accurate technique for the species and subspecies identification of *C. foetus* (Hum *et al.*, 1997) and many researchers have used this technique successfully in detecting *C. foetus* spp. DNA from clinical samples (Wang *et al.*, 2002; Van Bergen *et al.*, 2005). The present study also attempted to standardize genus, subspecies specific PCR and subspecies specific real time PCR for directly detecting antigens from clinical samples. The PCR primers based on *SapB2* gene and *ISCfe-1* gene were standardized at an

annealing temperature of 61.4°C for 30 seconds. One hundred and ten clinical specimens were subjected to PCR and all of them were found to be negative for the presence of genus specific as well as subspecies specific *Campylobacter* DNA. Positive controls showed respective amplicon size of 816bp, 435bp and 84bp in submarine agarose gel electrophoresis (Fig. 1-3).

Also, *SapB2* gene and *ISCfe-1* gene based real time PCR was standardized at an annealing temperature of 60.4°C and 61.4°C for 30 seconds using conventional PCR. Of the 160 samples, all the samples processed and tested were found to be negative. Positive controls showed an average T_m value of 86.4°C for *SapB2* and 83.4°C for *ISCfe-1* respectively (Fig. 4-7). Then, the amplicons were visualized in three per cent agarose gel which showed band approximately at 87 bp and 84 bp level respectively (Fig. 8).

According to Hosseinzadeh *et al.* (2013), any sample could contain a mixture of semen, faeces, urine, blood or other contaminants. Many of these contaminating materials may potentially interfere with DNA extraction techniques and are also inhibitory to PCR. This might be probable reasons for unsuccessful PCR results in the present study.

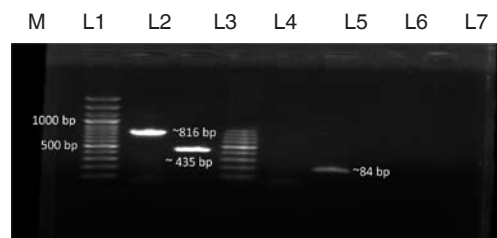


Fig.1 Agarose gel showing amplified product of *Campylobacter* genus and *C. foetus* subspecies specific PCR

M – 100 bp DNA ladder

L1 – *Campylobacter* genus (Positive control - 816bp)

L2 – *C. foetus* subsp. *foetus* (Positive control - 435bp)

L3 – 50 bp DNA ladder

L4 – Negative control

L5 – *C. foetus* subsp. *venerealis* (Positive control – 84bp)

L6 –L7 – Clinical samples

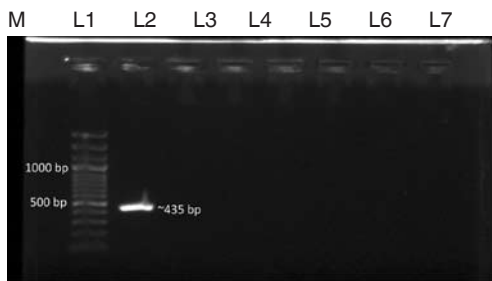


Fig.2 Agarose gel electrophoresis of PCR amplified product of *C. foetus* subspecies *foetus* specific PCR

M – 100 bp DNA ladder

L1 – *C. foetus* subsp. *foetus* (Positive control)

L2 – Negative control

L3 to L7 – Clinical Samples

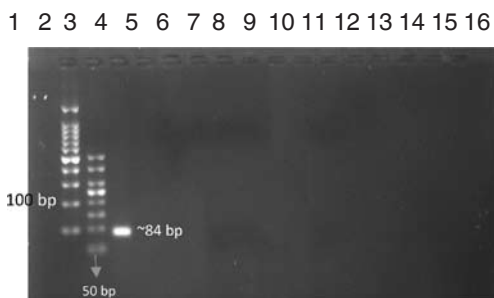


Fig.3 Agarose gel electrophoresis of PCR amplified product of *C. foetus* subsp. *venerealis* specific PCR

1 – 100 bp DNA ladder

2 – 50 bp DNA ladder

3 – *C. foetus* subsp. *venerealis* (Positive control)

4 – Negative control

5 to 16 – Clinical Samples

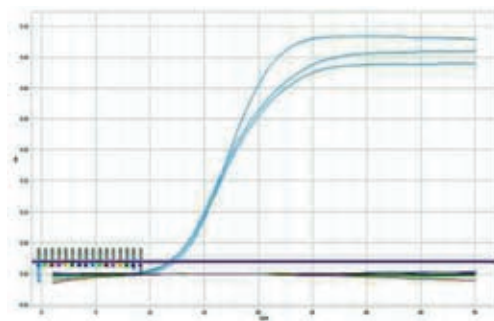


Fig. 4 Amplification plot of real time PCR assay for *SapB2* gene from positive control and clinical samples

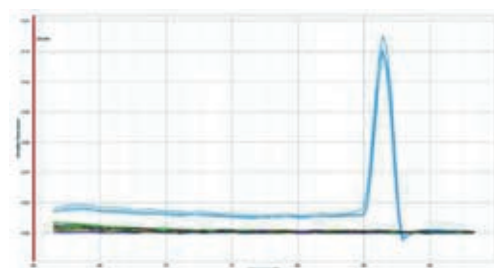


Fig. 5 Melt curve of real time PCR assay for *SapB2* gene from positive control and clinical samples

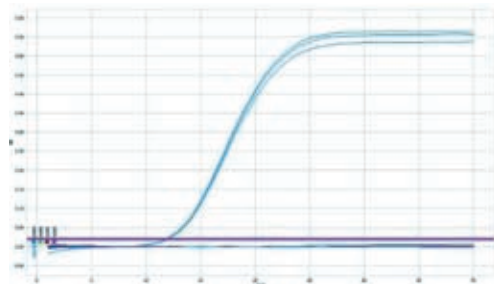


Fig. 6 Amplification plot of real time PCR assay for *ISCFE-1* gene from positive control and clinical samples

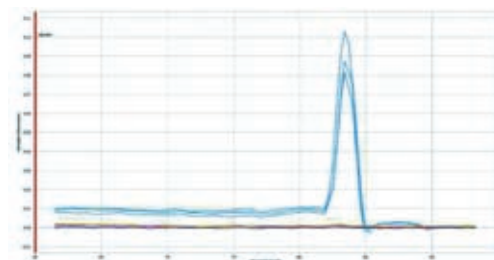


Fig. 7 Melt curve of real time PCR assay for *ISCFE-1* gene from positive control and clinical samples

Furthermore, the report of no campylobacteriosis case from 160 clinical samples processed might be due to insignificant endemic levels in the location, where the study was conducted. This assumption is supported by the results of Mani (2014), where he could not detect any *C. foetus* organism from the samples of infertility and abortion from bovines in the same area.

Similarly, ELISA was standardized for detecting antibodies from clinical samples. Initially the optimum concentrations of the antigen, antibody and IgG-HRP conjugate was calculated employing checker board analysis. It was observed that antigen concentrations (10

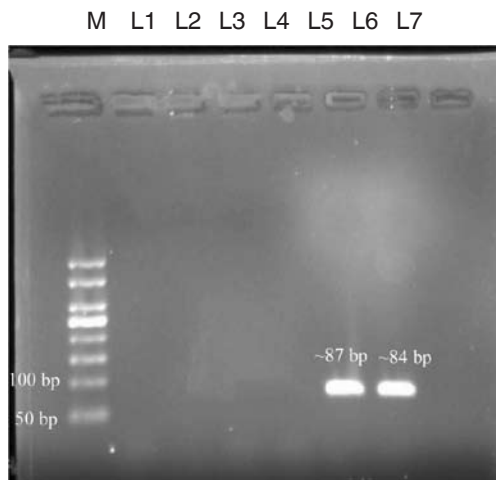


Fig. 8 Standardisation of *SapB2* and *ICSfe-1* gene using conventional PCR with product size 87bp and 84bp

M – 50 bp DNA ladder

L1-L4 – Negative control

L5 – *C. foetus* subsp. *foetus* (Positive control-87bp)

L6 – *C. foetus* subsp. *venerealis* (Positive control – 84bp)

µg /100 µL) of 50 ng per well for *SapB2* were found optimum at a test sera dilution of 1:100.

In order to determine the cut off value for demarcating the negative and positive sera samples, the ELISA was conducted employing the whole cell antigen antibody and IgG-HRP conjugate and the cut off values obtained were 0.12. A total of 50 suspected bovine serum samples were screened using the ELISA and all the serum samples were found to be negative. The high frequency of negative samples observed while performing ELISA might be due to factors such as better awareness regarding contagious disease among farmers, improved husbandry practices, climatic and geographic aspect of the area under study.

The work did not provide any proof of absence of the organism but the weight of evidence indicates that infection was highly unlikely to be widespread in herds. Artificial insemination is often considered as a simple control method for BGC, but is impractical for many herds where natural service takes place as a part of breeding. As per the breeding policy,

only artificial insemination is practiced in Kerala in bovines, which might be one of the factors that prevent extensive spread of BGC.

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Fourier transform infrared spectrophotometer with attenuated total reflectance (FTIR-ATR) for analysis of uroliths in caprine species

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Abstract

Pathophysiology of obstructive urolithiasis in goats are multifactorial involving management, nutritional and hormonal factors. Analysis of the composition of a particular urolith would aid in understanding the aetiopathogenesis of the calculi which can facilitate establishment of appropriate treatment and prevention protocols. Four cases of chronic obstructive urolithiasis in male goats presented to University Veterinary Hospital were surgically managed by tube cystotomy. The calculi extracted from the bladder were subjected to Fourier transform infrared spectrophotometer with attenuated total reflectance (FTIR-ATR) to identify the composition of the calculi. The infrared wavelength bandwidths of H-O-H stretching vibrations, H-O-H bending modes of vibrations of water molecules, N-H symmetric stretching vibrations, N-H bending vibration and N-H asymmetric bending vibration in NH₄⁺ unit's ionic phosphate were compared with the available reference spectrum of wavelength and were found to be identical and comparable with the standard infrared wavelength of struvite calculi.

Key words: Fourier transform infrared spectrophotometer, Attenuated total reflectance, Chronic obstructive urolithiasis, Goats

Urolithiasis is a clinical condition resulting from multiple congenital and/or acquired pathophysiologic processes that result in an elevated concentration of insoluble crystalloids in the urine (Dar, 2011). Pathophysiology of obstructive urolithiasis were identified to be multifactorial

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involving management, nutritional and hormonal factors. Sun *et al.* (2010) reported the most common aetiology of formation of urolithiasis as the supersaturation of the excretory solutes in the urine, increased concentrate feeding and lack of sufficient water intake and that the analysis of the composition of a particular urolith would aid in understanding the aetiopathogenesis of the calculi and further facilitated establishment of appropriate treatment and prevention protocols. Primiano *et al.* (2014) reported that FT-IR was found to be the gold standard test for analysis of urinary calculi and that it showed a high sensitivity thereby giving an accurate recognition of the composition of each stone.

Materials and Methods

Four male goats with chronic obstructive urolithiasis with an intact bladder were presented to University Veterinary Hospital, Mannuthy with history of stranguria. All animals had the history of being fed with high concentrate diet (cattle ration). The Ultrasonography evaluation revealed hypoechoic turgid bladder with hyper echoic uroliths forming sludge in the bladder floor that made wavy motion on gentle succession. (Fig-1) The animals underwent tube cystostomy by standard procedure documented by Gazi *et al.* (2014). The urinary calculi was collected by sieving the urine (Fig-2) The microanalysis of the sediments revealed uroliths similar to struvite crystals. (Fig- 3)

The urolith samples were serially numbered as A₁, A₂, A₃ and A₄ respectively. The calculi were thoroughly washed with deionized water to remove the adhered dirt and blood clots, dried at 37°C and stored for analysis of the mineral content. Analysis of calculi was done using Perkin Elmer Spectrum 2 Infra-red Spectrophotometer - USA using the principle of Fourier transform infrared spectrophotometer with Attenuated Total Reflectance (FTIR-ATR) at Central Instrumentation Laboratory, College of Veterinary and Animal Sciences, Mannuthy. To create a background blank in the machine, 300 mg of potassium bromide (KBr) was pressed under pressure to form a pellet and analysed. (Fig-3) Subsequently, 298 mg of KBr

and 2 mg of powdered uroliths were mixed and was pressed under pressure and the pellet was scanned and analysed to record the spectrum using FTIR- ATR machine and the observed spectrum was compared to the standard reference spectrum based on the observed transmittance and frequency of the waveforms.

Results and Discussion

The results of analysis of calculi in Fourier Transform Infrared Spectrometry with Attenuated Total Reflectance (FTIR-ATR) are depicted in Table 1. Analysis of the calculi from animal no. A₁, A₂, A₃ and A₄ using FTIR-ATR revealed a graph with an IR spectrum ranging from 4000 to 450 cm⁻¹. The H-O-H stretching vibrations of water of crystallization were at 3401.94 cm⁻¹ for animal no A₁, 3391.81 cm⁻¹ for animal no A₂, 3500 to 3350 cm⁻¹ for animal no A₃ and 3360.29 cm⁻¹ for animal no A₄. The H-O-H stretching vibrations of the cluster of water molecules were at 2231.52, 2346.67 and 2321.69 cm⁻¹ for animals' A₁ A₂ A₃ A₄ respectively. The H-O-H bending modes of vibrations ranged from 1440.19, 1434.90, 1434.68 and 1441.17 cm⁻¹ for animals' A₁ A₂ A₃ A₄ respectively. The N-H symmetric stretching vibrations were at 3401.94 cm⁻¹ for animal no A₁, 3391.81 cm⁻¹ for animal no A₂, 3500 to 3350 cm⁻¹ for animal no A₃ and 3360.29 cm⁻¹ for animal no A₄. The N-H bending vibration was at 1670.28, 1650.70, 1651.8 and 1654.67 cm⁻¹ for animals' A₁ A₂ A₃ A₄ respectively and the N-H asymmetric bending vibration in NH₄⁺ unit's ionic phosphate was at 995.16, 1000.4, 1000.48 and 1000.14 cm⁻¹ for animals A₁ A₂ A₃ A₄ respectively. These infrared wavelength bandwidths were compared with the available reference spectrum of standard wavelength of struvite calculi proposed by Bindhu and Thamby (2012). The infrared spectrum from animals are depicted in the figure 5-8.

The spectra contained a bandwidth comparable to that of the standard IR spectra of struvite crystals as proposed by Bindhu and Thamby (2012) who reported that in a pure magnesium ammonium phosphate spectrum, the broad envelop at 3270 cm⁻¹ correlates to the O-H and N-H stretching vibrations. The band at 2935 cm⁻¹ is due to NH₄⁺ ion and the

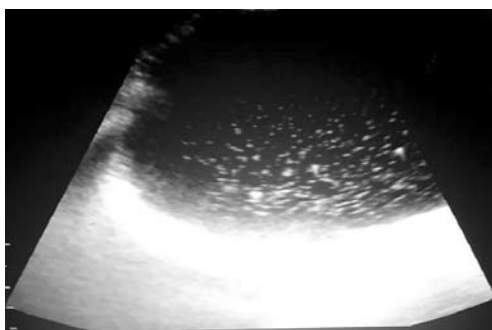


Fig-1 Ultrasonography to detect uroliths



Fig-2 Uroliths retrieved from urinary bladder by tube cystostomy



Fig-3 Microscopic examination of struvite cystals

band corresponding to 1666 cm^{-1} was assigned to N-H bending vibrations. The sharp yet weak envelop correlating to 1445 cm^{-1} could be due to N-O asymmetric stretching vibration. The absorption band occurring at 1010 cm^{-1} was assigned to ionic phosphate.



Fig-3 Prepared pellets of KBr and urolith for FTIR – ATR spectroscopy



Fig-4 Perkin Elmer Spectrum 2 Infra-red Spectrophotometer- USA

The determination of molecular composition, crystalline composition and the quantification of all stone components are helpful to establish the etiology of stones disease and consequently aid in the treatment of the clinical disease. Various methodologies exist for stone analysis. Fourier transform infrared spectroscopy (FT-IR) is becoming the gold standard for stone analysis as it is the most appropriate technique. The principle of FT-IR involves generating an infrared spectrum from the vibrational motion of the molecules. These vibrational frequencies are unique for each compound. This feature of the spectrometer is used to characterize inorganic compounds from organic ones present in the calculi. The band intensities are proportional to the compound concentration and hence qualitative estimations are also obtained. FT-IR spectroscopy leads to unambiguous information about the stone composition, both for main substances and trace elements, all essentials to guide therapy.

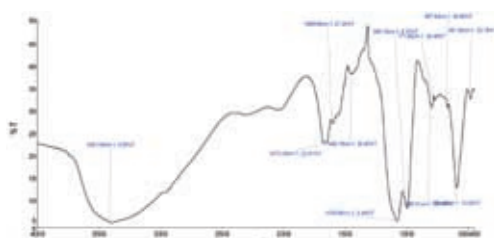


Figure 5: Infrared spectrum from goat A₁ showing struvite bands at 3401, 1440, 1670 and 995.16 cm⁻¹

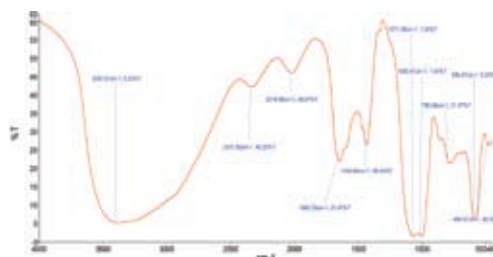


Figure 6: Infrared spectrum from goat A₂ showing struvite bands at 3391, 1434, 2331, 1650 and 1000.4 cm⁻¹

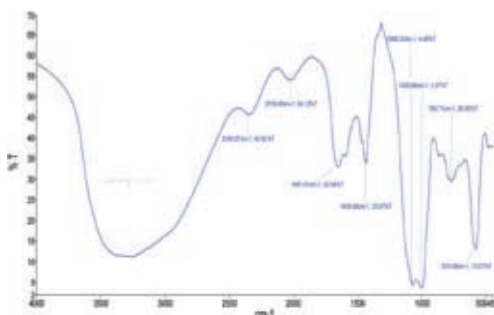


Figure 7: Infrared spectrum from goat A₃ showing struvite bands at 3500-3350, 1434, 2346, and 1000.48 cm⁻¹

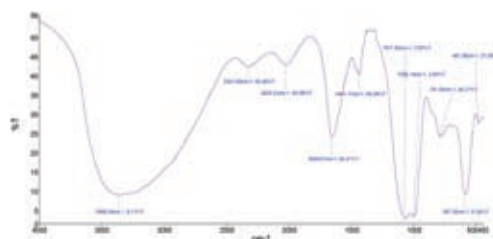


Figure 8: Infrared spectrum of goat A₄ showing struvite bands at 3360, 1441, 2321, 1654 and 1000.14 cm⁻¹

Wang *et al.* (2009) explained the preparation of the calculi before spectroscopy by washing the crystals twice in deionising water and air drying overnight. The authors recorded strong bands of wavelength 556.1, 986.4, 1066.7, 3201.7 cm⁻¹ and medium-strong bands at 1673.9, 2385.2 cm⁻¹ which was conclusive of magnesium ammonium phosphate crystals. Bindhu and Thambi (2012), in their study on microanalysis of the struvite crystal, reported that the standard IR spectra ranged within 4000 to 450 cm⁻¹ for pure struvite with the broad envelop at 3270 correlated to the O-H and N-H stretching vibrations. In their study, the band at 2935 cm⁻¹ was due to NH₄⁺ ion and the band corresponding to 1666 cm⁻¹ was assigned to N-H bending vibrations. The sharp yet weak envelop of 1445 cm⁻¹ was due to N-O asymmetric stretching vibration. The absorption band occurred at 1010 cm⁻¹ was assigned to ionic phosphate.

In the present study based on the alkaline pH of urine in all animals and the confirmed composition of struvite, medical

management was done with ammonium chloride at 300 mg/kg body weight per orally and the owners were advised to avoid feeding cattle feed to goats and provide ad libitum water.

Conclusion

Urolithiasis is a clinical disease affecting the feedlot animals fed on high concentrate diet (cattle ration). The most common urolith manifested in male goats is magnesium ammonium phosphate (struvite) calculi. The use of Fourier transform infrared spectroscopy has been highly specific for determining the composition of the urolith which invariably has aided in specific treatment protocols and preventive management strategies in caprine chronic obstructive urolithiasis.

Acknowledgement

Central Instrument Laboratory, CVAS, Mannuthy.

Table 1. Results of analysis of calculi in Fourier Transform Infrared Spectrometry with Attenuated Total Reflectance (FTIR-ATR)

Functional Group Assignments	Animal no.	Reported IR wavelength (cm ⁻¹)	Standard IR wavelength of pure struvite (cm ⁻¹) (Bindhu and Thambi, 2012)
H-O-H stretching vibrations of water of crystallization	A ₁	3401.94	3270
	A ₂	3391.81	
	A ₃	3500-3350	
	A ₄	3360.29	
H-O-H stretching vibrations of a cluster of water molecules	A ₁	-	2385
	A ₂	2331.52	
	A ₃	2346.67	
	A ₄	2321.69	
H-O-H bending modes of vibrations	A ₁	1440.19	1445
	A ₂	1434.90	
	A ₃	1434.68	
	A ₄	1441.17	
N-H symmetric stretching vibrations in NH ₄ ⁺ units		-	2935
N-H symmetric stretching vibrations	A ₁	3401.94	3270
	A ₂	3391.81	
	A ₃	3500-3350	
	A ₄	3360.29	
N-H symmetric stretching vibrations in NH ₄ ⁺ units		-	2935
N-H bending vibration	A ₁	1670.28	1666
	A ₂	1650.70	
	A ₃	1651.8	
	A ₄	1654.67	
N-H asymmetric bending vibration in NH ₄ ⁺ units Ionic phosphate	A ₁	995.16	1010
	A ₂	1000.4	
	A ₃	1000.48	
	A ₄	1000.14	

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Comparative evaluation of modified proximal perineal urethrostomy with direct guided urethral catheterisation and tube cystostomy for the management of obstructive urolithiasis in male goats



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Abstract

Twelve clinical cases of chronic obstructive urolithiasis in male goats were selected for the study with the objective to evaluate two surgical techniques - modified proximal perineal urethrostomy (MPPU) with direct guided urethral catheterisation (Group I) and tube cystostomy (Group II) for the surgical management. Ultrasonography was effective in assessing the urinary bladder and detection of uroliths. Functional patency of normal urethra was regained in five out of six animals of each group by third post-operative week. Direct access to the perineal urethra providing quick relief to the turgid bladder was identified as the major advantage of modified proximal perineal urethrostomy technique. Tube cystostomy technique provided direct visual assessment of urinary bladder, precise fixing of Foley's catheter and retrieval of uroliths. Even though this technique was found to be more invasive, it was identified as an effective approach for correcting cystorrhesis resulted from obstructive urolithiasis

Key words: Modified proximal perineal urethrostomy, tube cystostomy, obstructive urolithiasis

Urolithiasis is defined as the formation of uroliths as a consequence of multiple congenital and/or acquired pathophysiologic processes that resulted in the increased concentration of less soluble crystalloids in the urine (Dar, 2011). Many surgical procedures like amputation of urethral process, urethrotomy, urethrostomy, cystotomy, tube cystostomy and bladder marsupialization

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were documented by various authors for successful management of urolithiasis. (Ewoldt *et al.*, 2008).

Tube cystostomy has been considered a gold standard technique - due to its direct access to the urinary bladder whereas modified proximal perineal urethrostomy (MPPU) using direct guided urethral catheterisation technique is emerging as new technique to approach urethral access with a stylet guided catheter through a specific site between the pin bones at the level of about two cm below the anus (Nair *et al.*, 2020).

Materials and Methods

Twelve male goats with obstructive urolithiasis were presented to the Veterinary Hospitals were divided into two groups. Group I (six animals) were subjected to modified proximal perineal urethrostomy and Group II (six animals) were subjected to tube cystostomy.

The general condition of the goats, condition of urethral process, perineal urethral pulsation and ultra-sonographic evaluation of the urinary bladder was carried out on the day of presentation in all the goats as per the procedure documented by Braun *et al.* (1992.)

