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INDIAN JOURNAL OF VETERINARY AND ANIMAL SCIENCES RESEARCH
(Formerly Tamil Nadu Journal of Veterinary and Animal Sciences)

Vol. 46 **September - October 2017** **No. 5**

Review article

Full length articles

Short Communication

Review article

THE ROLE OF *BRUCELLA* VIRULENCE PROTEINS IN SUBVERSION OF HOST INNATE IMMUNE DEFENSES

Girish K Radhakrishnan
Laboratory of Immunology and Microbial Pathogenesis,
National Institute of Animal Biotechnology, Hyderabad

Abstract

Brucella spp. are infectious intracellular bacterial pathogens found in a wide range of mammals, includinghumans, causing abortion, infertility and undulant fever. Brucellaereplicate primarily in macrophages and dendritic cells and the mechanisms by which brucellae manipulate the intracellular milieu for theirsurvival remain poorly understood. *Brucella*spp. are considered as stealth pathogens as they efficiently evade or suppress host immune responses for their chronic persistence in the host. Identification and characterization of some of the virulence proteins of brucellae have gainedinsights on how brucellaemodulate the host immune responses for their survival. This review focuses on the modulation of host innate immune responses by the identified virulence proteins of *Brucella*spp.

INTRODUCTION

Brucellosis is a world-wide zoonotic disease that accounts for huge loss to the livestock sector and poses a serious threat to public health.Brucellosis is the most frequent zoonotic disease worldwide, with over 500,000 new human infections every year(1).The disease is caused by bacteria of the genus *Brucella*, a member of the α-2 Proteobacteria (1). *Brucella*spp. are Gram-negative, facultative, intracellular bacteria that can infect a wide range of domestic and wild animals as well as humans. Six classical species were initially recognized within the genus *Brucella* viz. *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, and B. neotomae* (2).Brucellaeinvade and replicate in professional phagocytic cells such as macrophages and dendritic cells as well as in non-professional phagocytes such as trophoblasts (3-5). Brucellaedo not encode classical virulence factors and the pathogenicity of brucellaemainly relies on their ability to adapt to the environmental conditions encountered intracellularly(6, 7). The mechanisms and virulence factorsof brucellae that mediate invasion and intracellular persistence have been poorly characterized.

Innate immunity is the first level of defense against an invading pathogen and pathogen recognition is mediated by a family of transmembrane receptors called Toll/interleukin-1 like receptors

(TLR) (8-10). TLRs are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/ IL-1 receptor-like (TIR) domain (10, 11). Recognition of pathogen derived components, such as lipopolysaccharide (LPS),by the LRR domain of TLRs leads to the formation of a signaling complex at the cytoplasmic face thatresults in the activation of transcription factors, including NF-κB, and the up-regulation of several pro inflammatory cytokines (10, 12). Microbial pathogens have evolved diverse strategies to subvert the innate and adaptive immune responses for their survival in the host(13, 14). Various studies have established that *Brucella* spp. successfully suppress or evade host innate and adaptive immune responses to persist in the host(15-17).TLR4 detectsLPS from Gram negative bacteria that leads to release of pro-inflammatory cytokines, which in turn activates potent innate immune responses(18). Studies have established that Brucellae are weak inducers of TLR4 signaling due to their unconventional LPS(19). In addition to this stealthy strategy, brucellae encode some virulence proteins that efficiently attenuatehost innate immune responses (20-23). This review focuses on *Brucella*-encoded virulence proteins that modulate host innate immune defenses for their chronic persistence in the host.

TIR domain-containing protein from *Brucella* (TcpB).