Fig-1. Modified Perineal Urethrostomy) using direct guided urethral catheterisation technique

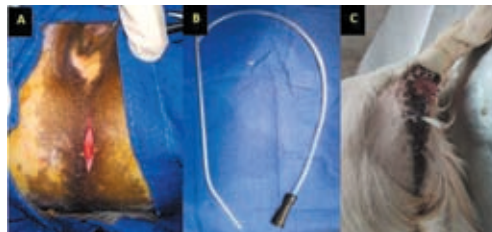
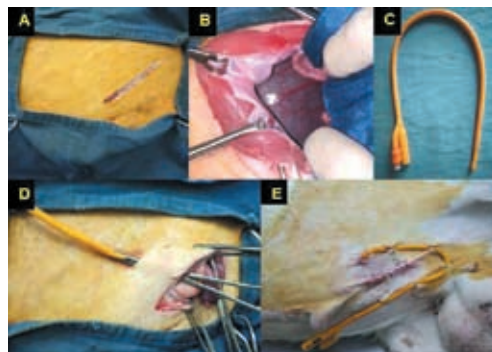


Fig-2. Tube cystostomy technique



In animals of group I, regional anaesthesia was achieved by caudal epidural anaesthesia using 2 % lignocaine hydrochloride, with animals restrained in sternal recumbency and tail fixed cranially and perineum elevated.

Table 1: Comparison of the surgical techniques of MPPU and Tube cystostomy in chronic obstructive urolithiasis in goats

Criteria	MPPU	Tube Cystostomy
Duration of surgery	15-30 min.	60-90 min.
Invasiveness	Less invasive	More invasive
Access to urethra	Direct	-
Access to the bladder	Indirect	direct
Status of the bladder	Only Intact	Both
Calculi retrieval	Inaccessible	Accessible
Outcome	Successful in five out of six animals	Successful in five out of six animals
Cost effectiveness	Effective	effective
Post operative Complications	Hemorrhage and frequent blockage of catheter	Dislodgement of Foleys' catheter and delayed functional patency of urethra
Average duration of achieving functional patency	13 days	20 days
Mortality	16.66 per cent	16.66 per cent

Modified catheter was fabricated and by passing a pre bent K-wire introduced into the lumen of an infant feeding tube of suitable size (Fig -1B). A one centimetre long incision commencing from two centimetres below the anal opening, between the pin bones was made to identify the pulsating urethra. (Fig- 1A). A modified stented catheter was introduced by gentle puncture into the urethral lumen to reach the bladder following which the K-wire was withdrawn and the catheter was fixed *in situ*. (Fig- 1C)

Animals of Group II were sedated by inj. xylazine hydrochloride at 0.05 mg/kg body weight intramuscularly and regional anaesthesia was achieved with inverted 'L' block using 2 % lignocaine hydrochloride. The animals were restrained in right lateral recumbency with the hind limbs extended backwards. Tube cystostomy was performed as per the technique described by Gazi *et al.* (2014). A three-inch oblique lower flank incision was made on the skin. Subcutaneous fascia and rectus abdominis muscles were separated followed by peritoneum to enter the abdomen (Fig- 2A). The bladder was isolated and exteriorized and an incision was made to retrieve the calculi and lavage the bladder (Fig- 2B). A ballooned Foley's catheter of size 16 FG made of rubber latex was used as an indwelling catheter for tube cystostomy (Fig-2C). A nick incision was made three centimetre lateral to the flank incision on the skin and a curved artery forceps was passed from the abdomen penetrating through all the muscle layers to facilitate the drawing of the catheter from outside to inside (Fig- 2D). A nick incision was made on the bladder away from the cystotomy closure and the tip of the catheter was inserted to the bladder. The balloon part of the catheter was then inflated with 5 ml of normal saline to keep the catheter tip locked inside the bladder and secured with purse-string sutures. The cystotomy closure was performed using double inversion sutures of Cushing's followed by Lembert pattern, abdomen closure was performed using Polyglactin- 910 sutures in a continuous pattern and skin sutures were performed using Nylon 1-0 in the interrupted pattern. The Foley's catheter was fixed to the skin and left *in situ* (Fig-2E) till functional patency of the urethra was regained.

Post-operatively, the animals were administered Inj. Enrofloxacin at the dose rate of 5 mg/kg body weight for seven days and Inj. tramadol at the dose rate of 2 mg/kg body weight for three days. Ammonium chloride at 300 mg/kg body weight was supplemented after confirming the alkalinity of the urine.

Results and Discussion

In Group I, the MPPU catheters were removed on second post-operative week in three animals and third post-operative week in two animals after confirming the functional patency of urethra, characterised by free flow of urine through the normal urethral opening. Even though urinary diversion was successfully achieved in all the animals in this group, only five out of six animals showed complete healing. One animal had severe operative site infection with perineal abscess and died on fifth post-operative week.

In group II, the urethral patency was restored in five out of six animals by the end of third to fourth post-operative week. Two animals had bladder rupture and catheter got dislodged with severe cystitis and uroabdomen. One animal died on fourth post-operative week.

Ultrasonography of the bladder revealed anechoic distended bladder with multiple hyperechoic sediments. Thinning of the bladder was appreciated in 10 goats and results were in accordance with Al-Lugami *et al.* (2017).

In animals of group-I, the pulsating perineal urethra was easy to access through a site below the anal opening between the pin bones in the procedure adopted. In the present study, bending of the MPPU catheter by passing a prebent K Wire into the lumen favored bypass of urethral diverticulum as reported by Fortier *et al.* (2004). Moderate perineal bleeding was seen as in three out of six cases out of which one animal had severe bleeding during and after the procedure. This could be due to the injury to the dorsal penile and external pudental artery running closely in alignment with the course of urethra as reported by Oman *et al.* (2019).

Direct lavaging of the urinary bladder

was possible by this technique which facilitated the alkaline pH correction by flushing the urinary bladder with two per cent boric acid solution. Five animals recovered by this technique by the end of three weeks and the catheters were removed after confirming the normal urethral patency. This was in accordance with Nair *et al.* (2020)

An oblique left lower flank incision performed in group I resulted in cystorrhesis in one and severe cystitis was detected in four animals. In cases of cystorrhesis, uroabdomen was noticed and the fluid was gradually relieved to avoid shock. An extension of the surgical site more caudally was done to identify the site of ruptured bladder.

The dislodgement of the catheter from the skin and bladder was another complication of the - group II animals. Delayed functional patency of urethra was noticed in three animals in which the animals were able to pass urine through normal opening by fourth post-operative week. Obstruction of Foley's catheter was found in one animal - which was relieved by flushing with normal saline and two per cent boric acid solution. Four animals started passing urine normally by second post-operative week while urethral patency was re-established by the end of three weeks in two animals.

Conclusion

Owing to the ease of access to the proximal perineal urethra, evident perineal pulsation, minimal surgical trauma and precise placement of the catheter in less time, modified proximal perineal urethrostomy could be a highly effective technique in managing caprine obstructive urolithiasis. Deliberate skill for the precise location of the urethra warrants the success of this technique. The correct selection of the size of the catheter, the landmark of the urethral puncture were the main challenges identified in this technique. Tube cystotomy offered the direct visualisation of the bladder and hence an effective fixation of Foleys' catheter was possible. Due to the direct access to the bladder, this procedure could be regarded as the only surgical option in cases of cystorrhesis due to obstructive urolithiasis. Partial dislodgement of the fixation of the

catheter and mild oedema at the surgical site were the only identified complications in tube cystostomy.

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Qualitative and quantitative analysis (GC-MS) of methanol extract of *Crataeva nurvala* stem bark



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Abstract

Medicinal plants are precious source of bioactive compounds which possess a range of beneficial properties and serve as the major source of medicine for a large proportion of population across the world. Since ancient times, *Crataeva nurvala* has been used as a vital herb in Ayurvedic system of medicine. In Unani system of medicine the bark of *C. nurvala* is used as an appetite stimulant and as an agent to decrease the secretion of bile and phlegm. In the present study, the methanol extract of stem bark of *C. nurvala* was analysed for preliminary phytochemicals and the chemical profiling of the extract was illustrated using gas chromatography mass spectrometry (GC-MS) analysis. The phytochemical analysis revealed that the plant extract contained alkaloids, steroids and triterpenoids. The GC-MS analysis determined the presence of different compounds of biological importance. The identification and characterisation of the phytoconstituents in the extract could pave the way for the discovery of new drugs for various ailments.

Keywords: *Crataeva nurvala*, triterpenes, alkaloids, steroids.

Medicinal plants are valuable source of naturally active phytochemicals which provide health benefits for humans and animals. These compounds commonly known as secondary plant metabolites have been attributed to have different biological properties which provide protection against various diseases. The analysis of the chemical constituents present in those plant extracts

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is strategic to comprehend many herbal drugs and their preparations which would further aid in discovering the actual values of folklore remedies.

Crataeva nurvala Buch-Ham., commonly known as Varuna, Neermathalam, Barna Chal, belonging to the family of Capparidaceae, is a moderate sized deciduous tree. A variety of medicinal properties have been reported for *C. nurvala* and its stem bark. It has been traditionally used in normalising blood flow, waste elimination, breathing problems, fever, metabolic disorders, joint lubrication and wound healing (Vashist *et al.*, 2020). Mekap *et al.* (2011) determined the antiurolithiatic activity of *C. nurvala*. Root and bark extracts of this tree are documented to be laxative, lithotripic and have been found to increase the appetite and biliary secretion (Malini *et al.*, 1995). The ethanol and aqueous extracts of the dried stem bark of *C. nurvala* have been found to possess significant anti-fertility effects in rats (Bhaskar *et al.*, 2009). The antidiarrhoeal activity of ethanol extracts of *C. nurvala* stem bark has been reported by Inayathulla *et al.* (2010). *Crataeva nurvala* stem bark extract exhibited antidiabetic activity against alloxan induced diabetic albino rats in the study done by Sikarwar and Patil (2010). Thus, the present study was carried out to evaluate the various phytochemical constituents present in the bark of methanol extract of *C. nurvala* which would be helpful to delineate the various biological activities shown by the stem bark.

Materials and methods

Plant collection and identification

The bark of *Crataeva nurvala* was collected from Valluvanad, Palakkad, Kerala (Figure 1 and 2). The collected plant material was identified and its authenticity was confirmed by Raw Material Herbarium and Museum (RHMD), NISCAIR, New Delhi, India.

Preparation of extracts

Freshly collected bark of *C. nurvala* were cleaned to remove adhering dust and then dried under shade. The dried bark was coarsely powdered using an electric pulveriser and the

powder obtained was extracted using a Soxhlet apparatus with methanol at 67°C. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40°C). The yield of the extract was calculated using the formula: Yield value (%) = Extracts obtained/ Total amount of crude drug × 100, and kept under refrigeration in an airtight container after complete evaporation of the solvent for further use.

Qualitative phytochemical analysis

The extracts were tested for the presence of bioactive compounds using methods described by Harborne (1998).

Tests for detection of steroids

Salkowski's test

Fifty milligrams of the extract were dissolved in 3 mL of chloroform. Few drops of concentrated sulphuric acid were added and the solution was allowed to stand. Formation of red colour directed the presence of steroids.

Liebermann Burchardt test

Fifty milligrams of the extract were mixed with 3 mL of chloroform. To this, five drops of acetic anhydride and 1 mL concentrated sulphuric acid was added along the sides of the test tube. Development of a reddish ring at the junction of two layers confirmed the presence of steroids.

Tests for detection of alkaloids

One gram of the extract was mixed with 5 mL of ammonia and then extracted with an equal volume of chloroform. To this extract, 5 mL of dilute hydrochloric acid was added. The acid layer obtained was further tested with the following reagents for the presence of alkaloids.

Dragendorff's test

Eight drops of Dragendorff's reagent was mixed with 1 mL of acid extract. Development of a reddish brown precipitate indicated the presence of alkaloids.



Fig. 1. Herbarium of *Crataeva nurvala* Buch-Ham



Fig. 2. Bark of *Crataeva nurvala* Buch-Ham.

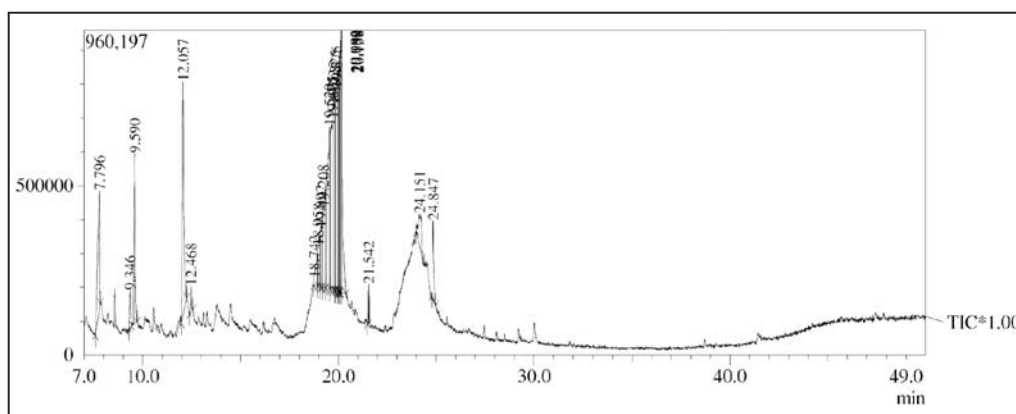


Fig. 3. GC-MS chromatogram of methanol extract of *C. nurvala* stem bark

Mayer's test

To 1 mL of the acid layer, eight drops of Mayer's reagent were added. Development of a cream coloured precipitate indicated the presence of alkaloids.

Wagner's test

One millilitre of Wagner's reagent was added to 1 mL of the extract. Development of reddish brown colour precipitate indicated the presence of alkaloids.

Hager's test

To 1 mL of the acid extract, eight drops of Hager's reagent were mixed. Formation of yellow precipitate specified the presence of alkaloids.

Tests for detection of glycosides

Benedict's test

Approximately 50 mg of the extract was mixed with 1 mL of water and then 5 mL of Benedict's reagent was added to it. Formation of brown or red precipitate indicated the presence of reducing sugars.

Test for detection of phenolic compounds

Ferric Chloride Test

Five milligrams of the extract was dissolved in 1 mL of water and five drops of 10 per cent ferric chloride were added to it. Development of bluish black colour specified the presence of phenols.

Tests for detection of tannins

Ferric chloride test

Treated two milligrams of the extract with 3 mL of one per cent ferric chloride solution. Development of a blue-black or a brownish green colouration showed the presence of tannins.

Tests for detection of flavonoids

Ferric chloride test

Treated 2 mL of the methanol extract (0.5 gram extract in 10 mL methanol) with four drops of neutral ferric chloride solution. Formation of green colour indicated the presence of flavonoids.

Test for detection of diterpenes

About five milligrams extract was mixed with 3 mL of five per cent copper acetate solution. Formation of green colour showed the existence of diterpenes.

Tests for detection of triterpenes

Salkowski test

Mixed 3 mL of chloroform to three milligrams of extract and it was shaken with 3 mL concentrated sulphuric acid. Development of yellow colour in the lower layer on standing indicated the presence of triterpenes.

Tests for detection of saponins

Froth test

Approximately 200 mg of the extract was shaken with 5 mL of water. Continuation of foam produced for ten minutes designated the occurrence of saponins.

GC-MS Analysis

The active phytochemical principles of methanol extract of *C. nurvala* was analysed using GC-MS system of Centre for Analytical Instrumentation- Kerala (CAI-K), Kerala Forest Research Institute (KFRI), Peechi, Kerala. The GC-MS analysis was carried out on Gas

chromatography Mass Spectrometer (Shimadzu GC-MS, Japan, QP2010SE) with a mass range of 1.5- 1000 m/z. Helium at a flow rate of 1 mL/min was used as the carrier gas. The oven temperature was maintained at 80°C for 4 min and then increased to 280°C in 6 minutes. The injector temperature was 260°C and total analysis time was 50 minutes. Aliquot of the extract (0.4 µL) was injected into the chromatographic column after obtaining a clear baseline. The interpretation of the mass spectrum of GC-MS was guided using the database of the National Institute of Standards and Technology (NIST 11) and WILEY 8. The spectrum of the unknown compounds was related with the spectrum of the known compounds. The name and molecular weight of the compounds of the tested materials were ascertained.

Results and Discussion

Yield of the extract

The methanol extract of *C. nurvala* yielded 12.93 per cent with reference to starting dry material.

Qualitative phytochemical analysis

The qualitative phytochemical screening of methanol extract of stem bark of *C. nurvala* showed the presence of steroids, triterpenoids and alkaloids. Phytochemical screening of methanol extract of stem bark of *C. nurvala* revealed the presence of steroid and terpenoids as well as alkaloids, phenolics, flavanoids, tannins and saponins (Hade *et al.*, 2016) which supported our results. Sodipo *et al.* (2000) have reported that alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity. Huang *et al.* (2016) isolated six phytosteroids and nine known triterpenoids from the leaves of *Chisocheton cumingianus* in which chisopanoids E and F exhibited potent cytotoxicities towards MCF-7 with IC₅₀ values of 3.24 ± 1.39 and 8.85 ± 4.73 µM, and were further proved to prevent the cell proliferation, mainly by inducing apoptosis. Haque *et al.* (2008) isolated two terpenoids, phragmalin triacetate and lupeol from ethyl acetate extract of stem bark of *C. nurvula* by chromatographic

techniques. Jain *et al.* (2016) suggested that terpenoids were capable of inhibiting NFκB through different mechanisms. Khatun *et al.* (2015) evaluated the antioxidant, anthelmintic, antimicrobial and phytochemical assessment of ethanolic extract of *C. nurvala* leaves and displayed the presence of alkaloids, flavonoids, reducing sugar, saponins, steroids, tannins. The above mentioned phyto constituents are described to exhibit various pharmacological activities.

GC- MS analysis

The results of GC-MS analysis of methanol extract revealed the presence of twenty-one compounds. The GC-MS chromatogram of twenty-one compounds is depicted in Figure 3. Thymine, 3-hydroxy-2,3-dihydromaltol, 5-hydroxymethylfurfural, n-methyl-3-hydroxymethyl pyrrolidine-2-one, cytidine, methyl pentofuranoside, undecane, 6,6-dideutero-5-methyl-, 2,4-ditert-butylphenol and 3-deoxy-d-mannoic lactone were the major compounds.

Balamurugan *et al.* (2019) have done the chemical profiling of methanol bark extract of *C. nurvala* using GC-MS technique. The study revealed the presence of 8 components such as lup-20 (29)-en-3ol, 2-hydroxy-4methoxybenzaldehyde, methoprene, 1'-acetoneaphthone, 1, 2-bis (Trimethylsilyl) benzene, pivalate, cyclotrisiloxane, limonen-6-ol and 4-hexadecen-6-yne.

The recognized major compounds in our study possess some significant biological activities for future drug development. Zhao *et al.* (2013) showed that 5-hydroxymethylfurfural (5-HMF) induced apoptosis and G0/G1 cell cycle arrest in human melanoma A375 cells. Takuli *et al.* (2020) elucidated the antioxidant and antibacterial activity of *Woodwardia unigemmata* (Makino) along with chemical characterization which revealed the presence of 3-hydroxy-2,3-dihydromaltol in GC-MS analysis. Azizi *et al.* (2006) performed fast gas chromatography/ time of flight mass spectrometry (TOF-GCMS) which identified N-methyl-3-hydroxymethylpyrrolidin-2-one from the oil extract of *Pithecellobium jiringan* jack seeds which was found to abolish excess free

radicals and counteract oxidative damage. Su *et al.* (2005) evaluated the antioxidant activity of methanol extract of *Morinda citrifolia* (Noni) fruits and the purification of its butanol soluble partition of methanol extract contained isolates like cytidine. Shaheed *et al.* (2018) identified methyl pentofuranoside, also known as alpha-d-mannofuranoside, from methanolic fenugreek seed extract and determined its antibacterial activity against *Streptococcus agalactiae*, *Escherichia coli*, *Enterococcus cloacae* and *Proteus mirabillis*. Gas chromatography mass spectroscopic analysis exhibited the presence of undecane, 6,6-dideutero-5-methyl- in *Nigella sativa*, *Allium sativum*, *Propolis* and *Olea europaea* mixture which was depicted as antibacterial and antifungal agent (Bintang *et al.*, 2018). Chuah *et al.* (2015) suggested that 2,4-di-tert-butylphenol induced oxidative stress through the generation of reactive oxygen species, which cause lipid peroxidation and membrane damage in root tissues and chloroplast in leaf tissues, thus leading to increased levels of antioxidant enzymes. Shobana *et al.* (2009) in their study identified compounds such as 3-deoxy-d-mannoic lactone and thymine from two varieties of garlic (*ophioscordon* and *sativum*) which was found to possess antibacterial activity against enteric pathogens. The aforesaid isolated compounds from the methanol extract of *C. nurvala* stem bark seemed to own the reported biological activity and further study of these phytoconstituents may demonstrate the medicinal importance in future. The biological activities of other compounds have not been reported so far and more study of these phytoconstituents might validate the significant medicinal features in forthcoming.

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Effect of supplementation of rumen protected choline and methionine on milk yield and composition of early lactating dairy cows



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Abstract

An experiment was conducted on early lactating dairy cows to study the effect of rumen protected choline (RPC) and methionine (RPM) on milk yield and composition. Fifteen crossbred dairy cows in early lactation (within 10 days of calving) were selected and randomly allotted to any one of the following three dietary treatments, T₁ (Control)- with compound feed mixture containing CP- 20% and TDN- 68% , T₂- T₁+20g RPM and 20g RPC, T₃- with compound feed mixture containing CP- 17%, TDN- 68% + 20g RPM and 20g RPC. All the experimental animals were fed as per ICAR feeding standards (ICAR, 2013). Results revealed no significant difference ($p>0.05$) in milk yield and 4 per cent fat corrected milk (FCM) yield between the three treatment groups. Among the milk constituents, animals in T₃ had significantly higher milk fat ($p<0.05$), SNF ($p<0.05$), protein ($p<0.05$) and total solids ($p<0.01$) compared to those in T₁ and T₂. Milk urea nitrogen levels did not differ significantly among the three treatments and were within the normal range. The study showed that milk composition could be effectively improved by supplementing feed with rumen protected forms of choline and methionine in combination at lower dietary protein level without any reduction in milk yield.

Key words: Rumen protected choline, rumen protected methionine, early lactating dairy cows

India has ranked first among the world's milk producing nations since 1998 and the nation also has the largest bovine population in the World. Milk production in India during the period 1950-51 to 2018-19, increased from 17 million tonnes to 187.7 million tonnes (DAHD, 2020). The per

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capita availability of milk in the country which was 130g/d during 1950-51 also increased to 394g/d in 2018-19 (NDDDB, Anand). Data sheds light on sustained growth in the availability of milk and milk products for India's growing population.

During early lactation, the daily nutrient intake in cows is low and insufficient to meet the demands of milk production and the animal will be in negative energy balance. The amount of energy required for maintenance of body tissues and milk production often exceeds the amount of energy available from the diet, thus forcing mobilization of body fat reserves to satisfy energy requirement. The negative energy balance in early lactation affects peak milk yield and overall lactation yield. The level of non-esterified fatty acids (NEFA) increases in plasma as a consequence of body fat mobilization leading to hepatic lipidosis (Garg *et al.*, 2012).

Choline, a component of phospholipid and methyl donor plays an important role in very low density lipoprotein synthesis and thereby contributes to fat export from the liver. Fat metabolism can be improved with the help of choline for better energy production. This also helps in improving milk production. Evidence suggests that the dietary supply of choline in early lactating dairy animals may be inadequate, even though choline can be synthesized by the animals (Pires and Grummer, 2008). As dietary choline gets degraded rapidly in the rumen, it must be supplemented in the protected form (Elek *et al.*, 2008). Therefore, rumen protected form of choline has been developed to deliver choline to the small intestine for effective absorption.

Methionine is an essential amino acid and building block for protein and is considered as one of the two most limiting amino acids for milk production and milk protein synthesis in lactating dairy cows. Six per cent of the available choline in the body is derived from methionine and 28 per cent of the body's methionine is used for choline synthesis. Hence, the use of protected choline in the ration would help to spare the methionine, that could be used for milk production.

Materials and methods

Location of study

This study was conducted at the experimental animal shed of the University Livestock Farm & Fodder Research and Development Scheme (ULF&FRDS), College of Veterinary and Animal Sciences, Kerala, India.