TcpB is a TIR domain-containing protein encoded by the chromosome 1 of *Brucella* spp. This virulence protein is also termed as Btp1 or BtpA. TcpB constitutes 250 amino acids where the C-terminal 130 amino acid domain exhibits significant similarity to the TIR domain of eukaryotes. The existence of TIR domain-containing proteins in bacterial pathogens were first identified by Newman *et al.* 2006 by searching the bacterial genome database for proteins with homology to TIR domain-containing eukaryotic proteins(24). Subsequently, they performed detailed characterization of the TIR domain-containing protein from *Salmonella enterica* serovar Enteritidis (termed as TlpA). TlpA was shown to attenuate TLR4-mediated NF-κB activation and to induce caspase-1 activation and IL-1β secretion(24). Furthermore, TlpA was demonstrated to contribute to the virulence of *Salmonella* in macrophages and mice(24). TlpA shared significant sequence similarity with the *Brucella* TIR domain-containing protein, TcpB. Therefore, it was envisaged that TcpB may also suppress TLR4-mediated signaling. Subsequently, Salcedo *et al*. 2007 demonstrated the ability of TcpB to suppress the TLR2 signaling, which down-modulated maturation of *Brucella*-infected dendritic cells(23). TcpB specifically inhibited TLR2 and 4 signaling and it did not affect the signaling mediated by TLR9(23). Further characterization of TcpB was carried by Cirl *etal.* 2008where they have shown that TcpB is capable of suppressing secretion of TNF-α and IL-6 by mouse macrophages(20). In this paper, TcpB was reported to interact with the myD88 that functions as an adaptor protein for TLR signaling expect for TLR3(20, 25). These studies concluded that TIR domain-containing proteins attenuate TLR signaling through myD88 where it may prevent

the interaction between myD88 and the respective TLRs.

A novel property of TcpB was reported by Radhakrishnan *et al*. 2009that provided insights into the probable mechanism of action of TcpB (21). This study demonstrated that TcpB mimicsthe properties of another TLR adaptor protein TIRAP(21). TIRAP has been identified as an adaptor protein for myD88-dependent pathway for TLR2 and TLR4 receptors (11-13). TIRAP is primarily localized to the plasma membrane using its phosphatidylinositol 4,5-bisphosphate binding domain and functions as a sorting adaptor to deliver myD88 to activated TLR2 and TLR4(26). TcpB shared 20% identity and 53 % similarity with TIRAP. Their subsequent studies revealed that TcpB mimics the phosphatidylinositol phosphate (PIP) -binding property of TIRAP, and both the proteins interacted with PIP through a cationic motif at the N-terminus(21). A mutation in the PIP-binding domain of TcpB affected its NF-κB suppression activity. Furthermore, it has been demonstrated that TcpB requires an intact TIR domain for its functional activity as a point mutation in the BB-loop region of TcpB ($TcpB^{G158A}$) abolished its NF-κB suppression activity(21). Based on these studies, it was hypothesized that TcpB may target TIRAP to inhibit TLR2 and TLR4-mediated signaling. Subsequently, Sengupta *et al.*2010demonstrated that TcpB induced the targeted polyubiquitination and degradation of TIRAP(22). TcpB did not promote ubiquitination and degradation of other TLR adaptor protein including myD88. It has also been shown that TcpB specifically targeted the signally competent form of TIRAP for degradation. These studies further confirmed that TcpB targets TIRAP to subvert the TLR2 and 4 signaling. TcpB is a cell permeable protein and the permeability is attributed to its N-terminal protein transduction domain(27). Studies have shown that TcpB could be efficientlyinternalized by macrophages and the endogenous TcpB could inhibit the degradation of IκB, which resulted in the attenuation of NF-κB activation(27).

A recent paper by Jakka *etal.* 2017demonstrated that TcpB induces degradation of inflammatory caspases that leads to the attenuation of non-canonical inflammasome activation in macrophages(28). Inflammasomes are essential components of innate immunity, which is the first line of defense against invaded microorganisms(29). Inflammasomes are comprised of Pathogen Recognition Receptors, ASC adaptor molecule and caspase-1(29). In the non-canonical inflammasome activation, murine caspase-11 and its human orthologue, caspase-4 serve as the receptor that recognizes intracellular LPS from Gram – ve bacterial pathogens(30). TcpB interacted with human caspase-4 and promoted ubiquitination and degradation of caspase-1, 4 and 11 that attenuated LPS-induced non-canonical inflammasome signaling(28). It appears that TcpB suppresses both intra- and extracellular LPS–mediated inflammatory responses.