Experimental Animals, Feeding and Management

Fifteen crossbred dairy cows in early lactation (within ten days of calving) were selected from University Livestock Farm and Fodder Research and Development Scheme, Mannuthy. They were divided into three groups of five animals each based on milk production and were randomly allotted to the three dietary treatments viz., T₁, T₂ or T₃. The experimental animals, individually identified by numbered ear tags, were individually tethered with nylon ropes in a well-ventilated stall with uniform management practices and facilities for individual feeding throughout the experimental period of 90 days. Antiseptic solution was sprayed at regular intervals on the floor of the shed to ensure maximum hygiene. All the experimental animals were fed according to ICAR, 2013 guidelines with compound feed mixture (CFM) in mash form depending on the treatment group as follows; T₁ (Control) - CFM containing crude protein (CP) - 20 per cent, total digestible nutrients (TDN) - 68 per cent, T₂- T₁+ 20g rumen protected methionine (metiPEARL; , Kemin Industries Pvt. Ltd.) and 20g rumen protected choline (choliPEARL; Kemin industries Pvt. Ltd.) and T₃- CFM containing CP- 17 per cent, TDN- 68 per cent+ 20g RPM and 20g RPC. Supplements were mixed with CFM thoroughly, to ensure complete intake. All supplemental products used in the study were procured from local markets.

The roughage used to feed the animals was hybrid napier. Clean fresh drinking water was offered to all the animals *ad libitum*. Ingredient compositions of compound feed mixture used for the three treatments groups of experimental animals are given in Table 1.

Table 1. Composition of compound feed mixture used in the three treatment groups

Ingredient	Percentage composition of compound feed mixture		
	T1	T2	T3
Maize	29.0	29.0	29.0
Rice polish	6.0	6.0	13.0
Deoiled rice bran	18.0	18.0	16.0
Corn gluten fibre	17.5	17.5	15.5
Coconut cake	10.5	10.5	15.5
Alfalfa	16.0	16.0	8.0
Calcite	1.5	1.5	1.5
Salt	0.5	0.5	0.5
Mineral mixture	1.0	1.0	1.0
Total	100.0	100.0	100.0
Rumen protected methionine (g)	-	20.0	20.0
Rumen protected choline (g)	-	20.0	20.0

Table 2. Chemical composition¹ of the rations fed to experimental lactating cows (%)

Parameter	Dietary treatments			Green grass
	T ₁	T ₂	T ₃	
Dry matter	90.31±0.61	90.31±0.61	90.53±0.49	15.00±0.33
Crude protein	20.93±0.14	20.93±0.14	17.06±0.58	10.58±0.50
Ether extract	3.03±0.13	3.03±0.13	3.29±0.09	2.17±0.20
Crude fibre	7.62±0.24	7.62±0.24	7.15±0.32	29.83±0.23
Total ash	10.58±0.42	10.58±0.42	10.62±0.28	9.55±0.32
Nitrogen free extract	57.81±0.16	57.81±0.16	61.85±0.08	47.86±0.47
Acid insoluble ash	1.71±0.16	1.71±0.16	1.90±0.02	1.36±0.23
Calcium	0.88±0.12	0.88±0.12	0.88±0.10	0.52±0.07
Phosphorus	0.53±0.13	0.53±0.13	0.53±0.13	0.23±0.06
Neutral detergent fibre (NDF)	52.86±0.36	52.86±0.36	47.15±0.25	64.84±0.16
Acid detergent fibre (ADF)	14.39±0.15	14.39±0.15	16.74±0.04	43.06±0.07

¹Values expressed on DM basis, average of six values

The animals were milked twice a day, at 5 A.M. in the morning and at 2 P.M. in the afternoon. The data on milk yield recorded was used to calculate the daily milk yield for each animal throughout the experimental period. Morning and evening milk samples were collected from individual animals every fortnight and pooled samples were analysed for milk composition.

Milk Analysis

The collected milk samples were pooled and analysed for total solids, protein (AOAC, 2016) and fat (IS: 1224, 1977). From the above data, the solids not fat (SNF) was calculated. The milk urea nitrogen (Bector *et al.*, 1998) was also analysed.

Table 3. Fortnightly average milk production¹ of lactating cows maintained on three experimental rations

Fortnight	Daily milk production (kg)			p-value
	T1	T2	T3	
1	12.23±1.53	12.63±1.04	10.57±1.16	0.49 ^{ns}
2	12.71±1.29	12.71±1.39	11.23±1.22	0.66 ^{ns}
3	12.58±1.02	12.33±1.43	11.41±1.14	0.78 ^{ns}
4	11.91±1.02	12.25±1.37	10.94±1.03	0.71 ^{ns}
5	11.92±0.92	12.11±1.36	10.97±1.02	0.75 ^{ns}
6	11.98±0.95	11.76±1.31	10.80±1.06	0.74 ^{ns}
Mean±S.E.	12.22±1.12	12.29±1.31	10.98±1.10	0.55 ^{ns}

¹Mean values are based on five replicates with S.E.; ns- non significant

Table 4. Fortnightly average 4 per cent FCM yield of lactating cows maintained on three experimental rations

Fortnight	Daily milk production (kg)			p-value
	T1	T2	T3	
1	11.30±1.34	12.03±0.92	10.57±1.10	0.67 ^{ns}
2	11.66±1.04	11.99±1.26	11.22±1.25	0.90 ^{ns}
3	11.51±0.80	12.15±1.45	11.31±1.14	0.87 ^{ns}
4	10.95±0.86	11.86±1.26	10.89±1.02	0.78 ^{ns}
5	11.19±0.87	11.72±1.24	11.08±0.95	0.90 ^{ns}
6	11.32±0.97	11.33±1.22	11.02±1.02	0.97 ^{ns}
Mean±S.E.	11.32±1.08	11.89±1.11	11.01±1.01	0.74 ^{ns}

¹Mean values are based on five replicates with S.E.; ns- non significant

The experimental design used in the study was completely randomised design. Data obtained on various parameters were analysed statistically (Snedecor and Cochran, 1994) and the comparison of means was done using IBM Statistical Production and Service Solutions (SPSS), version 24.0.

Results and Discussion

The per cent chemical composition of the ration fed to experimental lactating cows is shown in Table 2.

Milk production

Consolidated data on fortnightly average milk production and fortnightly average 4 per cent FCM yield of the lactating cows maintained on three treatments T1, T2 and T3 are given in Table 3 and 4, respectively. The average daily milk production of experimental

lactating cows maintained on dietary treatments T1, T2 and T3 were 12.22±1.12, 12.29±1.31 and 10.98±1.10 kg, respectively. Statistical analysis of the data revealed that there was no significant difference in average daily milk production of lactating cows maintained on the three dietary treatments. Tamura *et al.* (2018) observed that early lactating dairy cattle supplemented with 8g/day RPM top dressed on the TMR having 14.5per cent C.P did not have any significant effect on milk yield and both the groups had a milk production of around 40 kg/day. Similarly, Pawar *et al.* (2015) reported no significant difference in milk yield of crossbred lactating cows supplemented with 54 g/head/day RPC (16.93±1.57 kg/day) mixed with the basal ration when compared with the control (15.38±0.88 kg/day). These results were in accordance with those of the present study. Milk yield and 4 per cent FCM yield were not significantly improved in the above studies which may be because the

Table 5. Fortnightly average milk composition¹ of lactating cows maintained on three experimental rations, kg

Parameter	Treatment	Fortnights						Mean±S.E.
		1	2	3	4	5	6	
Fat (%)	T ₁	3.52 ^b ±0.08	3.49 ^b ±0.11	3.47 ^b ±0.13	3.48 ^b ±0.12	3.60 ^b ±0.17	3.63 ^b ±0.20	3.53 ^b ±0.14
	T ₂	3.71 ^b ±0.17	3.65 ^b ±0.15	3.91 ^b ±0.27	3.81 ^b ±0.18	3.80 ^b ±0.11	3.77 ^b ±0.08	3.78 ^b ±0.18
	T ₃	4.01 ^a ±0.16	3.98 ^a ±0.13	3.94 ^a ±0.12	3.97 ^a ±0.12	4.10 ^a ±0.17	4.18 ^a ±0.24	4.03 ^a ±0.15
SNF (%)	T ₁	8.00 ±0.10	7.81 ^b ±0.07	7.71 ±0.02	7.75 ^b ±0.06	7.85 ±0.06	7.76 ±0.11	7.81 ^b ±0.07
	T ₂	8.19 ±0.18	8.03 ^{ab} ±0.09	7.88 ±0.21	7.77 ^{ab} ±0.08	7.78 ±0.09	7.69 ±0.03	7.89 ^{ab} ±0.12
	T ₃	8.30 ±0.09	8.21 ^a ±0.06	8.06 ±0.02	7.96 ^a ±0.02	7.91 ±0.03	7.82 ±0.04	8.04 ^a ±0.04
Total solids (%)	T ₁	11.43 ^b ±0.05	11.51 ^b ±0.03	11.58 ^b ±0.18	11.46 ^b ±0.12	11.55 ^b ±0.16	11.54 ^b ±0.20	11.51 ^b ±0.07
	T ₂	11.68 ^b ±0.12	11.79 ^b ±0.07	11.68 ^b ±0.15	11.79 ^b ±0.16	11.59 ^b ±0.10	11.60 ^b ±0.07	11.68 ^b ±0.12
	T ₃	12.86 ^a ±0.19	12.71 ^a ±0.08	12.46 ^a ±0.09	12.39 ^a ±0.10	12.37 ^a ±0.13	12.43 ^a ±0.07	12.54 ^a ±0.10
Protein (%)	T ₁	2.93 ^b ±0.04	2.85 ±0.03	2.80 ^b ±0.02	2.84 ^b ±0.03	2.83 ±0.03	2.83 ±0.04	2.85 ^b ±0.04
	T ₂	2.94 ^b ±0.02	2.89 ±0.02	2.84 ^b ±0.03	2.86 ^b ±0.01	2.86 ±0.02	2.82 ±0.01	2.87 ^b ±0.03
	T ₃	3.04 ^a ±0.03	2.93 ±0.05	2.93 ^a ±0.02	2.92 ^a ±0.02	2.91 ±0.02	2.84 ±0.03	2.92 ^a ±0.04
MUN (mg/dL)	T ₁	13.63 ±0.10	13.74 ±0.09	13.82 ±0.08	14.06 ±0.11	14.15 ±0.12	14.31 ±0.14	13.95 ±0.07
	T ₂	13.11 ±0.23	13.23 ±0.26	13.30 ±0.22	13.54 ±0.32	13.63 ±0.31	13.78 ±0.30	13.43 ±0.22
	T ₃	11.98 ±0.17	12.09 ±0.19	12.17 ±0.22	12.41 ±0.17	12.50 ±0.23	12.65 ±0.22	12.30 ±0.24

^{a,b}Values in the columns bearing different superscripts differ significantly ($p < 0.05/0.01$)

animals selected for the experiment were only medium producers rather than high yielders.

Milk composition

Data on composition of milk collected fortnightly from the lactating cows maintained on the three experimental rations are shown in Table 5.

The average milk fat (%) content in milk from animals fed on the three dietary treatments T₁, T₂ and T₃ were 3.53±0.14, 3.78±0.18 and

4.03±0.15 per cent, respectively. The per cent fat in milk was higher ($p < 0.05/0.01$) in T₃ when compared to T₁ and T₂. However, no significant differences ($p > 0.05$) were observed between T₁ and T₂. Titi *et al.* (2013) conducted studies on Holstein heifers fed with TMR containing 14 or 16 per cent CP supplemented with 0, 15 or 25 g/day RPM and they observed a significantly higher milk fat content in the group fed with the ration containing 14 per cent crude protein with 25g/day RPM (3.61±0.01 per cent) compared with the non-supplemented group and the supplemented group with higher CP in ration

and the values were 3.42 ± 0.01 per cent and 3.55 ± 0.01 per cent for the non-supplemented group and the supplemented group with higher CP in ration, respectively.

The improvement in milk fat content may be due to the role of methionine in increasing the *de novo* synthesis of both short and medium chain fatty acids in the mammary gland as well as choline, both of which are essential for the synthesis of phospholipids that are in turn, required for the synthesis of chylomicrons and very low-density lipoproteins.

The average milk solids not fat (SNF) content of milk from animals maintained on the three dietary treatments T₁, T₂ and T₃ were 7.81 ± 0.07 , 7.89 ± 0.12 and 8.04 ± 0.04 per cent, respectively. The SNF content (%) of milk was higher ($p < 0.05/0.01$) in T₃ when compared to T₁, while no significant differences ($p > 0.05$) were observed between T₁ and T₂ and, T₂ and T₃ in this regard. Ahmed *et al.* (2016) observed that the supplementation of RPM@ 7g/day RPM in combination with 20g/day RPL had significantly improved milk SNF content (9.91 ± 0.07 per cent) when compared to the control (9.51 ± 0.16 per cent) in early lactating Nili Ravi buffaloes. Similarly, Rahmani *et al.* (2014) concluded that feeding TMR top dressed with 90g/day RPC to early lactating Holstein cows had significantly improved the SNF content of milk (8.87 ± 0.05 per cent) when compared to the control (8.67 ± 0.04 per cent). These results were in accordance with the results obtained in the present study.

The average milk total solids (%) content of milk were 11.51 ± 0.07 , 11.69 ± 0.12 and 12.54 ± 0.10 per cent in T₁, T₂ and T₃ groups, respectively. The average total solids (%) in milk was higher in T₃ when compared to T₁ and T₂, while no significant difference ($p > 0.05$) was observed between T₁ and T₂ groups. The increase in milk total solids resulted from a combined increase in fat and protein concentration. Sheikh *et al.* (2014) reported a significantly higher milk total solids content (13.54 ± 0.15 per cent) in Karan Fries lactating dairy cows on supplementation with rumen protected methionine and choline @7 and 60g/day, respectively along with the basal ration when compared with the control (12.92 ± 0.08 per cent).

The average milk protein content in milk was 2.85 ± 0.04 , 2.87 ± 0.03 and 2.92 ± 0.04 per cent in T₁, T₂ and T₃ groups, respectively. The average milk protein content increased significantly ($p < 0.05/0.01$) in T₃ when compared to T₁ and T₂, while no significant difference ($p > 0.05$) was observed between T₁ and T₂. These findings were in accordance with those made by Davidson *et al.* (2008), who observed a significantly higher milk protein content (2.77 ± 0.06 per cent) in early lactating dairy cows upon supplementation with RPM and RPC @ 40 and 45g/day, respectively top dressed on the TMR having 17.6 per cent CP when compared to the control (2.60 ± 0.06 per cent). Choline is a source of methyl groups and it also acts as a methyl donor in transmethylation reactions which may be the reason for the improvement in the milk protein content in the supplemented groups.

The average milk urea nitrogen (MUN) (mg/dL) content in milk from animals maintained on the three dietary treatments T₁, T₂ and T₃ were 13.95 ± 0.07 , 13.43 ± 0.22 and 12.30 ± 0.24 mg/dL, respectively. Statistical analysis of the data revealed no significant difference ($p > 0.05$) in MUN content of the milk of lactating cows maintained on three different dietary treatments. The present observations were similar to those made by Zhou *et al.* (2016) in transition dairy cows where the authors reported that dairy cows supplemented with RPM (0.08% DM of TMR) and 60g/day RPC didn't show any significant difference (12.82 ± 0.40 mg/dL) in milk urea nitrogen content when compared with the control (12.65 ± 0.40 mg/dL).

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Influence of different levels of dietary protein on serum biochemical parameters of dairy cows during the transition period



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Abstract

A study was carried out to find the influence of different dietary protein levels on serum biochemical parameters of dairy cows during the transition period. Twenty cross bred cows, three weeks prior to their expected date of calving were selected from University Livestock Farm and Fodder Research and Development Scheme (ULF&FRDS), College of Veterinary and Animal Sciences, Mannuthy as experimental animals. The cows were randomly allotted to two dietary treatments – T1 (12 per cent CP (crude protein) TMR (Total Mixed Ration)) and T2 (14 per cent CP TMR). After calving, half of the animals in T1 were allotted to T3 (16 per cent CP TMR) and remaining half to T4 (18 per cent CP TMR). Similarly half of the animals in T2 were allotted to T3 and remaining half to T4. All these rations were iso-caloric. The feeding trial was carried out for a period of 3 weeks prepartum and 45 days postpartum. The serum biochemical parameters such as serum glucose levels were significantly higher ($p < 0.05$) for groups receiving treatments T2 & T4 (84.87 ± 2.64 mg/dL) and total protein levels were significantly lower for groups receiving treatments T1 and T3 and the levels of all other biochemical parameters under observation remained unaffected in all treatment combinations, but the values of NEFA (non-esterified fatty acids) were below the normal range. Furthermore, there was no occurrence of metabolic diseases in any of the treatment groups, indicating that dietary treatments were sufficient to meet the requirement of the animals. Dietary protein levels did not have any significant effect on serum metabolites studied in the present work.

Key words: Total mixed ration, Transition period, Dietary protein

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The transition period in dairy cows is defined as the time period from three weeks prior to parturition to three weeks after parturition. Animals in the transition period require nutrients for maintenance, foetal growth and production. Judicious feeding during the prepartum period will ensure sufficient body reserves to meet the high nutrient demands of early lactation (Goff and Horst, 1997).

Protein and energy are the two nutrients that are critical during the transition period. Even though much work has been done to address the negative energy balance during the transition period, research regarding negative protein balance are scanty. During the transition period and early lactation, dry matter intake will not be sufficient enough to meet the requirements, leading to onset of metabolic disorders. Serum biochemical parameters during this period could be used as indicators for the detection of metabolic disorders (Roche, 2013). In such a scenario, the metabolic responses to different levels of dietary protein in transition cows and influence of dietary protein levels in pre and postpartum crossbred cows were evaluated in the present study.

Materials and methods

This study was conducted at the University Livestock Farm and Fodder Research

Station (ULF & FRDS), College of Veterinary and Animal Sciences, Mannuthy, Kerala, India.

Preparation of total mixed rations

Four types of total mixed rations were formulated using commonly used ingredients along with paddy straw as a source roughage.

The four experimental rations were: T1 - 12 per cent CP TMR, T2 - 14 per cent CP TMR, T3 - 16 per cent CP TMR and T4 - 18 per cent CP TMR. The chemical composition of each of the four rations used are presented in the Table.1

Feeding trial

Twenty dairy cows, around 325-340 kg body weight with 11-12 L of average milk production in previous lactation, three weeks prior to their expected date of calving were selected and randomly assigned to two groups in a completely randomised block design and were then randomly allotted to dietary treatments, T1 and T2. After calving, five animals from each group were allotted to T3 and the remaining five to T4 making four experimental groups. The feeding trial was carried out for a period of 3 weeks before the expected date of calving and 45 days after calving. All the experimental animals were fed as per standards (ICAR, 2013)

Table 1. Ingredient composition of paddy straw based TMR offered to experimental animals maintained on four dietary treatments

Ingredients	T1(kg)	T2(kg)	T3(kg)	T4(kg)
Maize	27	20	20	14
Rice Polish	9	10	9	10
Tapioca Starch waste	4	4	4	2
De-oiled rice bran	7	10	8	12
CGF	10	13	9	15
Coconut Cake	5	5	9	9
Alfalfa	5.5	8.5	14	15
Straw	31	28	24	20
Calcite	0	0	1.5	1.5
Salt	0.5	0.5	0.5	0.5
Mineral Mixture	1	1	1	1
Total	100*	100*	100*	100*

*To every 100 kg of complete feed, 10g of Vitamin AD3E supplement (containing 10,00,000 I.U of Vitamin A, 2,00,000 I.U of Vitamin D3 and 1,00,000 I.U of Vitamin E) were added

Biochemical Analysis

Blood samples were collected from all the experimental animals on the 14th day before the expected date of calving, on the day of calving and on the 21st day after calving. Serum samples were analysed for total protein (Jong and Veeter, 1950), albumin (Bromocresol green method), glucose (GODPAP methodology), Non esterified fatty acids (NEFA) and beta hydroxy butyrate (BHB) (Hosaka *et al.* 1981) using standard kits.

Results and discussion

Prepartum biochemical profile

Biochemical parameters of the experimental animals recorded two weeks before the expected date of calving and on the day of calving are listed in Table 2

Serum glucose levels of the animals fed with T1 and T2 were 75.18 ± 3.62 & 84.89 ± 2.11 mg/dL and 63.81 ± 3.06 & 67.92 ± 2.19 mg/dL, respectively at two weeks prior to the expected date of calving and on the day of calving. There was a decrease in serum glucose levels in both groups on the day of calving. This could be due to reduced feed intake at the time of calving. A decreased serum glucose level (46.33 mg/dL) at calving was also reported by Abdel *et al.* (2016). Valiente (2018) observed elevated serum glucose levels 20 days prior to calving.

Serum total protein levels for the groups receiving treatments T1 and T2 before calving were 7.40 ± 0.14 and 7.62 ± 0.24 mg/dL respectively and those on the day of calving were 8.61 ± 0.39 and 8.90 ± 0.27 mg/dL respectively. Farahani *et al.* (2017) also reported that the serum total protein levels of 7.06 mg/dL in animals fed conventionally.

Table 2. Biochemical parameters of experimental cows maintained on two experimental diets two weeks before and at calving

Parameter ¹	2 weeks before calving			At calving		
	T1	T2	P value	T1	T2	P value
Serum glucose (mg/dL)	75.18 ± 3.62	84.89 ± 2.11	0.08	63.81 ± 3.06	67.92 ± 2.19	0.02
Total protein (g/dL)	7.40 ± 0.14	7.62 ± 0.24	0.04	8.61 ± 0.39	8.90 ± 0.27	0.18
Albumin (g/dL)	4.28 ± 0.07	4.29 ± 0.15	0.97	3.71 ± 0.13	3.68 ± 0.15	0.88
NEFA (ng/dL)	0.09 ± 0.02	0.13 ± 0.01	0.01	0.18 ± 0.01	0.17 ± 0.01	0.97
BHBA (ng/dL)	0.28 ± 0.01	0.30 ± 0.01	0.81	0.31 ± 0.01	0.32 ± 0.01	0.82

¹Mean values are based on ten replicates with SE

Table 3. Biochemical parameters of experimental cows maintained on two experimental diets after 21 days of calving

Parameter ¹	Treatment combination				P value
	T1T3	T1T4	T2T3	T2T4	
Serum glucose (mg/dL)	79.05 ± 1.25^{ab}	74.37 ± 2.16^b	78.03 ± 1.98^b	84.87 ± 2.64^a	0.019
Total protein (g/dL)	7.30 ± 0.17^b	8.88 ± 0.36^a	8.61 ± 0.39^a	8.90 ± 0.27^a	0.006
Albumin (g/dL)	4.28 ± 0.15	4.14 ± 0.09	3.82 ± 0.24	4.17 ± 0.30	0.886
NEFA (ng/dL)	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.860
BHBA (μg/dL)	0.31 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.29 ± 0.01	0.832

¹Mean values are based on five replicates with SE

Mean \pm SE of different treatment having different alphabets (a-b) as superscripts differs significantly with in a row at $p < 0.05$

Albumin levels for the groups receiving treatments T1 and T2 before calving were 4.28 ± 0.07 and 4.29 ± 0.15 g/dL respectively and those on the day of calving were 3.71 ± 0.13 and 3.68 ± 0.15 g/dL respectively. A decrease in albumin could be associated with an increase in globulin. Globulin is known as a positive acute phase protein and its liver synthesis is commonly increased during the inflammatory condition (Bertoni *et al.*, 2008). Higher globulin may be associated with some degree of inflammation during calving. These values are in accordance with those reported by Seifi *et al.* (2005).

The NEFA levels in cows receiving 12 per cent CP prepartum increased from 0.09 ± 0.02 two weeks before calving to 0.18 ± 0.01 ng/dL at the day of calving while in those animals receiving 14 per cent CP, it increased from 0.13 ± 0.01 to 0.17 ± 0.01 ng/dL and these changes were similar between the groups. The increased NEFA levels on the day of calving could be due to fat mobilization, but the increase was not up to the level for causing a negative energy balance. This could be because, the feed given was sufficient to meet energy requirements. These values were in agreement with those of Ospina *et al.* (2010) and who observed pre partum NEFA concentrations of 0.27 mEq/L. Similar increase in NEFA levels (0.17 to 0.29 mmol/L) on the day of calving was also observed by Huzzey *et al.* (2011). In contrast, Perumbilly *et al.* (2019) observed a higher value for NEFA (0.702 ± 0.18 mmol/L) during two weeks before calving in cross bred dairy cows.

BHB levels were similar during the two weeks before and on the day of calving for all treatment groups. In contrast to values observed in this study, BHBA levels of 5.73 and 6.42 mg/dL for primigravid and multiparous cows fed during the prepartum period with moderate protein diet (12.7% CP, 36% rumen undegradable protein) was reported by Santos *et al.* (2001).