BtpB

Brucellae encode a second TIR domain-containing protein termed as BtpB, which is composed of 292 amino acids in *B. abortus* 9-941(31). BtpB was

shown to inhibit TLR2, 4,5 and TLR9 – mediated signaling(31). BtpB interacted with myD88 and it was hypothesized that BtpB targets myD88 for the suppression of TLR signaling. The BtpB was shown to translocate into the host cells and to interfere with the maturation of dendritic cells. Mice infection studies suggested that BtpB contributesto the virulence and control of local inflammatory responses.

VceC

VceC has been identified as the substrate of Type IV secretion system of *Brucella*spp.(32). It has been demonstrated that brucellae secrete VceC in the infected macrophages(32). The VceC protein of *B. abortus* contains 418 amino acids, with a proline-rich central domain. C-terminal 20 amino acid region is essential for translocation of VceC into the host cells(32).A recent study by Marijke Keestra-Gounder *etal*. 2016reported that VceC is involved in the induction of ER stress that leads to inflammation and production of IL-6(33). The nucleotide-binding oligomerization domain (NOD)-like receptors are innate immune receptors that detect tissue damage or microbial infection, which leads to inflammatory responses(34). NOD1 and NOD2 signalling pathways are essential for VceC-dependent induction of pro-inflammatory responses(33). Mice infection studies showed that VceC inflammation contributes to placentitis and viability of pups during the *B. abortus* infection(32).

In summary, brucellae encode essential virulence proteins that modulate the host inflammatory responses for their chronic persistence in the host. TcpB and BtpB suppresshost innate immune defenses where as VceC induces pro-inflammatory responses in the host. These virulence factors are translocated into the infected cells and play vital role in the virulence of *Brucella* spp.The activation or suppression of pro-inflammatory responses of host through the virulence proteins may help brucellaeto successfully complete various stages of their infection cycle for chronic persistence in the host.

ACKNOWLEDGEMENTS

GRacknowledges funding from the Department of Biotechnology, Ministry of Science and Technology, Government of India, through the National Institute of Animal Biotechnology (NIAB), Hyderabad,and Dr. Sathya Velmurugan, NIAB, for proofreading the review article.

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Full Length Research Articles

ANTIMICROBIAL SUSCEPTIBILITY OF *ESCHERICHIA COLI* ISOLATED FROM CHICKEN MEAT

Kalaiselvi L*, Karthick Venkatesh P, Ramesh S and K.V.Venkateswaran
Department of Veterinary Pharmacology and Toxicology
Madras Veterinary College, Chennai-600 007,
Tamil Nadu Veterinary and Animal Sciences University

ABSTRACT

The study was conducted to evaluate the antimicrobial resistance in *Escherichia coli* strains isolated from chicken meat. One hundred and five chicken meat samples were collected from retail broiler outlets in Chennai, Tamil Nadu, India and processed for bacteria isolation. Thirty one *E. coli* strains were isolated and the isolated strains were tested for antimicrobial susceptibility. All isolates showed resistance to two or more antimicrobials. The isolates exhibited resistance to ampicillin (93.55%), ampicillin-sulbactam (67.74%), amoxicillin-clavulanate (100%), erythromycin (77.42%), tetracyclines (61.29%) and co-trimaxazole (64.52%). Multidrug resistance was observed in 29 (93.55%) isolates. The results highlight the prevalence of multidrug resistance *E. coli* strains which is a serious threat to public health. Hence, the surveillance system should be strengthened and antimicrobial drug usage in the poultry industry should be optimized to prevent emergence and spread of antimicrobial resistance.