A perusal of serum biochemical parameters before and on the day of calving indicated that dietary protein levels had no significant effect on serum metabolites either

two weeks prior to calving or on the day of calving.

Post partum biochemical profile

Biochemical parameters of experimental animals recorded after 21 days of calving are listed in Table 3

Serum glucose levels for the groups receiving treatment combinations T1T3, T1T4, T2T3 and T2T4 were 79.05 ± 1.25 , 74.37 ± 2.16 , 78.03 ± 1.98 and 84.87 ± 2.64 mg/dL respectively. The animals receiving T2 and T4 had higher serum glucose levels. Glucose levels of 3.03 and 3.23 mmol/L after 11 and 21 days of calving (Reynolds *et al.*, 2003) and 55 to 70 mg/dL after 1 week of calving (Zaworski *et al.*, 2014) were reported for dairy cows.

Total protein levels for the groups receiving treatment combinations T1T3, T1T4, T2T3 and T2T4 were 7.30 ± 0.17 , 8.88 ± 0.36 , 8.61 ± 0.39 and 8.90 ± 0.27 respectively. Albumin levels for the groups receiving treatment combinations T1T3, T1T4, T2T3 and T2T4 were 4.28 ± 0.15 , 4.14 ± 0.09 , 3.82 ± 0.24 and 4.17 ± 0.30 g/dL. Total protein levels were significantly lower for groups receiving treatments T1 and T3. In contrast, higher serum total protein levels (7.07 mg/dL) were reported by Farahani *et al.* (2017).

NEFA levels for the groups receiving treatment combinations T1T3, T1T4, T2T3 and T2T4 were 0.12 ± 0.01 , 0.13 ± 0.01 , 0.12 ± 0.01 and 0.13 ± 0.01 ng/dL. Low NEFA levels indicated an absence of negative energy balance after calving. In contrast to this observation, higher levels of NEFA concentration (were 0.59 mmol/L) in dairy cows 25 days after calving was reported by De Souza *et al.* (2019).

BHBA levels for the groups receiving treatment combinations T1T3, T1T4, T2T3 and T2T4 were 0.31 ± 0.01 , 0.28 ± 0.01 , 0.32 ± 0.01 and 0.29 ± 0.01 μ g/dL respectively which were lower than the values reported by Fiore *et al.* (2017) and Perumbilly *et al.* (2019). This clearly indicates that the energy requirement for the animals was met from the feed given.

Levels of all biochemical parameters, except total protein (lowest in T1 and T3) and serum glucose (highest in T2 and T4), under observation remained unaffected in all treatment combinations, but the values of NEFA (Non esterified fatty acids) were below the normal range. There was no interaction between pre- and postpartum CP levels on the serum concentrations of total protein ($P = 0.68$), albumin ($P = 0.80$), globulin ($P = 0.82$), or albumin:globulin ratio ($P = 0.67$) between the treatment groups.

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Assessment of quality in specific fractions of Large White Yorkshire boar semen



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Abstract

Boar semen is voluminous and ejaculated as jets or fractions of pre-sperm, sperm rich (SRF) and post-sperm rich fractions. Recent studies have reported more resilient characteristics of sperm in initial portions of SRF towards cold shock and cryopreservation. The present study was conducted to assess the quality of specific fractions of SRF, namely, first 10mL of SRF (F1) and rest of SRF (F2) in Large white Yorkshire (LWY) boar semen. Ejaculates were collected using gloved-hand technique and were subjected to quality assessments of volume, pH, sperm progressive motility, concentration, plasma membrane integrity, abnormality, acrosome integrity and sperm membrane cholesterol. Upon statistical analysis, significant differences were noticed in volume, pH, sperm concentration and sperm membrane cholesterol between fractions of the ejaculate.

Keywords: Boar semen, sperm rich fraction, F1, F2

Qualities like better-feed conversion efficiency, early maturity, short generation interval, high fecundity, relatively smaller capital investment and a faster economic return to the farmers makes commercial piggery lucrative to farmers. For a sustainable livestock based industry, genetic enhancement is inevitable, towards which artificial insemination (AI) has played a significant role.

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The process of cryopreservation has aided in realising the full potential of AI programmes. Unfortunately, boar semen have not been as resilient as other species like bull, leading to reduced fertility with cryopreserved boar semen and hence lack of popularity of AI. This has affected the breeding and genetic enhancement of the species as not been as successful as in other species like bulls. One of the major reasons for poor boar sperm cold shock resistance is its low cholesterol to phospholipid ratio of 0.26 (Parks and Lynch, 1992).

Boar semen is voluminous and ejaculated as jets in broadly three fractions as (i) pre-sperm (ii) the SRF and, (iii) the post-sperm-rich (PSRF) which differ in sperm concentration and proportion of accessory sex gland secretions (Mann and Luwak-Mann, 1981). Recent studies have reported that the sperm fortuitously in F1 were resilient to cooling, freezing and thawing because of its lower bicarbonate level (leading to better sperm membrane cholesterol content) and presence or absence of specific proteins. Hence a study was carried out to assess quality of boar semen fractions (F1 and F2).

Materials and methods

Four adult Large White Yorkshire (LWY) boars aged 18-24 months reared at the Centre for Pig Production and Research, KVASU, Mannuthy were used for the study. A total of 66 semen ejaculates were collected using gloved hand technique at twice a week frequency with an interval of three to four days between collections from the same boar. Ejaculates were collected as fractions, with the first 10 mL of SRF being designated as F1 and the remaining SRF as F2. During the collection, a Buchner funnel was used to separate the gel mass and the semen fractions were transferred to an insulated container for transportation to the laboratory for preliminary evaluation.

The fresh semen collected was evaluated for volume, pH and concentration for 66 ejaculates. However, sperm progressive motility, plasma membrane integrity and abnormality, acrosome integrity and sperm membrane cholesterol content were evaluated

for 26 ejaculates. The volume of semen fractions were assessed using a graduated test tube for F1 and a class A glass cylinder for F2. The pH of the fractions was assessed using pH meter (CyberScan pH510, Eutech instruments). For progressive motility assessment, 25 µL of 1:10 diluted semen (with PBS) was taken on a clean, grease free glass slide, covered with a clean cover slip and examined under 400× magnification of a phase contrast microscope (Olympus, Model: Magnus MLX, India) with bio-therm stage facility maintained at 37°C. Semen samples with a minimum of 70 per cent sperm progressive motility alone were selected for further analysis. The sperm concentration of each fraction of boar semen ejaculate was determined by using a Neubauer counting chamber as per Salisbury *et al.* (1985). The sperm plasma membrane integrity was assessed using SYBR-14/PI (Live/dead® sperm viability kit L7011 Invitrogen, Thermo Fisher Scientific, USA) staining as per De Ambrogi *et al.* (2006). Sperm fluorescing bright green with SYBR-14 were considered live, while those stained red with PI were considered dead. The morphological abnormalities of sperm in each fraction were assessed using eosin-nigrosin staining as per Campbell *et al.* (1953). The sperm acrosome integrity of sperm in each fraction was assessed by Giemsa staining technique as described by Watson (1975). Sperm membrane cholesterol content in each fraction was assessed as per Zlatkis *et al.* (1953) after harvesting live sperm with Percoll density gradient (Sigma Aldrich, USA). The washed pellets were resuspended in BTS and stored at -70°C until used for estimation of cholesterol. At the time of estimation of cholesterol, pellet of approximately 1000×10^6 sperm were taken in a 15 mL centrifuge tube and cholesterol was extracted by vortexing with 20 volumes of chloroform: methanol (1:1 V/V) mixture. The mixture was centrifuged at $800 \times g$ for 5 min and the supernatant evaporated to dryness under an atmosphere of nitrogen. At the time of estimation, the dried cholesterol was dissolved in one mL NP 40: isopropanol mixture (ratio 1:9) and its concentration estimated by cholesterol assay kit (Agappe Diagnostics limited, India; Product No: 11403002, Product No: 11403002).

Results and discussion

As F1 was defined as initial 10 mL of SRF, volume of F2 alone was analysed statistically. It was observed that F2 volume was significantly higher ($p < 0.05$) for boar 4 (Table 1). Siqueira *et al.* (2011) had recorded the volume of F1 as 10.74 ± 0.25 and rest of SRF as 37.37 ± 5.73 mL.

The overall mean pH of both F1 and F2 were found to be on the alkaline side (7.22 ± 0.03 and 7.41 ± 0.03). The pH was significantly ($p < 0.01$) lower in F1 than F2 (Table 2). Variations in pH among different fractions of same ejaculate for animals with a fractionated ejaculation were reported (Frunza *et al.*, 2008). The difference being ascribed to the contributions of accessory gland secretions. The lower pH of F1 could be due to the higher amount of epididymal fluid and lower proportion

of bicarbonate containing accessory sex gland secretions (Saravia *et al.*, 2010).

In the 66 ejaculates studied, overall mean sperm concentration in F1 and F2 was $1161.06 \pm 71.01 \times 10^6/\text{mL}$ and $706.67 \pm 51.75 \times 10^6/\text{mL}$, respectively (Table 2). The concentration was significantly higher in F1 when compared to F2 ($p < 0.01$). This is expected as F1 represents the initial portions of SRF, which has not been diluted with the accessory gland secretions (Saravia, 2008).

The other parameters were assayed for 26 numbers of ejaculates. The overall sperm progressive motility did not differ significantly between F1 and F2 (83.08 ± 0.96 and 81.35 ± 0.94 %, respectively, table 3). Pena *et al.* (2003) had also reported a lack of significant difference in progressive motility of sperm in F1 and F2 in Swedish Yorkshire boars. Kumaresan

Table 1. Semen volume (Mean \pm SE) in different fractions of Large white Yorkshire boar fresh semen ejaculate

Semen characteristics	Boar	F1	F2	F value (p value) for F2 Between boars
Semen Volume (mL)	1 (n=16)	9.90 ± 0.18	30.50 ± 2.76^a	2.776* (0.049)
	2 (n=17)	10.75 ± 0.32	30.18 ± 3.75^a	
	3 (n=16)	10.99 ± 0.52	27.50 ± 2.75^a	
	4 (n=17)	10.96 ± 0.29	41.68 ± 5.19^b	
	Overall (n=66)	10.66 ± 0.17	32.57 ± 1.98	

F1 - first 10 mL of sperm rich fraction; F2 - rest of the sperm rich fraction

ns - non significant; ** - significant at 0.01 level

Table 2. Hydrogen ion concentration and sperm concentration (Mean \pm SE) in different fractions of Large white Yorkshire boar fresh semen ejaculate

Semen characteristics	Boar	F1	F2	t - value (p - value) Between fractions
Hydrogen ion concentration	1 (n=16)	7.23 ± 0.04	7.36 ± 0.04	5.092** (0.000)
	2 (n=17)	7.30 ± 0.04	7.50 ± 0.07	
	3 (n=16)	7.21 ± 0.04	7.40 ± 0.05	
	4 (n=17)	7.14 ± 0.07	7.36 ± 0.05	
	Overall (n=66)	7.22 ± 0.03	7.41 ± 0.03	
Sperm concentration (millions/mL)	1 (n=16)	1281.88 ± 153.26	772.50 ± 108.44	5.172** (0.000)
	2 (n=17)	888.24 ± 107.08	550.00 ± 75.25	
	3 (n=16)	1433.75 ± 184.64	918.75 ± 145.45	
	4 (n=17)	1063.53 ± 82.31	601.76 ± 49.04	
	Overall (n=66)	1161.06 ± 71.01	706.67 ± 51.75	

Table 3. Sperm progressive motility, Sperm plasma membrane integrity (using SYBR-PI), abnormality and acrosome integrity and sperm cholesterol content (Mean \pm SE) in different fractions of Large white Yorkshire boar fresh semen ejaculate, expressed in per cent

Semen characteristics	Boar (n=3 each)	F1	F2	t – value (p - value) Between groups
Progressive Motility (in per cent)	1 (n=7)	85.71 \pm 2.02	82.86 \pm 2.14	1.284 ^{ns} (0.21)
	2 (n=7)	82.14 \pm 1.84	81.43 \pm 2.10	
	3 (n=6)	85.00 \pm 1.29	83.33 \pm 1.05	
	4 (n=6)	79.17 \pm 1.54	77.50 \pm 1.12	
	Overall (n=26)	83.08 \pm 0.96	81.35 \pm 0.94	
Plasma membrane integrity by SYBR14 - PI	1 (n=7)	86.81 \pm 2.40	85.86 \pm 2.24	0.796 ^{ns} (0.430)
	2 (n=7)	83.36 \pm 1.20	83.65 \pm 1.74	
	3 (n=6)	89.02 \pm 1.75	86.93 \pm 1.97	
	4 (n=6)	84.08 \pm 1.94	82.15 \pm 1.57	
	Overall (n=26)	85.76 \pm 1.00	84.66 \pm 0.97	
Sperm abnormality	1 (n=7)	4.29 \pm 0.51	5.50 \pm 0.73	0.984 ^{ns} (0.330)
	2 (n=7)	8.21 \pm 0.26	8.21 \pm 0.82	
	3 (n=6)	5.83 \pm 1.19	6.17 \pm 1.54	
	4 (n=6)	7.17 \pm 0.67	8.33 \pm 0.65	
	Overall (n=26)	6.37 \pm 0.45	7.04 \pm 0.52	
Acrosome Integrity	1 (n=7)	93.36 \pm 0.96	91.07 \pm 1.22	1.587 ^{ns} (0.120)
	2 (n=7)	89.57 \pm 1.13	88.86 \pm 1.07	
	3 (n=6)	89.83 \pm 1.31	88.08 \pm 1.56	
	4 (n=6)	91.50 \pm 1.74	90.08 \pm 1.79	
	Overall (n=26)	91.10 \pm 0.68	89.56 \pm 0.70	
Sperm membrane cholesterol (μg/billion sperm)	1 (n=7)	114.61 \pm 3.16	99.60 \pm 3.71	6.319 ^{**} (0.000)
	2 (n=7)	116.10 \pm 2.13	104.99 \pm 1.80	
	3 (n=6)	116.13 \pm 3.58	104.81 \pm 3.95	
	4 (n=6)	129.09 \pm 2.60	105.65 \pm 3.15	
	Overall (n=26)	118.71 \pm 1.77	103.65 \pm 1.59	

F1 - first 10 mL of sperm rich fraction

F2 - rest of the sperm rich fraction

ns – non significant, ** - significant at 0.01 level

et al. (2011) used CASA to analyse sperm progressive motility in F1 and F2 of boar semen and found no variation in sperm progressive motility of the two fractions.

The overall sperm plasma membrane integrity in F1 and F2 were not significantly different with values 85.76 ± 1.00 and 84.66 ± 0.97 percent, respectively (table 3). SYBR-14/PI has been advocated for identification of viable sperm in different domestic animals

including swine, (Garner and Johnson, 1995). Saravia (2008) could not find any difference in sperm viability between the two fractions.

Similar observations were made with sperm abnormality and acrosome integrity too, as sperm in both F1 and F2, no significant differences could be appreciated (table 3). The etiology of sperm abnormality can vary widely, including hereditary causes, improper handling and storage of semen, genetic causes, etc.

(Cerovsky *et al.*, 2005). Lopez Rodriguez *et al.* (2012) suggested the importance of an intact acrosome in penetration of the oocyte and thus its role in fertilisation process. Both Wilson (2018) and Shylesh (2019) used the Giemsa staining technique to evaluate sperm acrosome integrity in LWY fresh semen ejaculates and recorded acrosome integrity of 90.63 ± 1.03 and 91.81 ± 0.86 per cent, respectively.

Though there were no significant differences in sperm parameters except concentration between F1 and F2, the sperm membrane cholesterol content in F1 and F2 fractions differed significantly ($p < 0.01$, $118.71 \pm 1.77 \mu\text{g}/10^9$ sperm in F1 and $103.65 \pm 1.59 \mu\text{g}/10^9$ sperm in F2). The basis of the higher cholesterol content in F1 or rather lower cholesterol content in sperm of F2 could be ascribed to the higher bicarbonate in F2 as recorded by Saravia (2008). The epididymal spermatozoa, post-exposure to high bicarbonate levels and specific proteins, undergo phospholipid scrambling at the apical region (Gadella and Harrison, 2002). The scrambling of phospholipids leads to cholesterol relocation at the sperm head, which precedes cholesterol extraction before capacitation. Leahy and Gadella (2015) suggested the role of bicarbonate and calcium ions in bringing about functional changes in the sperm lateral distribution of cholesterol and its efflux in the presence of albumin and enhanced membrane fluidity. In fresh ejaculates of LWY boar semen, Shylesh (2019) found average sperm cholesterol content of $110.98 \pm 1.64 \mu\text{g}/\text{billion}$ sperm, which ranged from 101.13 to 119.36 $\mu\text{g}/\text{billion}$ sperm.

Thus, there were no apparent changes brought about by the lower levels of sperm membrane cholesterol of F2 at fresh semen stage. However, the sperm of F1 might be more resistant to cold shock and stress of cryopreservation because of better cholesterol content. Hence, future cryopreservation studies could focus on utilizing sperm of F1 fraction, which had significantly high concentration (avoiding the need for centrifugation) and higher cholesterol content (thus better cryo-tolerance).

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Gross and histopathological lesions associated with tuberculosis in two sloth bears (*Melursus ursinus*) in India

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Abstract

Post-mortem examination of two sloth bears which died in Bannerghatta Bear Rescue Centre, Bengaluru, Karnataka, were performed. Both the animals were anorectic and had considerable weight loss before death. Representative lung tissue samples were subjected to histopathology and staining. The lung tissues of the animals had diffuse congestion and subpleural petechial hemorrhages. In addition, small nodules of varied diameters were seen scattered on the lung lobes of both animals. On histopathological examination, the lung tissue of one of the animals showed extensive proliferation of blood vessels. Congestion and subpleural hemorrhages were seen in both cases. Few macrophages and epithelioid cells were seen scattered adjacent to a bronchiole. Kinyoun's acid fast staining of the histological sections revealed numerous acid fast bacilli indicative of tuberculosis.

Key words: Tuberculosis, sloth bear, post-mortem

Sloth bear (*Melursus ursinus*) is one of the four species of bears which are found in India. They are classified as vulnerable species according to the International Union for Conservation of Nature and included in schedule I of the Indian Wildlife Protection Act, 1972. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* is a significant cause of morbidity and mortality in both wild

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and domestic animals worldwide (Lecu and Ball, 2011). Emergence of tuberculosis in sloth bears could be due to the spillover of *Mycobacterium tuberculosis* into this species because of their prolonged contact with humans. A significant number of sloth bears were poached by a community of people known as the Kalandars, and trained for street shows. There is inadequacy of diagnostic assays for detecting tuberculosis in these animals. Moreover, the affected bears appear apparently healthy and exhibit only mild or vague clinical signs in the terminal stages, which make diagnosis and treatment difficult. Hence diagnosis of tuberculosis in this species during post-mortem examination is important for considering preventive measures in live animals against TB.

Materials and methods

Two sloth bears, which died naturally at Bannerghatta Bear Rescue Centre, formed the sample of the study. Both the bears exhibited anorexia, isolation from the group and considerable weight loss before death. A thorough post-mortem examination of the animals was carried out and representative samples from lungs were collected in 10 per cent neutral buffered formalin for histopathology.

The formalin fixed samples were washed overnight, processed, cut at five micron thickness and stained with haematoxylin and eosin as per Suvana *et al.*, 2012. Special staining technique, Kinyoun's acid fast staining, was done to demonstrate the presence of the acid fast bacteria. The staining procedure is briefed below.

The tissue sections were deparaffinized and hydrated to distilled water after which it was immersed in Kinyoun's carbol fuchsin and incubated at 50°C for one hour. The slides were differentiated in two changes of one per cent acid alcohol after washing in running tap water. Counterstaining was done using methylene blue solution and the slides were dehydrated in 95 and 100 per cent alcohol sequentially and cleared in two changes of xylene. The stained sections, after drying completely, were mounted in DPX and viewed under oil immersion objective (100x) in a compound microscope.

Fluorescent staining technique using auramine was also performed to further confirm the presence of acid fast bacilli. Staining technique was done as per Suvana *et al.*, 2012 with slight modifications. The tissue sections were deparaffinized and hydrated to distilled water after which the slides were flooded with phenolic auramine – O and placed at 60°C for 10 minutes. It was then rinsed off with distilled water and decolourised using one per cent acid alcohol for 2 minutes. Counterstaining was done using Potassium permanganate solution for five minutes. The slides were then dehydrated in 95 and 100 per cent alcohol sequentially and cleared in two changes of xylene. The stained sections, after drying completely, were mounted in DPX and viewed under oil immersion objective (100X) of fluorescent microscope (Carl Zeiss®).

Results and discussion

On gross examination, the carcasses of both the animals appeared emaciated. Diffuse congestion along with subpleural petechial hemorrhages were evident in all lung lobes of the first animal (Fig. 1). This finding is in agreement with Pereira (2016) who also reported presence of areas of congestion and hemorrhage as post-mortem findings in TB affected sloth bears. Small nodules of two centimeter diameter were seen scattered on both lung lobes. The right cranial lobe showed two distinct dark red colored nodules of three to four centimeter diameter. This is in agreement with Hedau and Kamdi (2016) who reported the same in a sloth bear carcass suspected to have died of TB.

The second animal showed multiple nodules of two to three centimeter diameter scattered over the parenchyma of both left and right lung lobes (Fig. 2). In addition, left lung lobes had diffuse areas of congestion and mild edema. The present findings are in agreement with Hunter (2011) who reported that granulomas were characteristic lesions of primary TB in immune-competent individuals.

Microscopically, extensive proliferation of capillaries along with severe congestion and hemorrhages were found in the lung tissue sample of the first animal, suggestive

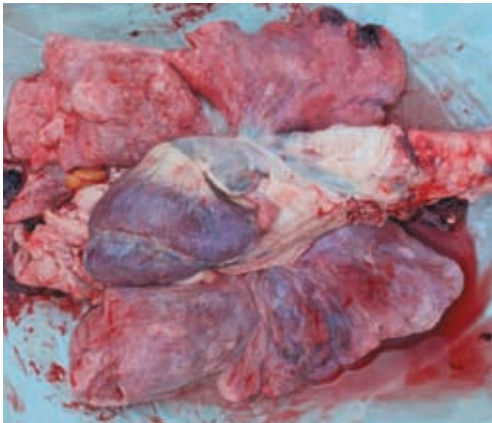


Fig. 1. Gross picture showing diffuse congestion and subpleural petechial haemorrhages in lung tissue

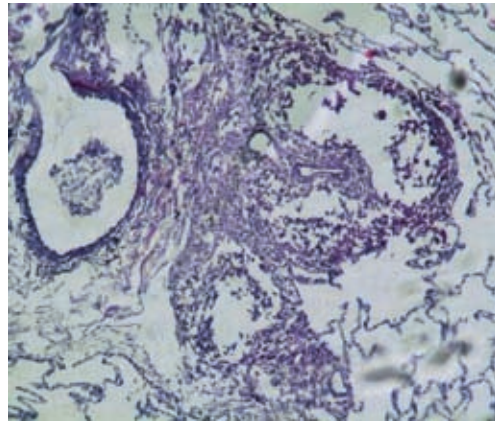


Fig. 4. Lung section with macrophages and epithelioid cells scattered adjacent to bronchiole, H and E (100x)

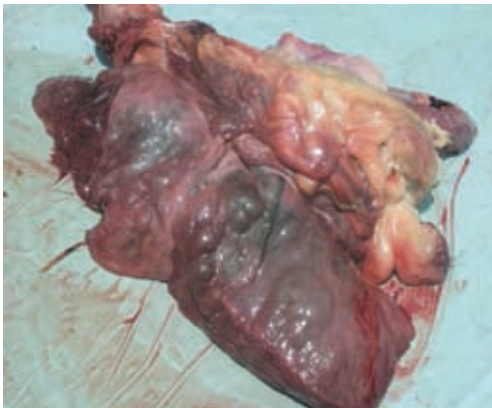


Fig. 2. Gross picture showing multiple nodular lesions in lung

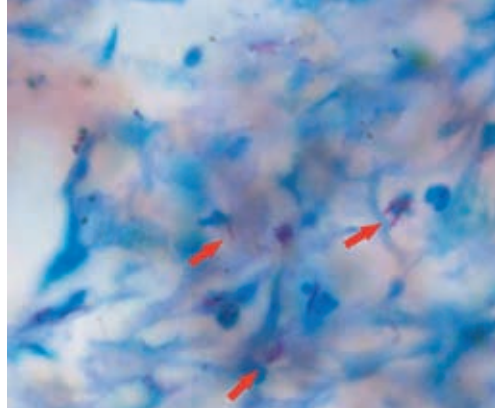


Fig. 5. Lung tissue with acid fast bacilli (arrow), Kinyoun's acid fast staining (1000x)

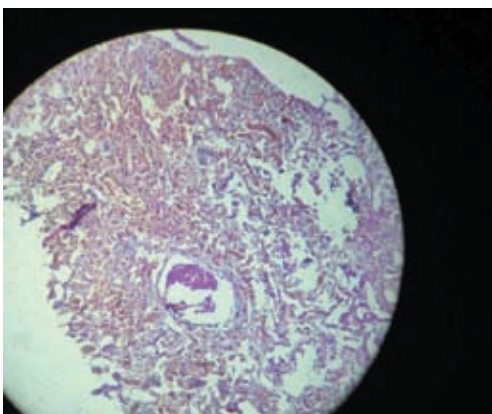


Fig. 3. Capillary proliferation with congestion and haemorrhage, H and E (100x)

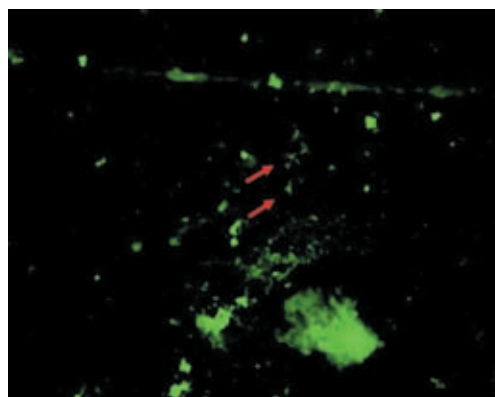


Fig. 6. Lung tissue with acid fast bacilli (arrow), Fluorescent staining (1000x)

of haemangioma (Fig. 3), whereas the lung tissue sample from second animal showed focal areas of macrophages and epithelioid cell proliferation adjacent to a bronchiole (Fig. 4). The typical tubercular granuloma with central caseous necrosis and surrounding inflammatory cells as described by Fefar *et al.* (2012) was not present in both the samples. However, Hernandez-Pando *et al.* (2000) had reported the presence of *M. tuberculosis* in lungs without typical histopathologic lesions of TB in case of human beings.

Both the tissue sections showed the presence of numerous acid fast bacilli when stained using Kinyoun's acid fast staining technique (Fig. 5). This is in agreement with Stephenson and Byard (2020) who reported that TB could be confirmed histologically by acid fast staining of suspected tissue sections. Rishikesavan *et al.* (2010) also confirmed TB in a captive male leopard by the presence of numerous acid fast bacilli in stained tissue impression smears.

Fluorescent staining for mycobacteria was also carried out in the tissue sections and both tissue samples showed the presence of numerous fluorescent acid fast bacilli when viewed under oil immersion objective (100X) of fluorescent microscope (Fig. 6). This is in agreement with Bodal *et al.* (2015) who reported that fluorescent staining was highly sensitive and can be used to confirm TB.

In the present study, both acid fast and fluorescent staining of histological sections showed identical results, which indicate that both these techniques are equally effective in confirming TB in post mortem samples. However, Kommareddi *et al.* (1984) reported that fluorescent staining using Auramine- O was simpler and had more sensitivity compared to Ziehl Neelsen (ZN) technique, at the same time, less specific than ZN technique.

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Seroprevalence of *Toxoplasma gondii* in aborted goats in Kerala



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Abstract

Toxoplasmosis, is a cosmopolitan zoonotic parasitic infection prevalent throughout the world affecting all warm blooded animals and man. A total of 72 serum samples from goats belonging to the organised, unorganised and small holder farms that had aborted recently or had a history of abortion were collected from the central districts of Kerala viz., Palakkad, Thrissur and Ernakulam. The samples were subjected to indirect Enzyme Linked Immunosorbent Assay (ELISA) for the detection of IgG antibodies of Toxoplasma gondii. Factors predisposing to the prevalence of infection such as age, grazing behaviour and stage of gestation were also taken into account during the study. Out of 72 serum samples examined, 31(43 per cent) were negative, 13 (18 per cent) were weakly positive and 28 (38.88 per cent) were highly positive. A higher prevalence was noticed in goats above four years of age, in those with regular grazing behaviour and in those that had aborted in the second stage of gestation. District-wise prevalence revealed relatively higher prevalence in Ernakulam district (62.5 per cent) compared to Thrissur (56.09 per cent) and Palakkad (53.33 per cent), even though the difference was not statistically significant.

Keywords: *Toxoplasma gondii*, Enzyme Linked Immunosorbent Assay, Goat, Kerala.

Toxoplasmosis is a wide spread zoonotic protozoan parasitic infection caused by *T. gondii* capable of infecting both mammals and birds (Dubey and Beattie, 1998). Felids are the definitive host and following infection they shed oocysts in the faeces. While most of the intermediate

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hosts remain asymptomatic, toxoplasmosis is acute in sheep and goats causing resorption, abortion, foetal mummification and still birth (Ahmed *et al.*, 2008). Goats become infected when they feed on pastures, concentrates or drinking water contaminated with *T. gondii* oocysts. Toxoplasmosis associated abortions significantly affect the reproductive status of the goats, eventually leading to decreased production and productivity. It also poses a major threat to humans because of its zoonotic importance. Reports on the prevalence of toxoplasmosis and *Toxoplasma* associated abortions in food animals especially in goats are scarce in India, although there are a few studies on the seroprevalence of the disease in this species. Due to the significance of *T. gondii* in public health and economic points of view, there is an urgent need for early and accurate detection of the disease. This will help to map the seroprevalence among goats so that *T. gondii* contamination can be avoided. It will also help to improve the economy of farmers by containment of *T. gondii* associated abortions in goats. Therefore the present work was carried out to assess the extent of *Toxoplasma gondii* infection in aborted goats from the central districts of Kerala by ELISA.

Materials and Methods

A total of 72 serum samples from goats belonging to the organised, unorganised and small holder farms that had aborted recently or had a history of abortion were collected from the central districts of Kerala viz., Palakkad, Thrissur and Ernakulam during the period from November 2019 to November 2020. Details of risk factors such as age, grazing behaviour of goats and stage of gestation were also collected to evaluate its significance on the presence of *T. gondii* antibodies. The serum samples were separated from the blood by centrifugation and stored at -20°C until further processing.

All the serum samples were subjected to indirect ELISA for the detection of IgG antibodies of *T. gondii* using a commercial ELISA kit (IDEXX Toxotest, Switzerland). ELISA results were read using spectrophotometer at 450 nm. Serum with sample to positive ratio (S/P) per cent which is equal or more than 100 per cent was considered as positive, serum with S/P per cent between 30 to 100 was considered as weakly positive and serum with less than 30 S/P per cent was considered as negative. The data was analysed statistically using Chi square test to ascertain the association of age, grazing behaviour and stage of gestation on the prevalence of *T. gondii* in goats.

Results and Discussion

Among the 72 serum samples examined, 31 (43 per cent) were found negative for the presence of *T. gondii* antibodies, whereas 13 (18 per cent) were weakly positive, and 28 (38.88 per cent) were detected to be highly positive for the presence of *T. gondii* specific antibodies (Table 1). The overall seroprevalence was determined to be 56.94 per cent. This is in accordance with the findings of Syamala and Devada (1999) who also recorded, 58.16 per cent seroprevalence in goats of Kerala.

A highly significant difference ($p < 0.01$) was noticed in the prevalence of *T. gondii* antibodies in goats above four years of age when compared to those of other age groups (Table 2). Thus it was inferred that the prevalence of *T. gondii* antibodies increased with increase in age. The ELISA results were in accordance with the study of Figueiredo *et al.* (2001), Figliuolo *et al.* (2004), Cavalcante *et al.* (2008), Kamani *et al.* (2010), Balea *et al.* (2012) and Hareendran (2017), who reported more number of animals above four years of age to be seropositive to *T. gondii* antibodies when compared to other age groups. This could be due to the continuous

Table 1. Seroprevalence of *T. gondii* in aborted goats by ELISA

Samples examined	Seronegative		Weakly seropositive		Seropositive	
	Number	Percentage	Number	Percentage	Number	Percentage
72	31	43	13	18	28	38.88

Table 2. Age-wise seroprevalence of *T. gondii*

Age(in years)	No. of samples examined	Seronegative		Weakly Seropositive		Seropositive		p value
		No.	%	No.	%	No.	%	
<2 year	20	18	90	1	5	1	5	0.000*
2-4 years	28	8	28.57	9	32.14	11	39.28	
>4 years	24	5	20.83	3	12.5	16	66.66	
Total	72	31	43	13	18	28	38.88	

*Highly significant (p<0.01)

Table 3. Seroprevalence of *T. gondii* in goats related to grazing

Grazing behaviour	No. of samples examined	Seronegative		Weakly seropositive		Seropositive		p value
		No.	%	No.	%	No.	%	
Regular grazing animals	58	21	36.20	11	18.96	26	44.82	0.046*
Non grazing animals	14	10	71.42	2	14.28	2	14.28	
Total	72	31	43	13	18	28	38.88	

*Significant difference (p<0.05)

Table 4. Seroprevalence of *T. gondii* based on the stage of gestation

Stage of gestation	No. of samples examined	Seronegative		Weakly seropositive		Seropositive		p value
		No.	%	No.	%	No.	%	
First stage (<2months)	22	8	36.36	6	27.27	8	36.36	0.000*
Second stage (2 to 3 months)	28	4	14.28	4	14.28	20	71.42	
Third stage (4 months and still birth)	22	19	86.36	3	13.63	0	0	
Total	72	31	43.05	13	18.05	28	38.88	

* Highly significant (p<0.01)

Table 5. District - wise seroprevalence of toxoplasmosis

District	No. tested	No. positive	% positive	p value
Thrissur	41	23	56.09	0.9*(ns)
Palakkad	15	8	53.33	
Ernakulam	16	10	62.5	

(Not significant - p>0.05)

and prolonged exposure of aged animals to oocysts of *T. gondii* in the environment over the years. Repeated exposure from soil, feed and fodder results in the development of antibodies in the serum with high titres. Sharif *et al.* (2006) opined that the prevalence of toxoplasmosis in aged animals could be due to the browsing behaviour of goats. Goats generally browse the top of the grass and shrubs rather than taking the lower parts of the plants.

During the present study, positive correlation was observed between the prevalence of *T. gondii* antibodies in the serum samples of goats and their grazing habits. Out of 58 regular grazing animals, 44.82 per cent (26/58) were highly positive for *T. gondii* antibodies. Significant difference (p< 0.05) was noticed between the animals that were let out for grazing than those that were staged indoors (Table 3). This finding corroborated

with that of Skjerve *et al.* (1998), where higher seroprevalence was noted in Norwegian lambs that were let out for grazing regularly. Similarly, Lahmar *et al.* (2015) noted a higher seroprevalence in Southern Tunisia sheep and goats that were let out for grazing.

Regular grazing makes the animals to come in close contact with the oocyst contaminated environment, which results in accidental ingestion. Repeated exposure to oocysts leads to infection and further development of serum antibodies. It is also worth mentioning that Kerala owing to its high humid weather, oocysts are protected against dessication thereby enabling their survival and sporulation.

A highly significant difference ($p < 0.01$) was noticed in the prevalence of *T. gondii* antibodies in goats that had aborted in the second stage when compared to the other stages (Table 4). Among the three stages of gestation, 71.42 per cent animals that had undergone abortion in the second stage of gestation were seropositive for *T. gondii*. According to Ahmed *et al.* (2008), foetal death was found to be caused by the multiplication of *T. gondii* in the placenta, rather than invasion of foetus by the parasite. The continuous multiplication of the parasite in the placenta and foetus could be enhanced by the local suppression of immune mechanism in the maternal placenta and immaturity of the foetal immune system. Moreover, it has been found that the inflammatory reactions induced in the placenta by *T. gondii* are capable of stimulating synthesis and release of prostaglandin F_{2α} which has a leuteolytic action leading to decreased progesterone level and subsequent abortion.

A relatively higher seroprevalence of toxoplasmosis was noticed in goats from Ernakulam district (62.5 per cent) followed by Thrissur (56.09 per cent) and Palakkad (53.33 per cent). Statistically, no significant difference could be noticed between the different districts and the positive results obtained (Table 5). The higher prevalence rate observed in Ernakulam could be due to the relatively less number of samples examined (16). Besides, the samples collected from Ernakulam were from goats

reared in households and unorganised farms and were let out for grazing regularly. The presence of cat population in the premises of these houses and farms could also contribute to the results obtained. This finding corroborated with that of Figliuolo *et al.* (2004), where higher prevalence was noted in the household goats.

A high seroprevalence of 56.94 per cent was detected in recently aborted goats or those that had a history of abortion. This indicates that infection of *T. gondii* is widespread in this area. Hence, it is imperative to carry out a mapping of seroprevalence in goats in a wider geographical region in the state of Kerala to contain *T. gondii* contamination in the food chain to control zoonotic transmission. Adequate awareness must be created among goat farmers on the managerial aspects in order to control toxoplasmosis in goats.

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Effect of dietary energy and lysine levels on cost of production and profit of meat production in “TANUVAS Namakkal gold Japanese quails”



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Abstract

Two biological trials were conducted to determine the energy and lysine requirements of “TANUVAS Namakkal gold Japanese quail” for production performance. The economic impact of different levels energy and lysine during the chick (0-2 weeks) and grower (3-5 weeks) phases was assessed using seven hundred and twenty straight-run, day-old chicks randomly grouped in nine treatments with four replicates of twenty chicks each. Three levels of energy (2800, 2900 and 3000 kcal/kg) and three levels of lysine (1.2, 1.3 and 1.4%) were tested for chick and grower phase of the first experiment. The net profit per bird (Rs. 8.49) was high in group T₁ (low energy and low lysine) and the net profit per kg live weight (Rs. 48.57) was high in T₂ (2900 kcal/kg and 1.2%). For the second experiment an energy level of 2700, 2800 and 2900 kcal/kg was fixed for chick and grower mash, lysine level of 1.2, 1.3 and 1.4% was fixed for chick phase and 1.1, 1.2 and 1.3% for grower phase and the crude protein level was fixed as 20.3 and 19.4 per cent for chick and grower mash respectively for the second trial. The net profit per bird (Rs. 7.18) was high in group T₆ and the net profit per kg live weight (Rs. 37.62) was high in T₆ (2900 kcal/kg and 1.3% lysine during chick phase and 1.2% lysine during the grower phase).

Key Words: Economical efficiency, energy, lysine, net profit, production performance

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Over the recent decades the population of Japanese quail has been replaced by new varieties developed by genetic selection for better body weight and superior egg production performance (Hussain *et al.*, 2013). One such evolution is the “TANUVAS Namakkal gold Japanese quail” which is superior compared to both the parent lines and is used for commercial egg production purpose.

Energy-protein ratio and the ratio of energy to other nutrients are important in the formulation of diets. In recent days, importance is given for fixing the best ratio of lysine to other amino acids rather than the crude protein content of the diet otherwise called as ideal protein concept. Excess protein increases the oxidation of amino acids as source of energy and nitrogen excretion leading to environment pollution; whereas the deficiency increases the catabolism of body protein and fat (Filho *et al.*, 2012). Hence, the present study was carried out to evaluate the production performance and its impact on economic efficiency fed with different dietary levels of metabolizable energy and lysine in “TANUVAS Namakkal gold Japanese quail”.

Materials and Methods

Two experiments were conducted in “TANUVAS Namakkal gold Japanese quail” from day-old to 5 weeks of age. Both the experiments were conducted using seven hundred and twenty each, straight-run, day-old quail chicks. They were weighed and randomly assigned into nine treatments with four replicates of twenty chicks each. Nine diets were formulated with three levels of energy and three levels of lysine in a 3 x 3 factorial design for both the experiments.

Three levels of dietary energy (2800, 2900 and 3000 Kcal/kg) and three levels of lysine (1.2, 1.3 and 1.4 per cent) during chick cum grower phase (0-5 weeks) were used for the experiment -I. Based on the best results of the first experiment the energy and lysine levels were fixed for the second experiment. For experiment -II, isonitrogenous diet was formulated with crude protein level of 20.3% in chick (0-2 weeks) and 19.4% in grower stage

(3-5 weeks) with three levels of dietary energy (2700, 2800 and 2900 Kcal/kg) and three levels of lysine (1.2, 1.3 and 1.4 per cent) during chick (0-2 weeks) phase and 2700, 2800 and 2900 kcal/kg and 1.1, 1.2 and 1.3% for the grower phase. The birds were reared in cages under standard and uniform management conditions throughout the experimental period. The quail chicks were fed with weighed quantity of experimental diets and they had free access to wholesome water.

Data on individual bird weight and total feed consumption in each replicate were recorded every week and mortality was recorded at the occurrence. The economics of feeding diets containing different levels of energy and lysine under cage system for chick cum grower (0-5 weeks) stage for both experiments was worked out using the prevailing feed cost and sale price of live bird and meat of Japanese quails.

Results and Discussion

The cost effectiveness of “TANUVAS Namakkal gold Japanese quail” from 1 to 5 weeks of age (chick cum grower) as influenced by different levels of energy and lysine in both the experiments are shown in Table 1 and 2.

The total feed cost per bird (Rs.11.87) and the cost of production per bird (Rs.17.87) was highest in the group T₈ (2900 kcal/kg and 1.4%) and the lowest total feed cost per bird (Rs.10.51) and the cost of production per bird (Rs.16.51) was observed in T₁ (2800 kcal/kg and 1.2%).

The net profit per bird (Rs. 8.49) was high in group T₁ and the net profit per kg live weight (Rs. 48.57) was high in T₂ (2900 kcal/kg and 1.2%). The net profit per bird (Rs.7.13) was lowest in group T₈ and the net profit per kg live weight (Rs.39.28) was lowest in T₇ (2800 kcal/kg and 1.4%).

Alagawany *et al.* (2014) reported that economic efficiency was higher in low protein (20%) and low lysine (1.05%) groups but not significant between the groups in Japanese quail which is similar to present results. In contrast Mahmood *et al.*, (2014) reported that

Table 1. Cost effectiveness of TANUVAS Namakkal gold Japanese quail from 1 to 5 weeks of age (chick cum grower) as influenced by different levels of energy and lysine (Experiment-I)

Treatment groups	Body weight (kg)	Total feed consumed (kg)		Cost of feed per kg (Rs.)		Total feed cost per bird (Rs.)	Cost of production per bird (Rs.)	Cost of production per kg live weight (Rs.)	Total income per bird (Rs.)	Net profit per bird (Rs.)	Net profit per kg live weight (Rs.)
		Chick	Grower	Chick	Grower						
T ₁ (2800, 1.2)	0.188	0.073	0.404	23.43	21.77	10.51	16.51	88.02	25.00	8.49	45.30
T ₂ (2900, 1.2)	0.173	0.071	0.396	23.85	22.48	10.60	16.60	96.05	25.00	8.40	48.57
T ₃ (3000, 1.2)	0.168	0.066	0.400	24.28	23.22	10.90	16.90	100.78	25.00	8.10	48.31
T ₄ (2800, 1.3)	0.182	0.070	0.398	23.85	22.54	10.63	16.63	91.32	25.00	8.37	45.99
T ₅ (2900, 1.3)	0.177	0.070	0.388	24.28	23.16	10.67	16.67	94.21	25.00	8.33	47.04
T ₆ (3000, 1.3)	0.168	0.069	0.390	24.70	23.89	11.04	17.04	101.71	25.00	7.96	47.54
T ₇ (2800, 1.4)	0.185	0.072	0.408	24.29	24.50	11.74	17.74	95.93	25.00	7.26	39.28
T ₈ (2900, 1.4)	0.170	0.073	0.401	24.76	25.08	11.87	17.87	105.03	25.00	7.13	41.88
T ₉ (3000, 1.4)	0.166	0.069	0.392	25.32	25.64	11.79	17.79	107.37	25.00	7.21	43.54

Chick cost : Rs.5/- chick
Miscellaneous cost : Rs.1/- bird

increasing energy (3000 kcal/kg) level in the diet had enhanced the production efficiency with minimum cost.

The net profit per bird and net profit per kg live weight in low and medium energy (2800 and 2900 kcal/kg) and low lysine (1.2%) groups may be due to high growth rate, better feed efficiency and less feed cost.

The results of this experiment recommend low energy and lysine (2800 kcal/kg and 1.2 %) for best economic efficiency during the chick cum grower phase.

In the second experiment, the total feed cost per bird (Rs.13.31) and the cost of production per bird (Rs.19.43) was highest in the group T₄ (2700 kcal/kg and 1.3% lysine during chick phase 1.2% lysine during the grower phase) and the low total feed cost per bird (Rs.11.82) and the cost of production per bird (Rs.17.82) was low in T₆ (2900 kcal/kg and 1.3% lysine during chick phase 1.2% lysine during the grower phase).

The net profit per bird (Rs. 7.18) and the net profit per kg live weight (Rs. 37.62) was

high in T₆ (2900 kcal/kg and 1.3% lysine during chick phase and 1.2% lysine during the grower phase). The net profit per bird (Rs.5.57) was low in group T₄ and the net profit per kg live weight (Rs.26.20) was low in T₁ (2700 kcal/kg and 1.2% lysine during chick phase and 1.1% lysine during the grower phase).

Mahmood *et al.*, (2014) reported that increasing energy (3000 kcal/kg) level in the diet enhanced the production efficiency with minimum cost which is in accordance to this experiment. On the contrary, Alagawany *et al.* (2014) reported that economic efficiency was higher in low protein and low (1.05 %) lysine groups in Japanese quails.

The net profit per bird and net profit per kg live weight in low and medium energy (2800 and 2900 kcal/kg) and low lysine (1.2%) groups may be due to high growth rate, better feed efficiency and less feed cost.

The results of this trial recommend low energy and lysine (2800 kcal/kg and 1.2%) for best economic efficiency during the chick cum grower phase.

Table 2. Cost effectiveness of TANUVAS Namakkal gold Japanese quail from 1 to 5 weeks of age (chick cum grower) as influenced by different levels of energy and lysine (Experiment-II)

Treatment groups	Body weight (kg)	Total feed consumed (kg)		Cost of feed per kg (Rs.)		Total feed cost per bird (Rs.)	Cost of production per bird (Rs.)	Cost of production per kg live weight (Rs.)	Total income per bird (Rs.)	Net profit per bird (Rs.)	Net profit per kg live weight (Rs.)
		Chick	Grower	Chick	Grower						
T ₁ (2700, 1.1)	0.217	0.125	0.540	20.44	19.92	13.31	19.31	88.91	25.00	5.69	26.20
T ₂ (2800, 1.1)	0.211	0.113	0.500	20.76	20.28	12.50	18.50	87.79	25.00	6.50	30.84
T ₃ (2900, 1.1)	0.195	0.107	0.486	21.12	20.61	12.29	18.29	93.83	25.00	6.71	34.42
T ₄ (2700, 1.2)	0.220	0.108	0.553	21.08	20.17	13.43	19.43	88.32	25.00	5.57	25.31
T ₅ (2800, 1.2)	0.203	0.096	0.502	21.56	20.55	12.39	18.39	90.40	25.00	6.61	32.50
T ₆ (2900, 1.2)	0.191	0.084	0.472	22.43	21.04	11.82	17.82	93.35	25.00	7.18	37.62
T ₇ (2700, 1.3)	0.197	0.075	0.489	23.20	21.98	12.47	18.47	93.88	25.00	6.53	33.16
T ₈ (2800, 1.3)	0.202	0.075	0.494	23.68	22.69	13.00	19.00	93.85	25.00	6.00	29.66
T ₉ (2900, 1.3)	0.199	0.074	0.469	24.40	23.21	12.71	18.71	93.80	25.00	6.29	31.56

Chick cost : Rs.5/ chick

Miscellaneous cost : Rs.1/ bird

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Mortality and morbidity pattern in goats under organized farm conditions of Kerala



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Abstract

A study was undertaken to find out the morbidity and mortality pattern in the goat population of Kerala. Factors such as cause, age, sex, breed, season and year were included in the study. Data were collected from 3925 goats maintained at The Goat and Sheep Farm, KVASU, Mannuthy for six years from 2011 to 2017 to assess the effect of different factors. Data pertaining to 2649 goats from 2013 to 2017 were analysed to find out the morbidity pattern among goats. The overall mortality rate was 11.76 per cent. Among the various causes of mortality, the results of this study indicated that mortality due to enteritis was the highest (40.60 %) followed by that due to pneumonia (22.88%) and acidosis (10.40%). The effect of age, season, sex and year on goat mortality were significant ($p < 0.05$). Mortality was the highest in the age group of 0.-3 months followed by 3-6 months. The incidence of mortality rates were 4.67, 5.09 5.27 and 5.26 per cent in pre-monsoon, south-west monsoon, post-monsoon and winter respectively. Mortality was more in females (64.11%) than males. Mortality was highest in cross bred goats followed by Malabari and Attappady black, but not significant. The proportional morbidity due to foot rot was the highest (35.28%) followed by enteritis (16.03%) and orf (7.65%). Highest morbidity was observed during south west monsoon (45.55%) followed by post monsoon (21.98%) and pre monsoon (18.14%). The results of this study suggest that proper management during first three months of age especially during south west monsoon was critical to minimise mortality among goats.

Key words: goat, morbidity, mortality

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Introduction

Goat is considered as a versatile animal, adapted to different agroclimatic conditions of the world with more than 95 percent of the goat population found in developing countries. India possesses 135.17 million goats which makes up around 26.4 percent of total livestock in the country, ranking second in goat population of the world (Livestock census–19th, 2012). Goat contributes 3.7 percent (4.8 MT) of the India's total milk (127.90 MT) production (Basic Animal Husbandry Statistics, 2013). Goat meat is chosen irrespective of religion or culture; therefore, goat meat has great demand in India. The productivity of goats under traditional production systems is very less.

Commercial goat production is gaining momentum, on account of good economic prospects of this system under the intensive and semi-intensive system. Goats contribute more than 52 percent of households' total income towards nutrition and food security of the rural families involved in this vocation (Kumar, 2007). Under organized farm conditions, mortality due to one reason or another is a matter of concern for producers, because it is directly linked with economic loss. Kumar *et al.* (2003) reported that estimated losses due to diseases in goats were 23.22 percent of net returns and 5.21 percent of gross returns. Economic losses due to mortality caused by various diseases in goats have been a major constraint in native flocks. The risk of disease further increases when goats are maintained in large flocks under the intensive system. To make goat farming profitable, in addition to good managerial practices, measures to ensure that the herd is made free from diseases and other health problems should also be ensured (Singh and Kumar, 2007).

Morbidity and mortality greatly affect economic returns from goat husbandry. Knowledge of disease pattern in different age groups, breeds, sex, year and seasons will be of immense help in health management to reduce mortality. Several studies have shown that on an average 20 percent of kids and 10 percent of adult goats die each year (Ershaduzzaman *et al.*, 2007). Therefore, the mortality and morbidity

pattern of the farm should be known in order to plan proper management and prevention strategies. Keeping these aspects in view, the present study was undertaken to ascertain factors affecting morbidity and mortality pattern in goats under organized farm conditions of Kerala, India.

Materials and Methods

Data in this study were obtained from University Goat and Sheep Farm, Mannuthy. Mortality data of 3925 goats for the time period 2011 to 2017 and morbidity data of 2649 goats for the time period 2013 to 2017. The data were classified as per their respective season, year, breed, sex and age of the animal. The calendar year was divided into 3 seasons: March-May (Pre-monsoon), June-September (South-West monsoon), October-November (Post monsoon) and December-February (Winter). The time period from birth to death was divided into three age groups kids (0-3 months), young goats (3-6 months) and adults (> 6 months). The breeds analysed include Attappady black, Malabari and cross-bred goats. The collected data were compiled and analyzed to study the incidence and effects of diseases in goats.

Proportional morbidity and mortality rates:

Among various diseases the proportional morbidity/ mortality (PMR) due to each disease was calculated by the formula:

$$\text{Morbidity Rate (P)} = \frac{\text{Number of animals affected by the specific disease}}{\text{Total number of animals affected by all the diseases}} \times 100$$

$$\text{Morbidity Rate (P)} = \frac{\text{Number of animals died due to the specific disease}}{\text{Total number of animals died due to all the diseases}} \times 100$$

Statistical analysis

The percent mortality rate, morbidity rate and their distribution pattern for different years and season were calculated. The effect of different factors such as year, season, age, sex and causes of death with both mortality rate and morbidity rate were analyzed by one way ANOVA using SPSS software version 20.0 using the following model (Snedecor and Cochran, 1994).

$$y_{ijklm} = \mu + A_i + B_j + C_k + D_l + E_m + e_{ijklm}$$

Where y_{ijklm} is the observed value of i^{th} treatment,

μ = population mean effect,

A_i = effect of year,

B_j = effect of season,

C_k = effect of sex,

D_l = effect of breed,

E_m = effect of age, and

e_{ijklm} is the error term

Results and Discussion

The overall mortality rate was 11.76 percent, which varied from 4.28 percent (2013) to 20.82 percent (2016). The lower mortality rate in the particular years could be due to small number of kids born, more kids being born in the favourable kidding season and better management conditions when compared to the years having higher mortality (Barbind and Dandewar, 2004 and Ramachandran *et al.*, 2006).

The major diseases instrumental in causing deaths in the present study were diarrhoea, pneumonia, acidosis and enterotoxaemia. The mortality due to enteritis (40.60 %) was the highest, followed by that due to pneumonia (22.88 %) and acidosis (10.40 %). The overall morbidity rate was 19.95 percent. Foot rot remained the most frequently contracted disease especially during the post monsoon period, followed by digestive and respiratory diseases and further by some specific diseases like orf, mastitis and coccidiosis.

Effect of season on mortality and morbidity

Different seasons of the year had a profound and significant effect ($p < 0.05$) on both mortality and morbidity. Season wise mortality within the year indicated that the highest mortality was during the South West monsoon (39.28 %) followed by pre monsoon (32.48 %) and post monsoon (18.47 %) season. Highest morbidity was during South West monsoon (45.54 %) followed by the pre monsoon (21.98 %) and post monsoon (18.14 %) seasons. Such fluctuation in survivability could be attributed to variations in climatic conditions and high incidence of some specific diseases in a particular season (Awemu *et al.*, 1999 and Dohare *et al.*, 2013).

Effect of Year on mortality and morbidity

The variable year had a significant effect ($p < 0.05$) on both mortality and morbidity. Relatively higher instances of death and disease incidence occurred during 2015 and 2016.

Effect of breed on mortality and morbidity

Breed also had a significant effect on mortality and morbidity. Disease incidence and deaths were relatively higher in Malabari goats when compared to other breeds.

Effect of Age on mortality and morbidity

Age had a significant effect on both mortality and morbidity. Deaths and risk of contraction of disease is always higher in kids and young stock when compared with the adult stock (Sabapara and Deshpande, 2010).

Table 1: Percent mortality and morbidity in different age groups of goats

Year	<3 Months	3-6 Months	>6 Months	Total Death	Stock	Mortality %	Morbidity %
2011	26	20	3	49	628	7.80	(not known)
2012	48	37	6	91	648	14.04	(not known)
2013	15	12	2	29	676	4.28	7.47
2014	45	34	5	84	652	12.88	7.74
2015	58	45	6	109	615	17.72	28.10
2016	46	35	5	86	413	20.82	45.99
2017	7	6	1	14	293	4.78	10.70
Total	245	189	28	462	3925	11.76	19.95

Table 2: Percent mortality and morbidity in different season

Season	Number of deaths	Number of disease incidence	Mortality %	Morbidity %
Pre monsoon	153	405	32.48	18.14
South West Monsoon	185	1017	39.28	45.54
Post Monsoon	87	491	18.47	21.98
Winter	46	320	9.77	14.34
Total	471	2233	100	100

Table 3: Percent mortality and morbidity in different years

Year	Number of deaths	Number of disease incidence	Mortality %	Average number of deaths	Morbidity %
2011	48	(not taken)	7.64 %	10.20 %	(not known)
2012	90	(not taken)	14.33 %	19.10 %	(not known)
2013	37	167	5.89 %	7.85 %	7.47
2014	84	173	13.37 %	17.84 %	7.74
2015	114	627	18.15 %	24.20 %	28.10
2016	84	1027	13.37 %	17.84 %	45.99
2017	14	239	2.23 %	2.97 %	10.70
Total	471	2233			

Table 4: Percent mortality and morbidity in different breeds

Breed	Number of deaths	Number of cases	Mortality %	Morbidity %
Attappadi black	128	564	27.18	25.26
Malabari	169	1277	35.88	57.19
Cross bred	174	392	36.94	17.55
Total	471	2233		

Table 5: Percent mortality and morbidity in different age groups

Age	Number of deaths	Number of disease incidence	Mortality %	Morbidity %
0 – 3 months	204	347	43.33	15.54
3 – 6 months	230	958	48.83	42.90
Above 6 months	37	928	7.84	41.56
Total	471	2233		

Table 6: Percent mortality and morbidity in males and females

Sex	Number of deaths	Number of disease incidence	Mortality %	Morbidity %
Male	169	99	35.89	4.44
Female	302	2134	64.11	95.56
Total	471	2233		

Effect of sex on mortality and morbidity

Sex had a significant effect on both mortality and morbidity. Higher mortality and morbidity were observed in female animals. The low mortality in male animals could be due to their lesser presence on the farm at a given time as there was a practice of disposing

male animals from the farm immediately after weaning (Singh *et al.*, 2004 and Singh *et al.*, 2008).

Conclusion

This study confirms that overall mortality rate averaged 11.76 per cent under

semi-intensive farm conditions. It was generally higher during south-west monsoon followed by pre monsoon and post monsoon seasons. Season-wise mortality followed a similar trend for all age groups. Mortality was higher in kids particularly those under 1 month of age than in young stock and adults. The disease which singly contributed major share to overall mortality included digestive diseases followed by respiratory diseases and parasitic diseases. It was revealed that diarrhoea and pneumonia were the major causes of morbidity, warranting improved hygiene and good management practices on goat farms should be taken to reduce the occurrence of diseases. Morbidity and mortality were significantly associated with age suggesting that more care and attention needed to be paid to kids of less than 3 months of age irrespective of the season. Mortality due to various diseases in kids and adults could be minimized by identifying the cause and giving proper treatment.

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Surgical management of congenital foetal hydrocephalus in a crossbred cow



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Abstract

A rare case of dystocia due to external congenital hydrocephalic foetus with ankylosis of forelimbs managed by ventral midline surgical approach in a crossbred cow is reported.

Keywords: Dystocia, hydrocephalus, monster.

Dystocia caused by various congenital foetal malformations have been reported in bovines and remains a challenging case especially for the field veterinarians due to the relatively lesser exposure that they have to such case presentations (Singh *et al.*, 2003, Singh *et al.*, 2013). Among the various congenital malformations, hydrocephalus occurs sporadically with an incidence of only 0.15 per cent in bovines (Long, 2001). Congenital hydrocephalus is a dropsical condition which may involve either the ventricles or the sub-arachnoid space of the brain (Noakes, 2009). This condition usually leads to foetal cause of dystocia at calving, causing economic loss to livestock farmers. Hence, the present study reports a rare congenital malformation diagnosed as foetal hydrocephalus in a crossbred cow. The aim of the current study is to disseminate knowledge about the morphology, diagnosis and successful management of congenital foetal hydrocephalus.

A pleuriparous full term crossbred cow was presented to the TVCC, LUVAS, Karnal for treatment of dystocia with the history of continuous straining and ruptured water bags nearly 12 hours before. On general clinical examination, the cow was dull and exhausted while clinical parameters were within the normal range. Per-vaginal examination revealed a fully dilated cervix with a foetus in anterior longitudinal presentation and dorso-sacral position. Detailed morphologic examination of the foetus revealed an enlarged head with a marked fluctuating swelling over frontal and occipital region with softening of cranial bones and without any foetal reflex, resting on ankylosed extended forelimbs. Ankylosis of the limbs could also have contributed to dystocia. The case was diagnosed as dystocia due to congenital foetal hydrocephalus. The cow was stabilized with supportive therapy

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consisting of Inj. Calcium borogluconate 400 ml slow intravenous, Inj. Ceftiofur sodium 2.2 mg/kg body weight, intramuscularly; Inj. Flunixin meglumine 15 ml, intramuscularly; fluid therapy, rumenotronics and vitamin B-complex injection. Under epidural anaesthesia using 2% lignocaine, per vaginal delivery was attempted. The birth canal was well lubricated with the liquid paraffin. An incision was made by a guarded knife on the swollen foetal head to drain the fluid present in the cranial cavity. Per-vaginal delivery of the foetus was attempted by gentle traction but was not successful. Caesarean section was performed by ventral mid line approach following the standard procedure (Schultz *et al.*, 2008) and a dead hydrocephalic male foetus was delivered (Fig. 1). Dissection of foetal head revealed the presence of straw-coloured fluid in sub-arachnoid space, affirming that the foetus was malformed and congenital external hydrocephalus was also confirmed. The cow was discharged on same day after with appropriate suggestions for follow up with a protocol consisting of antibiotics, anti-inflammatory, rumenotronics, anti-histaminics and multivitamins all accompanied by a laxative diet for the next five days. The incision line and suture healed at 12 days after surgery and the cow showed an uneventful recovery after 15 days of surgery (Agerholm *et al.*, 2015).

There are many factors which may be responsible for the accumulation of abnormal volume of the cerebrospinal fluid in the brain tissues such as possible intra-uterine infection of the foetus by viral agents like bovine viral

diarrhoea virus (Agerholm *et al.*, 2015) or this condition may also be due to the inheritance of a single autosomal recessive dominant gene with incomplete penetrance (Jabb and Kennedy, 1970; Purohit *et al.*, 2012). Other predisposing factors for this malformation may include deficiency of vitamin-A or any other brain lesions that may cause a disturbance in the normal flow as well as the reabsorption of the cerebrospinal fluid (Ferris *et al.*, 2011). The present case reported that congenital external hydrocephalus was morphologically characterized by a large sac containing serous fluid, hanging over the head and face. Similar observations have also been made earlier in cattle (Balasubramanian *et al.*, 1997; Sunil *et al.*, 2016; Saini *et al.*, 2019) and buffalo (Dhaliwal *et al.*, 1998) calves. It has also been documented that this type of congenital malformation is usually accompanied by lesions of the musculoskeletal system (Agerholm *et al.*, 2015) as evidenced in the present study as well. In severe cases of hydrocephalus, caesarean section is recommended because it is very difficult to relieve dystocia by mutation and forced traction (Selvaraju *et al.*, 2020).

The cause of death in hydrocephalic foeti may be due to pressure necrosis of the vital centres of the brain (Purohit *et al.*, 2012). If the hydrocephalic calf is born alive, the chances of long term survival are very low because of various lesions in the central nervous system. Congenital malformations involving central nervous system and musculoskeletal system are comparatively easier to diagnose when the foetus is in an anterior longitudinal presentation.

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Fig.1: Hydrocephalic foetus delivered by caesarean section.

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Molecular identification of *Haemonchus contortus* in goats

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Abstract

Haemonchus contortus commonly called the stomach worm or wire worm of ruminants inhabits the abomasum and is considered to be one of the economically important gastrointestinal strongyles in goats. In the present study, *H. contortus* was identified by PCR using the primers targeting partial 5.8S and partial internal transcribed spacer region 2 (ITS-2). Adult worms were identified morphologically and genomic DNA was extracted using DNeasy Blood and Tissue kit (QIAGEN, Germany). Gradient PCR protocol was standardised using the extracted genomic DNA. Ten-fold serial dilution of adult DNA was used to analyse the minimum detection limit and the products were amplified upto the tenth dilution. Cross reaction of primer sets was checked using the DNA extracted from predominant adult strongyles like *Oesophagostomum columbianum* and *Trichostrongylus colubriformis* and no cross reaction was seen at the optimum annealing temperature (60.7°C).

Keywords: *Haemonchus contortus*, goats, PCR, ITS-2

Haemonchus contortus belong to the Trichostrongylidae family and are commonly called the stomach worms or wire worms of ruminants. The adult worms attach to the abomasal mucosa of small ruminants and due to its haematophagous nature it causes anaemia, jaw oedema and even death in young ones. Adult worms are identified based on morphological features. But identification of nematode species based on features of the strongyle egg is difficult during coprological examination. Coproculture aids in species identification but it takes seven to ten days to identify the infective larvae. Hence, molecular identification was undertaken in this study as a tool for species

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level identification.

Abomasum of 19 animals brought for slaughter at the Corporation slaughter house, Kuriachira, Thrissur was collected and taken to the laboratory for further processing. An incision was made at the greater curvature of the abomasum and the contents were carefully washed on to a container. Abomasal contents were washed several times until the contents were clear so that the worms could be visualized easily. Adult worms were collected and stored in 1 X phosphate buffer solution (PBS) at -20°C. About three to five adult *H. contortus* worms were used for extracting genomic DNA using DNeasy Blood and Tissue kit (QIAGEN, Germany). The DNA thus obtained was stored at -20°C for further use. The concentration and purity of DNA extracted were assessed with nanodrop spectrophotometer and it was found to be 74 ng/μL. Primer sets targeting partial 5.8S and ITS-2 regions were designed using online software "Primer 3" Input Version 0.4.0, its suitability checked using the online software 'Sequence Manipulation Suite' Version 2 and its specificity checked using the software NCBI BLAST (www.ncbi.nlm.nih.gov). The designed forward and reverse primers were 5'-CACGAATTGCAGACGCTTAGA-3' and 3'-TCGTCGCCATACATGTCCT-5', respectively. Gradient polymerase chain reaction was standardised with initial denaturation at 94 °C for 5min followed by 39 cycles of denaturation (94 °C for 30s), annealing (57 to 63°C for 30s), extension (72 °C for 30s) and final extension at 72°C for 10min. The composition of reaction mixture is given in Table 1.

After performing gradient PCR, the amplicons were subjected to agarose gel electrophoresis in 1.5 per cent agarose gel at 80V, 400mA for 35 min and the gel was visualised in Gel Doc™ EZ imager and documented using Image lab software. The amplicons were purified and sequenced at AgriGenom labs Private Limited, Cochin using Sanger's di-deoxy nucleotide chain termination method. Bidirectional sequencing was done using both forward and reverse primers. Sensitivity and specificity of the primers were also assessed.

The adult worms were identified morphologically based on their characteristics like the presence of cervical papillae at their head end with small buccal cavity. The male tail end had a well developed bursa with elongate lateral lobes which was supported by an asymmetrical dorsal lobe. The dorsal lobe was placed on the left lateral lobe which was supported by an inverted Y shaped dorsal ray. The female worm had a barber's pole appearance which was due the coiling of white ovaries that were wound around the red intestine (Soulsby, 1982). Out of 19 abomasa collected, 12 were found to be positive for *H. contortus* which accounted for a 63.1 per cent infection in goats. *Haemonchus contortus* has been identified as the predominant strongyle species in goats in different places including Kerala (Deepa, 2005), North-West India (Kumar *et al.*, 2008) Malaysia (Chandrawathani *et al.*, 2009), Kashmir (Irfan-ur-Rauf-Tak *et al.*, 2013) and Ethiopia (Chalchisa *et al.*, 2015).

Table 1. Composition of reaction mix

Components	Quantity (μL)
10 X PCR buffer (without MgCl ₂)	2.5
dNTP (10 mM each)	0.50 (200μM each)
Primer forward	1 (10 pmol)
Primer reverse	1 (10 pmol)
Magnesium chloride (25 mM)	1.50 (1.5mM)
Taq polymerase (5 IU/μL)	0.20 (1U)
DNA template	5
Nuclease free water	13.3
Total	25

Single specific amplicons of ~300bp size were obtained at all the annealing temperatures *i.e.*, 57 to 63°C in gradient PCR. Yin *et al.* (2013) amplified *H. contortus* DNA targeting ITS-2 region and the products were obtained at 231 bp. The obtained sequences were subjected to homology analysis and 100 per cent identity obtained with other published *H. contortus* 5.8S rRNA gene and ITS sequences. The sequences were submitted to GenBank and assigned with accession number MW341458.1.

The sensitivity of *H. contortus* primer sets was checked using ten-fold serial dilution and the ability of primers to amplify minimum DNA concentration was analysed. The initial concentration of DNA used for sensitivity study was 4.7 ng/μL and ten-fold serial dilution was performed. PCR products were amplified upto tenth dilution which showed that the primer pairs could amplify DNA with minimum concentration of 4.7 ag/μL (attogram per microlitre) (Fig. 1).

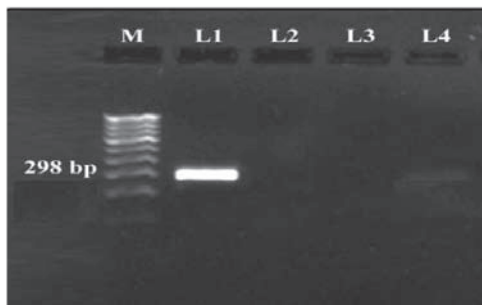


Fig. 1 Amplicons of *H. contortus*

Lane M: 100 bp ladder

Lane 1-10: Ten-fold serial dilution *H. contortus* DNA

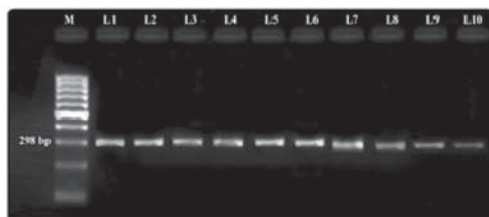


Fig. 2 Specificity of *H. contortus* primer

Lane M: 100 bp ladder

Lane 1: Adult *H. contortus* amplicon

Lane 2, 3: Adult *O. columbianum* and *T. colubriformis* DNA

Lane 4: NTC (No Template Control)

The specificity of primer was cross checked with DNA of other important strongyles like *Oesophagostomum columbianum* and *Trichostrongylus colubriformis* to detect cross amplification between species. Non-specific amplification was not detected between these species (Fig. 2), thus proving the species specificity of primers.

Summary

The study forms the basis for developing copro-polymerase chain reaction for detecting *H. contortus* infection in goats. Specific detection of this pathogen from clinical samples would aid in initiating timely control measures.

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Scanning electron microscopic study of ovary and oviduct of crossbred dairy cows with ovarian hypoplasia



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Abstract

Hypoplasia of ovary is one of the major causes of infertility in dairy cattle, which is characterized by absence of oestrus cycle, affecting livestock productivity and economics to a great extent. This study was conducted on the female genitalia collected from 100 dairy cows/heifers from the Meat Technology Unit, Mannuthy which included six animals culled on account of factors other than infertility with normal genitalia (control group) and remaining animals with a known history of infertility. Two animals of 22 months and 24 months of age showed bilateral ovarian hypoplasia. The history revealed that the animals were healthy, but had not shown oestrus. Grossly ovaries of the first animal appeared as pink-coloured, small, wrinkled, flattened, elongated structures without any follicles or CL. Second animal showed inactive, small, flat, streak-like left ovary without any cyclical structures. But the right ovary showed a single large corpus haemorrhagicum on the caudal end. Then ovarian tissue was fixed in 2 percent glutaraldehyde in 0.1 M phosphate buffer at pH 7.3. Under scanning electron microscopy, the ovary exhibited an uneven surface with clefts and grooves. The surface cells lost their normal appearance without any microvilli. Large round smooth germ cells were also observed on the ovarian surface. In the oviduct, the mucosa was lined by non-ciliated cells having rounded surface interspersed among shrunken cells. The epithelial cell surface was covered with bulbous processes. Ciliated cells were not observed in the present study. Ovarian abnormality is reported to be the main cause of altered morphology of the surface epithelial cells and change in the ultrastructure of oviductal mucosa.

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Key words: *Ovarian hypoplasia, Scanning Electron Microscopy, Dairy cows*

Infertility is one of the major problems confronting the dairy industry and is a frequent reason for culling. Features like anatomical defects, nutritional factors, improper herd management, hormonal disturbances and diseases affecting the reproductive system may disturb the balance and thus influence fertility. Among factors influencing the reproductive performance, anatomical abnormalities in the ovary are of great importance. Better understanding of the cellular differences and functions that occur in the female reproductive tract in domestic animals is required for the proper diagnosis and treatment of reproductive problems. Therefore, this work was undertaken to study the ultrastructural details of the ovary and oviduct of animals with bilateral ovarian hypoplasia and correlate it with functional processes.

The present study was conducted on the female genitalia collected from 100 dairy cows/heifers from the Meat Technology Unit, Mannuthy. This included six animals culled on account of factors other than infertility with normal reproductive system (control group) and the remaining animals with a known history of infertility. Two animals of 22 months and 24 months of age showed bilateral ovarian hypoplasia condition. The history revealed that the animals were healthy, but had not shown oestrus. After recording the morphological and morphometrical features of the genitalia, the ovaries and oviducts were separated and washed in normal saline and the adipose tissue surrounding the ovaries was removed by careful dissection. The fixation of tissues was carried out in 2.5 per cent glutaraldehyde prepared in 0.1M phosphate buffer at 4 °C. After fixation, the tissues were washed in 0.1 M PBS for 10 min each. The samples were dehydrated through serial dilutions of ethanol (50 %, 60 %, 70 %, 80 %, 90 % and 100 %) for 45 min each. The samples were air dried and mounted on sample stub with double sided carbon adhesive tape. The samples were sputter coated with a thin layer of gold for making it conductive using an automated sputter coater. Finally, the tissues were analysed using scanning electron

microscope (Hitachi 3000 N) at an accelerated voltage of 15kV and microphotographs were taken.

Among the 100 animals under study, two animals of 22 months and 24 months

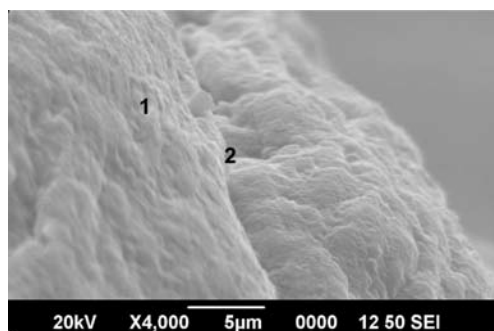


Fig. 1. SEM image of squamous epithelial cells of the ovary. SEM x 4000

1. Squamous epithelial cells
2. Ridges

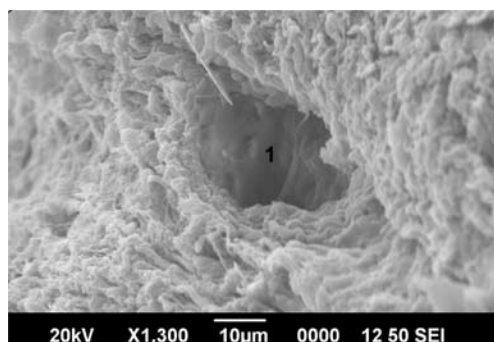


Fig. 2. SEM image of the surface of the ovary. SEM x 1300

1. Epithelial Cleft

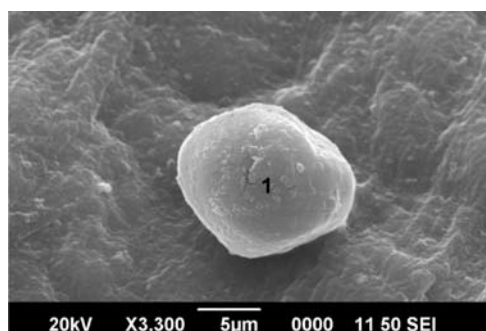


Fig. 3. SEM image of germ cells. SEM x 3300

1. Germ cells

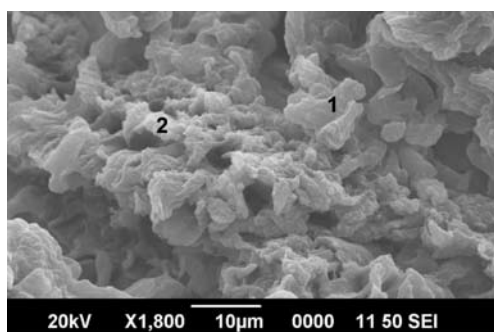


Fig. 4. SEM image of mucosal folds of the oviduct. SEM x 1800

1. Non - ciliated epithelial cells
2. Ridges

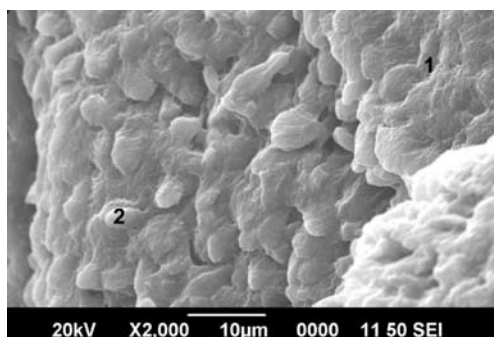


Fig. 5. SEM image of bulbous processes on the surface of non-ciliated cells of oviduct. SEM x 2000

1. Non - ciliated epithelial cells
2. Bulbous process

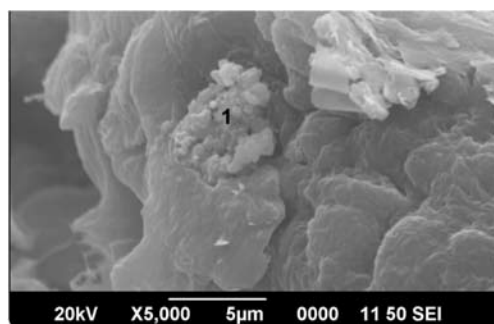


Fig. 6. SEM image of secretory material of the oviductal epithelium. SEM x 5000

1. Secretory material

of age showed bilateral ovarian hypoplasia condition constituting an incidence rate of two per cent. Ovaries of the first animal appeared as pink-coloured, small, wrinkled, flattened, elongated structures without any follicles or

corpora lutea. Morphological examination of the ovaries of the second animal showed inactive, small, flat, streak-like left ovary without any cyclical structures. But the right ovary showed a single large corpus haemorrhagicum on the caudal end. No follicular structures could be located grossly. These animals did not exhibit any oestrus signs thereby being completely infertile and was considered as bilateral ovarian hypoplasia.

In scanning electron microscopy (SEM), the ovary was characterized by an uneven surface with clefts and grooves (Fig. 1). The surface cells lost their normal appearance and were arranged more closely. This is comparable to the findings of Hartanti *et al.* (2019) who stated that the surface epithelium of the adult and foetal ovary was dynamic which altered by repair of the tunica albugenia and surface epithelium or during cell death. Gillett *et al.* (1991) explained two types of epithelial cells on the surface of ovary in human and named as Type A and Type B cells. Type A cells were cuboidal, whereas Type B cells were flat squamous type with broad and flat apices.

In between the tightly packed epithelium some invaginations/clefts could be located (Fig. 2). Hartanti *et al.* (2019) also observed clefts and grooves in the surface of the developing foetal ovary who related this as the opening of ovigerous cords to the surface of ovary. The stroma originating from the mesonephros extended laterally below the ovarian surface. At this point, some germ cells were trapped on the surface. Such germ cells which were larger than the surface epithelial cells could be observed on the ovarian surface in the present study also which was characterized by round shape with smooth surfaced appearance (Fig. 3). The overall features of hypoplastic ovary resembled the foetal ovary in the present study which suggests that the development of the ovary might have arrested in the foetal stage that led to the condition of ovarian hypoplasia.

The mucosa of oviduct was thrown into folds and lined by non-ciliated cells (Fig. 4). The surface of these cells was seen as rounded in appearance and some cells had a shrunken appearance also. The epithelial cell

surface was covered with bulbous processes which were uniform in size and elliptical in shape without any microvilli (Fig. 5). These are in accordance with the observations of Abe and Oikawa (1993) in bovines, Kumar *et al.* (2008) in buffalo cows and Tienthai *et al.* (2009) in Thai swamp buffalo cows who also found numerous bulbous processes of non-ciliated cells in the epithelium of oviduct during the luteal phase. Between the mucosal folds, secretory material was observed as secretory droplets (Fig. 6). Similarly, Pathak *et al.* (2012) also noticed large amount of secretory materials on the surface of secretory cells during the luteal phase in ovine oviducts. Tienthai and Sajjarengpong (2007) could observe thin, smooth and uniform cilia for oviductal epithelium which were absent in the present study. This could be correlated to the functionally inactive ovary.

Acknowledgement

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Histo-morphology of genitalia in crossbred dairy cows with kinked cervix



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Abstract

The study was conducted on genitalia collected from 100 dairy cows/heifers from the Meat Technology Unit, Mannuthy. The animals brought for slaughter at Meat Technology Unit, Mannuthy were from herds of five different farms (University and Government Undertaking farms) in Kerala state. This included six animals culled on account of factors other than infertility with normal reproductive system (control group) and the remaining animals with a known history of infertility. In total, seven animals showed kinked cervix condition. Cervix was evaluated morphometrically and histologically. Grossly, the cervix was hard, kinked and S-shaped, with a mean length of 9.64 ± 1.19 cm. Average number of annular rings in the cervical canal was 4.14 ± 0.26 with an average diameter of 1.74 ± 0.18 cm. At the external os, diameter of the cervix was less, while at the uterine end, diameter was more. The opening of each cervical ring showed a misalignment instead of a straight line. Histologically, the cervix was lined by simple columnar epithelium with signs of degeneration and desquamation. However, the submucosal layer was extensively infiltrated by collagen fibres. In uterus, there was a significant difference in the number of endometrial glands and height of the glandular epithelial cells which was less when compared to the control group. Muscular layer outer to connective tissue layer was thin and uneven. Alterations of the glandular structures negatively influence the viability of spermatozoa due to the lack of secretory products. This fibrous, collagen rich kinked cervix limits the smooth deposition of the semen into the uterus during artificial insemination and the insufficiently dilated cervix affects its contractibility leading to dystocia and subfertility.

Key Words: Kinked cervix, Histomorphology, Dairy cows

Regular and successful reproduction is the key for profitable cattle production. The

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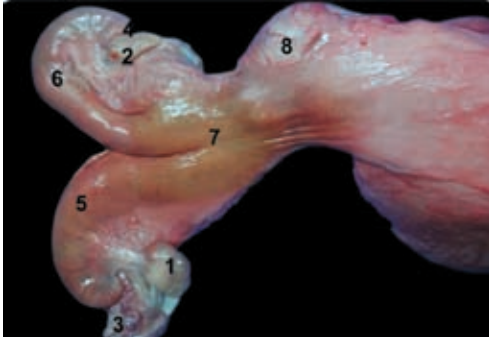
reproductive performance depends upon the normal structure and function of the genitalia. Any structural and functional abnormalities in the reproductive system may interrupt reproductive efficiency. Mullerian ducts are the primordia for development of internal reproductive tracts in females and differentiate into fallopian tubes, uterus, cervix and upper part of the vagina (Kaufman and Bard, 1999). Cervix is a self-contained organ in the reproductive tract, which represents the anatomical and functional barrier between the vagina and the uterus. The knowledge of biometrical status of cervix is essential to perform artificial insemination and while dealing with infertility problems. Kinked cervix is one of the known inherited causes of infertility in dairy cattle in which the cervix region will be in the form of S-shaped structure (Jagir *et al.*, 2009). Collagen is one of the most important components that give rigidity to the cervix and its content changes significantly according to the physiological status of the animal. Reliable information on the parameters of anatomical abnormality of cervix in crossbred dairy cows is scanty. Hence, the present study was designed to explore the morphological, histological and histochemical aspects of genitalia with kinked cervix in crossbred cattle.

The present study was conducted on the female genitalia collected from 100 dairy cows/heifers from the Meat Technology Unit, Mannuthy. The animals brought for slaughter at Meat Technology Unit, Mannuthy were from herds of five different farms (University and Government Undertaking farms) in Kerala state. This included six animals culled on account of factors other than infertility (like wart, abscess, laminitis etc) with normal reproductive system (control group) and the remaining animals with a known history of infertility. In total, seven animals showed kinked cervix condition. Morphology and morphometry of various parts of the genitalia viz., ovary, oviduct, horns, body of the uterus and cervix were noted. Morphometric measurements were recorded with the help of Vernier Calipers and non-stretchable thread. The patency of the oviduct was assessed through the perfusion of 0.9 per cent sodium chloride solution with 1 per cent methylene blue. Methylene blue stained the entire passage of oviduct during its course through the genital

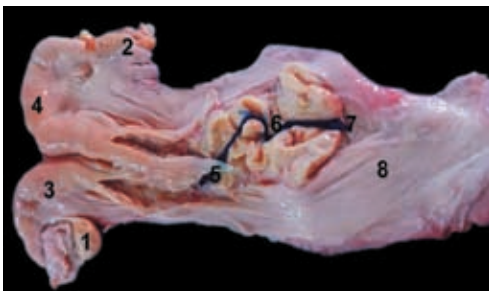
tract. Tissue samples from different regions were fixed in 10 per cent neutral buffered formalin. Standard procedures were adopted for histoarchitectural and histochemical studies. Histologically in the cervix H&E, Masson's trichrome stain and Verhoeff's elastic stain were employed (Luna, 1968). Histochemically, presence of carbohydrates, mucin, phosphates and lipids were demonstrated by Mc Manus method, per iodine acid - alcian blue stain, Gomori's method and osmium tetroxide stains. The micrometrical parameters like thickness of each layer of ovary, oviduct, uterine horn, body of uterus and cervix were measured using Leica Application Suite software. The parameters of normal and anatomically defective samples were compared statistically.

Among the 100 female genitalia collected, cervical abnormality was the most common type found, giving an estimated incidence of seven per cent. Animals were in good physical condition with no defects of the external genitalia. On gross examination, genital structures were observed as normal except the cervix. In all cases, cervix was very hard, twisted and bent in the form S-shaped structure (Fig. 1).

Average measurements of the left ovary viz. length, width and thickness were recorded as 3.23 ± 0.18 cm, 2.24 ± 0.27 cm and 1.56 ± 0.21 cm, respectively and that of the right ovary were 3.68 ± 0.29 cm, 2.27 ± 0.19 cm and 1.90 ± 0.12 cm, respectively. The mean weight of left and right ovaries was 7.94 ± 1.06 g and 8.94 ± 1.14 g, respectively. The ovaries were rounded to oval in shape and functionally active with follicular structures of various sizes. The left and right oviducts were tortuous, wavy, attached with mesosalpinx and opened at the fimbria. In one genitalia, left oviduct showed a tubal obstruction near to utero-tubal junction. The left and right oviducts had an average length of 26.07 ± 0.98 cm and 27.21 ± 0.90 cm, respectively. Mean length of left uterine horns was measured as 31.64 ± 1.77 cm and thickness as 0.27 ± 0.02 cm. Right uterine horn was having a length of 30.50 ± 1.62 cm and thickness of 0.30 ± 0.02 cm. Body of the uterus was 3.29 ± 0.26 cm long with a thickness of 0.34 ± 0.03 cm.

Fig. 1. External appearance of genitalia

1. Left ovary, 2. Right ovary, 3. Left oviduct
4. Right oviduct, 5. Left uterine horn
6. Right uterine horn, 7. Body of uterus
8. Cervix

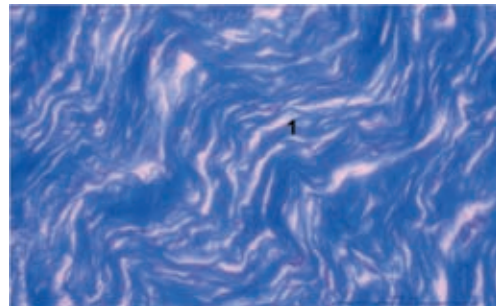
Fig. 2. Bent cervical lumen in kinked cervix

1. Left ovary 2. Right ovary,
3. Left uterine horn, 4. Right uterine horn
5. Internal orifice, 6. Cervical canal
7. External orifice, 8. Vagina

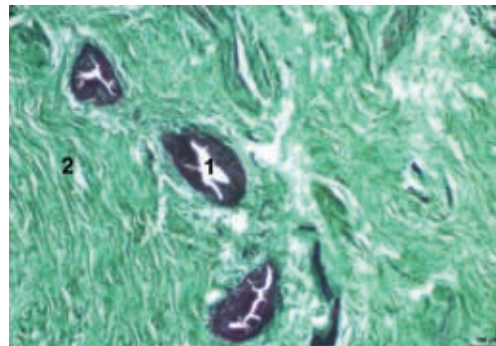
Fig. 3. Cross section of ovary. H&E. x 100

1. Antrum, 2. Cumulus oophorus, 3. Oocyte
4. Zona pellucida, 5. Membrana granulosa
6. Theca interna, 7. Theca externa

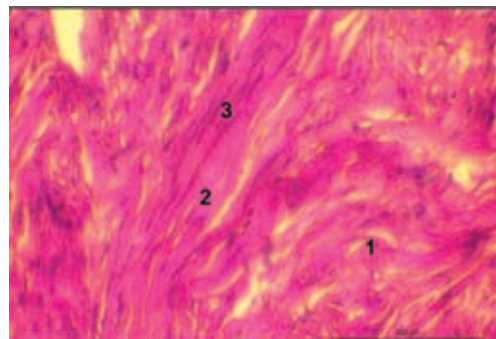
The mean length and number of cervical rings were found to be 9.64 ± 1.19 cm and 3-5, respectively. The os externa was rigid and appeared to be rounded with a diameter of 1.74 ± 0.18 cm. Length and diameter of cervix had a positive correlation with the age and body

Fig. 4. Longitudinal section of cervix. Masson's trichrome method x 400

1. Collagen fibres

Fig. 5. Longitudinal section of cervix. Masson's trichrome method x 100

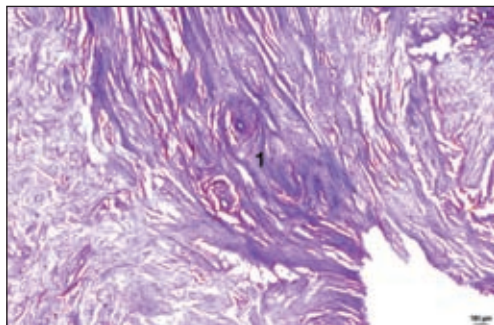
1. Cervical gland, 2. Collagen

Fig. 6. Longitudinal section of cervix. Verhoeff's elastic stain x 400

1. Tunica muscularis
2. Collagen fibres
3. Elastic fibres

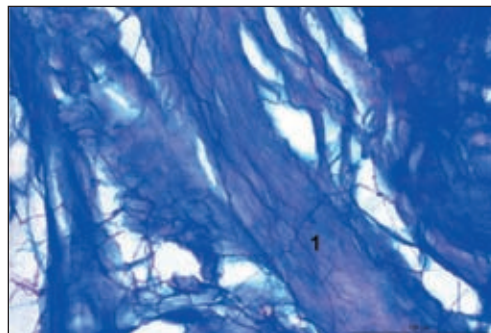
weight (Khaton *et al.*, 2015). At the external os, the diameter of the cervix was less, while at the uterine end, the diameter was more in control animals. Khaton *et al.* (2015) observed the maximum length of cervix as 5.64 ± 0.24 cm in Local \times Holstein Friesian cross. Similar results

Fig. 7. Longitudinal section of cervix. PAS Mc Manus method x 400



1. Collagen fibres

Fig. 8. Longitudinal section of cervix. PAS alcian blue method x 400



1. Connective tissue

of Khaton *et al.* (2015) was also reported by Memon (1996), Kunbhar *et al.* (2003) and Bello *et al.* (2012) in bovines, Thari cows and African zebu cattle respectively. According to Varner *et al.* (1985), cervical diameter had a positive correlation with age and conical shape of the cervix was associated with a lower pregnancy rate in all breeds of cattle. Grossly, cervical rings were thicker and harder compared to the control group. The opening of each cervical ring showed a misalignment instead of a straight line (Fig. 2). Jagir *et al.* (2009) reported this condition as one of the inherited causes of infertility. Kunbhar *et al.* (2003) noted that in Thari cows, uterus was the most affected region which included infectious diseases followed by the cervix. The hardness of cervix might be due to lacerations during calving followed by artificial insemination causing infections and fibrosis.

Histologically, in the ovary, cortex and medulla were clearly defined and follicles of different stages including Graafian follicles, regressing corpora lutea and corpus albicans were observed in the cortical region (Fig. 3). This is in agreement with the findings of Fair *et al.* (1997) and Eurell and Frappier (2006) in bovines. Statistically the cortical thickness showed significant difference when compared to the control animals. In the oviduct, tunica mucosa was composed of lamina epithelialis and lamina propria which blended with the submucosa forming lamina propria-submucosa. The wall of the oviduct was thinner compared to the control animals with thin muscular and serosal coats showing significant difference.

Uterine epithelium was of simple columnar type with oval to elongated basophilic nucleus. In the lamina propria, collagen and elastic fibres were abundant with sparsely distributed reticular fibres. Myometrium was composed of inner circular and outer longitudinal layers. The main difference was noticed in the number of endometrial glands and height of the glandular epithelial cells which was less in affected animals compared to the control group.

In the genitalia with kinked cervix, the main change noticed in the cervical wall was the abundance of fibrous tissue. Mucosa was thrown into numerous folds and was lined by simple columnar epithelium which showed signs of degeneration and desquamation towards their free surface. In the normal cervix, mucosa was highly folded to form primary, secondary and tertiary folds lined by simple columnar epithelium with goblet cells. The submucosal layer was extensively infiltrated by collagen fibres which was confirmed by Masson's trichrome staining (Fig. 4). The stromal layers exhibited only a few glandular structures and in the deep stromal layer, smooth muscle fibres were interspersed with connective tissue (Fig. 5) as reported by Eurell and Frappier (2006) in domestic animals. The tunica muscularis was thin and uneven and was formed of inner circular and outer longitudinal layers. Bundles of dense connective tissue with numerous collagen fibres and a few elastic fibres were infiltrated into tunica muscularis (Fig. 6). This is in accordance with the reports of Breeveld-Dwarkasing (2002) in bovines. In the normal cervix, elastic fibres were more prominent in between the muscle bundles.

Tunica muscularis was thicker when compared to the control group at one percent level of significance. Morphological alterations of the glandular structures of the cervix negatively influenced the transport of spermatozoa (Adams, 1995) because of the change in secretory products of the glands. Variation in the structural arrangement of collagen network of the cervical connective tissue (Leppert, 1995) formed a major cause of loss of tensile strength of the cervix.

Histochemically in the cervix, presence of carbohydrates, mucin, phosphates and lipids was less when compared to that of control group (Figs. 7 and 8). According to Breeveld-Dwarkasing (2002), relative concentration of various carbohydrates, glycosaminoglycan and hyaluronic acid in the interstitial matrix may contribute to the regulation of the visco-elastic properties of the connective tissue.

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Process standardization for alginate encapsulation of potentially probiotic *Pediococcus pentosaceus* DM101



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Abstract

The main objective of this study was to standardise the procedure for encapsulation of potentially probiotic breast milk isolate *Pediococcus pentosaceus* DM101 using sodium alginate by extrusion method. The encapsulation parameters were optimized by comparing the encapsulation efficiency obtained with varying concentration of sodium alginate (0.5, 1.0, 1.5 and 2.0%), calcium chloride (0.05, 0.1 and 0.2M) and gelling time (5, 10, 20 and 30 minutes). Encapsulation efficiency was ascertained by finding the survival percentage after exposing the encapsulated cells to acid stress (pH 2.0 for 3h). Encapsulation carried out using two per cent sodium alginate and 0.1M CaCl_2 following 20 min gelling time was found to confer maximum protective effect. Encapsulation efficiency of alginate beads prepared under optimized conditions was found to be 81.37 ± 2.44 per cent. The results endorse alginate encapsulation as a means to confer a protective shielding effect thereby facilitating effective probiotic delivery.

Key-words: Probiotic, Optimization, Sodium alginate, *Pediococcus pentosaceus*

The consumption of probiotics in adequate amounts, provide health benefits to the host by improving their intestinal microbial balance. The survivability and functionality of probiotics in food products are very much dependent on the processing methods adopted, additives used, storage conditions and intrinsic parameters of the food product. Though inclusion of probiotic bacteria in food products does enhance their value as functional foods, ensuring the stipulated number of cells

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is a challenging in commercial food products. Food matrices and storage conditions have a direct and significant impact on viability of cells and so there is a requirement for a compatible system to ensure the required number of cells at the time of consumption. Apart from the availability of the viable probiotic cells at the time of consumption, the bioavailability of these cells within the host also must be given due consideration. A compatible food system that will safeguard the probiotic cells within the product as well as during gastrointestinal transit will be very much advantageous. Encapsulation is a promising technique for protecting probiotic bacteria in adverse conditions posed within the product prior to consumption as well as after consumption within the gastrointestinal tract. Alginate along with calcium and barium is frequently used for encapsulation of biologically active components as it forms a stable gel by cross linking with divalent cations as in the egg-box model proposed by Pankongadisak *et al.* (2014). Process optimization of alginate encapsulation will ensure effective probiotic delivery at the target site thereby improving health benefits. The encapsulation parameters chosen for optimization of encapsulation process were sodium alginate concentration, CaCl_2 concentration and gelling time.

The probiotic microorganism selected for this study was *Pediococcus pentosaceus* DM101 (NCBI accession number: MK774704), a human milk isolate stored in the culture stock of Department of Dairy Microbiology. This isolate with a log phase of 19h at 37°C has been reported to possess remarkable acid tolerance, bile tolerance and good auto-aggregation potential (67.9 per cent) during its probiotic characterization. With an IC 50 value of 20.78mg/ml, the antioxidant activity of the isolate has also been established (Rappai *et al.*, 2020). The freshly activated cells of *Pediococcus pentosaceus* DM101 in the log phase (19h) were harvested by subjecting to refrigerated centrifugation (Velocity 18R Refrigerated, Dyna MICO) at 12000rpm for 10min at 4°C. The cell pellet obtained from 1ml De Man Rogosa and Sharpe (MRS) broth was dispensed in minimum quantity of sterile distilled water prior to mixing with encapsulating solution. Encapsulation of bacteria was done

in sodium alginate by extrusion method. The highly concentrated cell suspension (from 1 ml culture broth) with a viable count of 10^9 cells was mixed with one milliliter of sodium alginate solution. This mixture was then carefully added drop by drop using a sterile dropper (diameter 2mm) into sterilized CaCl_2 solution (Hi-media) maintained at a temperature of 29°C. Upon contact with the solution, spherical gels were formed immediately. The beads so formed were collected, washed with 0.1 per cent peptone water and stored at 4°C till further assays.

The optimization of encapsulation parameters were done by finding the survival percentage of cells in beads prepared by varying encapsulation parameters: concentration of alginate (0.5, 1.0, 1.5 and 2.0 per cent), concentration of CaCl_2 (0.05, 0.1 and 0.2 M) and gelling time (5, 10, 20 and 30 min). Survival percentage was determined by comparing the initial number of viable cells and number of surviving cells in the beads after exposure to pH 2.0/3h in phosphate buffered saline (PBS) (Kiran *et al.*, 2015).

$$\text{Survival percent} = \left\{ \frac{\text{Log CFU/ml at 3h} \times 100}{\text{Log CFU/ml at 0h}} \right\}$$

The strategy of varying one parameter at a time, keeping others unaltered was employed. Once a particular parameter was found to show maximum survival percent, that parameter was fixed for designing the next trial for optimization. In all experiments free cells was served as control. Accordingly after finalizing the concentration of alginate, concentration of CaCl_2 and then the gelling time were fixed. All the beads prepared with the cells from one milliliter broth constituted the sample for plating. The beads were aseptically homogenized in normal saline using sterile mortar and pestle to ensure complete release of the cells. Appropriate dilutions of this were pour plated in MRS agar and incubated at 37°C for 48h (Harrigan, 1998). The efficiency of encapsulation was evaluated by finding the encapsulation yield (EY). Encapsulation efficiency was evaluated by looking into the number of viable cells in the encapsulate to that of master culture. This was done immediately after encapsulation.

$$EY = (N/N_0) \times 100$$

Where N_0 is the number of viable bacteria in culture and N is the number of viable bacteria in beads.

The observations indicated that the survivability improved progressively with increase in alginate concentration. With the fixed gelling time of 30 min and 0.1M CaCl_2 concentrations, the percentage survival of encapsulated *Pediococcus pentosaceus* DM101 with varying alginate concentrations: 0.5, 1.0, 1.5 and 2.0 per cent were 59.74, 51.31, 57.50, and 91.97 per cent respectively. This observation is in agreement with the reports of Mandal *et al.* (2006). Low alginate concentration yield beads with poor gel strength that are less capable of withstanding simulated gastric conditions. Such beads have a sticky nature and are difficult to handle (Jankowski *et al.*, 1997). But increasing the alginate concentration beyond a limit makes the bead preparation difficult due to its high viscosity (Belalia and Djelali, 2014)

In this study, an alginate concentration of two per cent was sufficient to maintain a survivability of more than 90 per cent. The optimization of strength of CaCl_2 solution was done by keeping alginate concentration and gelling time constant at two percent and 30 min respectively. The survivability of encapsulated *P. pentosaceus* DM101 at CaCl_2 concentrations; 0.05, 0.1, and 0.2M were 60.24, 66.91 and 56.78 per cent respectively. Calcium chloride (0.1M) yielded maximum number of survivors in the experimental conditions followed in our study. From the observations, it can be inferred that influence of CaCl_2 concentration is comparatively less than that of alginate concentration on the viability of the encapsulated cells. Tanaka *et al.* (1984) also reported that the effect of CaCl_2 on the diffusion characteristics of the beads is quite negligible. In our study more than 90 per cent survivability was observed for a gelling time of both 20 and 30 min. Percentage survivors were more or less same for 5 and 10 min of gelling time. The maximum survivability of 98.25 per cent was obtained when gelling time was 20 min. Chandramouli *et al.* (2004) opined to increase

the gelling time for improving the number of viable cells in encapsulates, when hardened in 0.1M CaCl_2 .

Summary

The encapsulation of *Pediococcus pentosaceus* DM101 was optimized with two per cent alginate, 0.1M CaCl_2 and 20 min gelling time. The encapsulates/ beads prepared following the optimized procedure exhibited an encapsulation efficiency of 81.37 ± 2.44 per cent. Alginate concentration was found to have more influence on the encapsulation efficiency than other parameters. The survivability of encapsulated cells in simulated gastric pH endorses the efficacy of encapsulation technique to achieve targeted delivery of probiotics. Process sophistication of the conventional techniques is essential for improving the encapsulation efficiency and further *in-vivo* tests are needed to confirm the benefits of functional foods developed using bioactive components in the encapsulated form.

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Successful utilization of triphasic silica containing ceramic coated hydroxyapatite (HASi) for the treatment of comminuted tibial fracture in a goat: a case report



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Abstract

A one year old female Sirohi crossbred goat was presented with a history of non weight bearing lameness on right hind limb. Radiography revealed comminuted fracture of tibial mid diaphysis. The comminuted fracture fragments and bone devoid of any soft tissue were surgically removed and the resultant segmental defect was reinstated with triphasic silica containing ceramic coated hydroxyapatite (HASi) after stabilising the fracture fragments with 2.7mm dynamic compression plate and screws. The animal recovered with normal limb ambulation after the eighth post-operative week.

Keywords: Comminuted, segmental defect, scaffold, HASi

Goats are one of the major livestock reared in Southern India, especially in Kerala where both male and female goats have equal value. Lameness is considered as the third largest cause for economic loss for farmers. It reduced the resale value of goats while slaughtering and reduced breeding efficiency of bucks.

Comminuted fractures are difficult to treat because of loose bone fragments which make it difficult for anatomical reconstruction of the fractured fragments (Houlton and Dunning, 2005).

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Removal of such fragments resulted in large sized defects which lead to the formation of non unions or delayed unions.

Emara *et al.* (2015) stated that autogenous bone grafts are the gold standard and the choice of treatment of segmental defects. The major drawbacks of these grafts are donor site morbidity and unavailability of sufficient graft material. Allografts and xenografts are remedy for the drawbacks of autograft, but graft rejection and transmission of other diseases are the major disadvantages. Introduction of biodegradable synthetic bone substitute has revolutionized the scenario of treating the segmental defects.

Ceramic-based bone substitutes are reported to be the best biologically accepted materials applied to bone healing (Nascimento, *et al.* 2007). Hydroxyapatite (HA) ceramics are commonly used bone grafts owing to its osteo-conductivity and biocompatibility.

However, the disadvantage of plain hydroxyapatite is its slow rate of biological interaction. Addition of silicon (Si) with HA was found to increase the speed and quality of bone repair process. Based on these traits, a novel graft composite with porosity has been developed by incorporating tri-phasic silica containing ceramic-coated hydroxyapatite (HASi) scaffold. The porosity of the material aids in the osteogenesis through the pores and facilitate faster bone healing.

This paper describes the successful use of triphasic silica containing ceramic coated hydroxyapatite for the treatment of comminuted tibial fracture in a goat and post surgical observations over a period of eight weeks.

A one year old female Sirohi crossbred goat weighing 21kg was presented to the Department of Veterinary Surgery and Radiology, Teaching Veterinary Clinical Complex (TVCC), College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala with a history of the goat falling from a height and being lame on the right hind limb. On further examination, the goat was exhibiting non-weight bearing lameness of the right hind limb. On physical examination of the affected

limb, pain and crepitus could be felt over the mid-diaphyseal tibial region.

All physiological parameters were within normal range.

Radiography with orthogonal views revealed a comminuted mid-diaphyseal fracture of the tibia along with short splinters (Figure 1 and 2).

Corrective surgery using the biomaterial graft was resorted to. The animal was premedicated with Meloxicam @ 0.2 mg/Kg body weight subcutaneously followed by Nalbuphine @ 1mg/Kg body weight and Xylazine @ 0.05mg/Kg body weight intravenously. Anaesthesia was induced by administering Midazolam @ 0.2mg/Kg body weight and Ketamine @ 5mg/Kg body weight



Figure 1: Preoperative radiograph showing a mid-diaphyseal comminuted fracture of tibia (Lateral view)



Figure 2: Cranio-caudal view



Figure 2: Biograft material filled into the fracture gap after fracture fixation with plates and screws.

intravenously. Anaesthesia was maintained by giving ketamine-midazolam top-up at 1:1 (v/v) ratio, whenever required.

The tibia was approached through a cranio-medial incision. The peroneus tertius muscle, long digital extensor muscle and tibialis cranialis muscle were surgically separated and undermined to reach the fracture site. The splinters devoid of soft tissue attachment were removed from the site and fracture ends were surgically debrided. The resultant segmental defect was reinstated with bioceramic graft material HASi (Figure 2) after application of a 2.7mm 8 hole dynamic compression plate in buttress mode to stabilize the fracture fragments.

Muscles and sub-cutaneous tissue were sutured using suture material PGA of size 0 in a continuous suture pattern and skin was sutured in horizontal mattress pattern by using nylon. Modified Robert Jones bandage was applied for external immobilisation for a period of eight weeks.

Immediate post-operative radiographs showed a good apposition, alignment and no angulation (*i.e.*, the fracture fragments remained intact by the apparatus and showed no deviation from their position) between the fracture fragments, biomaterial graft and the implant (Figure 3).



Figure 3: Immediate post operative radiograph.



Figure 4: Radiograph at the end of 8th post operative week.

Partial weight bearing was observed on the affected limb on the fourth week of observation. There was complete weight bearing and improvement in lameness by the end of 8th week observation (Kaler *et al.* 2009). Normal limb ambulation was regained by the animal over the next four weeks.

Radiographs were evaluated and observed on the 2nd, 4th, 6th and 8th post-operative weeks which are as follows:

Fourth and sixth week radiography revealed a mild degree of osteolytic activity (Nair *et al.* 2009). This was indicative for osteoclastic bone resorption that took place during the initial phase of fracture healing process (Hobbs, 2003).

Periosteal reaction was observed on sixth week radiography. Because of a rigid fixation, periosteal callus was minimum (Hobbs, 2003) but periosteal bridging could be detected on eighth week radiograph.

Initial stages of bone resorption were observed on eighth week radiograph with marked areas of radiolucent zone around the graft on account of osteogenesis (Nair *et al.* 2009).

Signs of remodelling were observed at the end of the study. The graft and the fracture ends had good apposition throughout the study. The radiopacity of the biomaterial increased significantly throughout the study and was found integrated with host bone by the end of eight weeks (Figure 4).

Increase in the radiopacity of the biomaterial is a clear indication of the progressive healing of fracture which substantiates the osteoconductive and osteointegrative properties of the graft.

The study suggested that HASi bone graft substitutes could be successfully employed in clinical conditions of segmental defects such as high-energy trauma causing comminuted fractures which requires bone grafting due to the loss of bony fragments/segments.

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Non-invasive blood pressure monitoring in an anaesthetised calf - a case report



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Abstract

A six month old cross bred calf was presented with a swelling on lateral abdomen and diagnosed with lateral abdominal hernia, which underwent herniorrhaphy under general anaesthesia formed the subject of the study. The animal was administered with meloxicam @ 0.5 mg/kg body weight intravenously for pre-emptive analgesia. Thirty minutes later, xylazine @ 0.02 mg/kg and butorphanol @ 0.05 mg/kg, were administered intravenously. Upon sedation, the animal was administered with a loading dose of lignocaine @ 1 mg/kg body weight intravenously. Immediately following the loading dose of lignocaine, anaesthesia was induced using guaiphenesin @ 50 mg/kg and ketamine @ 2mg/kg intravenously. Following induction, anaesthesia was maintained using the prepared guaiphenesin-ketamine-lignocaine-butorphanol anaesthetic mixture as continuous rate infusion @ 3 ml/kg/hr. Indirect and direct blood pressure measurements were monitored and recorded. The non-invasive blood pressure values for systolic, diastolic and mean blood pressures did not differ significantly ($p > 0.05$) from those monitored invasively.

Keywords: Anaesthesia, non-invasive blood pressure, invasive blood pressure, calf, intravenous anaesthesia

Every anaesthetic drug causes changes in the homeostasis of the animal hence warranting constant monitoring of anaesthetised patient. Of the many parameters measured, blood pressure is the key to understand patients' cardiovascular status. Hypotension, a common

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drawback of most anaesthetic drug, if left unmonitored can lead to detrimental effects for the patient during and after surgery. Arterial blood pressure can be measured by two techniques - invasive (direct) and non-invasive (indirect) methods. Invasive blood pressure (IBP) monitoring is reported to be the most accurate method for blood pressure monitoring (Afshar *et al*, 2005). High cost of equipment, requirement of technical skill in catheterising arteries and calibrating transducers are the constraints involved in usage of IBP in field conditions. Non-invasive blood pressure (NIBP) monitoring is an alternative to invasive blood pressure monitoring. It is economical and requires minimal technical knowledge with easy applicability in field practice. But the debate on reliability of blood pressure values monitored non-invasively keeps away the field practitioner from depending on non-invasive blood pressure monitoring for routine practice. The present study plays on record a comparative evaluation of blood pressures monitored non-invasively to that monitored invasively by arterial catheterisation.

A six month old cross bred calf weighing 48.5 kg, presented to the Teaching Veterinary Clinical Complex, Pookode with a swelling on lateral abdomen and diagnosed with lateral abdominal hernia, classified as ASA class I which underwent herniorrhaphy under general anaesthesia formed the subject of the study. Following fasting, the animal was administered with meloxicam @ 0.5 mg/kg body weight intravenously for pre-emptive analgesia. Thirty minutes later, xylazine @ 0.02 mg/kg and butorphanol @ 0.05 mg/kg, were administered intravenously. Upon sedation, the animal was administered with a loading dose of lignocaine @ 1 mg/kg body weight intravenously. Immediately following the loading dose of lignocaine, guaiphenesin @ 50 mg/kg and ketamine @ 2mg/kg intravenously were used for induction of anaesthesia. Intubation using suitable size endotracheal tube was carried out and then connected to a fresh oxygen supply using a Boyle's apparatus. Following induction, anaesthesia was maintained using the prepared guaiphenesin-ketamine-lignocaine-butorphanol anaesthetic mixture as continuous rate infusion @ 3 ml/kg/hr so as to deliver

guaiphenesin @ 150 mg/kg/hr, ketamine @ 6 mg/kg/hr, lignocaine @ 3 mg/kg/hr and butorphanol @ 21 mcg/kg/hr. Indirect blood pressure measurements were made using an NIBP cuff of width approximately 40 percent of the circumference of the base of the tail, wrapped around the tail base and connected to the multipara patient monitor for monitoring the non-invasive blood pressure throughout the anaesthesia. The NIBP cuff was applied over the coccygeal artery. A 22G catheter was placed into the auricular artery, following aseptic preparation of the site and was then secured using tape. The catheter was then connected to a blood pressure transducer (disposable) through a heparin saline packed tubing (Fig. 1). Care was taken to place the transducer at the same level as that of the heart. The apparatus, with respect to the local atmospheric pressure, was then zeroed. NIBP and IBP were monitored and recorded at every 15 minute till recovery.

The non-invasive mean blood pressure values after induction and every fifteen minutes thereafter till recovery ranged from 78 to 117 mmHg with a mean \pm SE of 93.50 ± 5.86 mmHg, while the invasive mean blood pressure values varied from 93 to 108 mmHg with a mean \pm SE of 102.83 ± 2.79 mmHg. The non-invasive diastolic blood pressure values after induction and every fifteen minutes thereafter till recovery ranged from 65 to 91 mmHg with a mean \pm SE of 74.3 ± 4.43 mmHg, while the invasive diastolic blood pressure values varied from 79 to 94 mmHg with a mean \pm SE of 85.66 ± 2.53 mmHg. The non-invasive systolic blood pressure values after induction and every fifteen minutes thereafter till recovery ranged from 104 to 151 mmHg with a mean \pm SE of 124.6 ± 7.33 mmHg, while the invasive systolic blood pressure values varied from 115 to 130 mmHg with a mean \pm SE of 123.5 ± 2.70 mmHg. The non-invasive blood pressure values for systolic, diastolic and mean blood pressures - did not differ significantly ($p > 0.05$) from those monitored invasively. The co-efficient of variation for the values studied were less than 20 per cent, suggesting that the experiment was reliable.

The values obtained for invasive and non-invasive blood pressure are illustrated in Table 1.

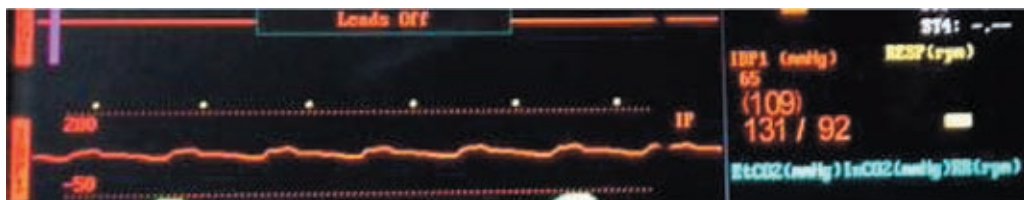
Table 1: NIBP and IBP values obtained during anaesthesia

Time	Non-invasive blood pressure monitoring			Invasive blood pressure monitoring		
	Systolic	Diastolic	Mean	Systolic	Diastolic	Mean
After induction	151	91	117	122	82	102
15 minutes later	141	64	81	130	91	108
30	122	82	102	132	94	112
45	111	67	90	118	80	98
60	119	77	93	124	88	104
75	104	65	78	115	79	93
Mean	124.6	74.3	93.50	123.5	85.66	102.83
SE	7.33	4.43	5.86	2.70	2.53	2.79

This study compared the efficiency of invasive and non-invasive methods of blood pressure monitoring in large animals. According to Glen (1970), the NIBP cuff placement over the tail was found to be the most effective for the measurement of non-invasive blood pressure in cattle. Our findings of non-significant difference between NIBP and IBP validates the use of NIBP in farm settings as IBP requires experienced technicians. The findings are consistent with studies conducted in small animals which show that NIBP and IBP have minimal variations (Olsen *et al.* 2016). The comparative findings

from the present study were in accordance with those reported by Nout *et al.* (2002) in foals. But it is in contradiction to the findings by Aarnes *et al.* (2014) where he concluded from his study on 38 cattle that NIBP is not an efficient alternative to IBP and this could be attributed to the tight fit of NIBP cuff around the tail used for the present study.

Blood pressure measurement is crucial for monitoring in animals. Invasive blood pressure monitoring is the gold standard for monitoring blood pressure but lack of expert technicians and equipment can hinder its usage in the field conditions. Non-invasive blood pressure measurement can be taken as an alternative to invasive blood pressure measurement and can be utilised to monitor changes in a healthy animal undergoing surgery. It should be noted that NIBP is a reliable alternative which do not differ significantly from IBP and could be used for routine practice.

**Fig. 1:** Ear cannulation for IBP measurement**Fig. 2:** IBP reading

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A case study on therapeutic management of a cross-bred downer cow



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Abstract

A five year old cross bred cow, 24 hours post calving was presented to Teaching Veterinary Clinical Complex (TVCC), Pookode, with the history of recumbency 24 hours post calving. The serum biochemical parameters of calcium, phosphorus and potassium on the first day of presentation were 7.93 mg/dL, 3.82 mg/dL and 3.67 mEq/L, respectively. Tentatively the condition was diagnosed as downer cow with metabolic origin. The cow was treated with calcium borogluconate, flunixin meglumine and inj. Vitamin B₁, B₆, B₁₂, sodium acid phosphate, vitamin ADE, Selenium, Biotin liquid for 7 days. Along with this, potassium chloride was administered for two days. The supportive treatment was done with lifting of cow using cow sling. Sand bedding was provided along with physiotherapy of rolling of the recumbent cow alternate ways six times a day. The serum biochemical parameters became normal on 8th day and the cow recovered by 13th day.

Key words: Downer cow, cow sling, Potassium Chloride

Downer cows are the cows which are recumbent for more than 24 hours (Constable *et al.*, 2017). Delay in treatment and management results in pressure induced myositis and neuritis, and leads to poor prognosis. A case of successful recovery of a cross bred downer cow is reported. The cow was presented to TVCC, Pookode, 24 hours post calving with a history of sternal recumbency, crawling on forelimbs and inability to stand. The cow was able to hold its head up, exhibited wagging of tail with perineal response (Van Metre *et al.*, 2001). No musculo-skeletal abnormalities were observed on physical examination. The cow was alert and clinical examination

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revealed normal temperature (100.9° F), respiration (24/minute), rumen motility (2/5 minutes), elevation of heart rate (86 beats / minute). Haematological parameters viz:- Total erythrocyte count (TEC) 8.77×10^6 / μ L, total leukocyte count (TLC) 9.3×10^3 / μ L, different leukocyte count (DLC) viz:- granulocyte 24 per cent, monocyte six per cent and lymphocyte 70 per cent, haemoglobin (Hb) 10.89 g/dL and packed cell volume (PCV) 26.3 per cent respectively. Serum biochemical parameters viz :-calcium (7.93 mg/dL), phosphorus (3.82 mg/dL) and potassium (3.67 mEq/L) were below the normal. Muscle weakness might have been due to reduced calcium which resulted in recumbency leading to downer cow syndrome. Hypophosphatemia may be due to increased activity of parathyroid hormone and loss of phosphorus through milk. Hypokalemia might be attributed to reduction in the resting potential of cell membrane which resulted in decreased excitability of neuromuscular tissue and muscle weakness (Radostits *et al.*, 2007).

The cow was treated with 225 ml of 25 per cent calcium borogluconate as slow IV, once daily for two days (Constable *et al.*, 2017), potassium chloride @ 120 gm twice daily orally for three days (Constable *et al.*, 2013 ; Beder *et al.*, 2020), flunixin meglumine @ 1.1 mg/ kg body weight once daily IV, for three days (Jesse *et al.*, 2016) and Inj. vitamin B₁, B₆ and B₁₂ -15 ml daily IV, sodium acid phosphate @ 60g, orally once daily and supportive treatment by vitamin ADE, Selenium, Biotin liquid @ 10 ml orally, once daily for seven days. Soft bedding was provided with sand and the animal was lifted using cow sling once daily. The recumbent cow was rotated alternatively six times a day, the skin wounds due to recumbency were cleaned daily and herbal wound healing ointment was applied locally. Green grass, dairy cattle feed, drinking water were provided as per requirement of the animal. Physiotherapy was given with body massage to improve the blood circulation and neurological reflexes (Huxley *et al.*, 2010). During the course of treatment, the cow was milked twice daily. The animal recovered eventually on 13th day, similar observation was reported by Poulton *et al.*, 2016.



Fig: 1 Downer cow on sling

Summary

In this study, the post-parturient downer cow with reduced serum calcium, phosphorus, potassium was treated with calcium borogluconate, flunixin meglumine @ 1.1 mg/ kg body weight i/v for three days, Inj. Vitamin B₁, B₆, B₁₂, sodium acid phosphate and vitamin ADE, Selenium, Biotin liquid for 7 days and potassium chloride for two days along with the routine physiotherapy, lifting the cow and massaging which made the animal to stand on its feet on 8th day. The serum calcium, phosphorus and potassium increased to be back to normal level and the cow recovered on 13th day.

It was concluded that while attending a recumbent cow without delay, with accurate diagnosis and appropriate treatment along with the supportive care such as physiotherapy, providing soft bedding with sand, changing recumbency posture by turning and lifting the animal daily using body sling with support to stand would result in complete recovery.

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