Key Words: *Escherichia coli* – Poultry - Antibacterial activity – Zone of Inhibition - Resistance

INTRODUCTION

Antimicrobials are used in livestock and poultry industry for treatment of various infectious diseases and as growth promoters. The global consumption of antibiotics in food animals was estimated to be 63,151 (±1,560) tons in 2010. India accounts for 3% of the global consumption and is the fourth highest in the world, behind China (23%), the United States (13%) and Brazil (9%) (Boeckel, et al., 2015). In poultry industry, antibiotics are used for disease treatment, disease prevention and for growth promotion. In a survey conducted among poultry farmers in India, it was reported that 67% of them used antibiotics as growth promoters, rather than solely for disease treatment and prevention (Brower et al., 2017). The drugs which are commonly used as growth promoters include oxytetracycline, chlortetracycline, bacitracin, furazolidone, virginiamycin, tiamulin, tylosin,and enramycin.

Development and spread of antimicrobial drug resistance is a global problem with serious health and economic

* Corresponding author: Email: kalaiselvivet@gmail.com

consequences. The imprudent use of antimicrobials is one of the major driving forces for selection and emergence of drug resistant pathogens. The emergence of resistant bacteria in livestock and poultry is considered to be a major public health threat since it can be transmitted to human through food chain or by direct contact and the infections caused by these drug resistant pathogens are very difficult to treat. *Escherichia coli* is a commensal organism of the gastrointestinal tract and many strains are highly pathogenic causing several diseases in poultry and human. The pathogenic strains of *E. coli* cause respiratory and septicaemic diseases in poultry including fibrinous air sacculitis, polyserositis, chronic granulomatous inflammation. Colibacillosis caused by *E. coli* is one of the leading causes for increased mortality and economic loss in poultry industry (Huang *et al*., 2009). Antibiotics are very important for treatment of these infections and some drugs are added as feed supplements. The frequent use of the drugs can lead to development of multidrug resistant isolates. The usage of antibiotics selects resistance not only in pathogenic strains but also in endogenous commensal organisms. The development of resistance is a growing concern also from a zoonotic point of view and needs attention. The emergence of antimicrobial resistance should be monitored continuously and the resistance data obtained from these studies will be highly useful to formulate recommendations for rational antibiotic use to prevent emergence of resistance. Besides that, *E. coli* is highly capable of acquiring and transferring antimicrobial resistance genes and it is considered to be good bio-indicator model for surveillance of antimicrobial resistance (Aljah *et al.,* 2007). The main objective of the study was to isolate *Escherichia coli* strains from poultry meat and to evaluate their susceptibility and resistance patterns to antimicrobials.

MATERIALS AND METHODS

Sample collection

One hundred and five chicken meat samples were collected from the retail outlets in Chennai, Tamil Nadu, India. The source of broiler for these retail outlets includes both organized and unorganized farms. Fresh meat samples were collected immediately after slaughter in sterile plastic envelopes. They were stored at 4° C until processed.

Isolation and identification of *E. coli*

One gram of minced meat sample was inoculated into 9 ml of sterile nutrient broth and incubated overnight at 37°C. The nutrient broth samples which showed growth were then inoculated into two selective medias, *viz*., MacConkey agar plates and Eosin Methylene Blue agar plates. The isolated strains were confirmed by the following biochemical tests: Oxidase, TSI, Urease, Motility, Catalase, Indole, Simmons Citrate, Methyl Red and Voges Proskauer.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for the isolated organisms were performed as per Kirby–Bauer disk diffusion method as recommended by the Clinical Laboratory Standards Institute guidelines (CLSI, 2015). The following commercially available antimicrobial disks (HiMedia Labs, India)

were used: Ampicillin (10 µg/disc); Ampicillin + Sulbactam (10/10 µg/disc); Amoxycillin + Clavulanic acid (20/10 µg/disc); Ceftriaxone (30 µg/disc); Gentamicin (10 µg/disc); Enrofloxacin (10 µg/disc) ; Erythromycin (15 µg/disc); Tetracyclines (30 µg/disc); Chloramphenicol (30 µg/disc); Co-Trimoxazole (1.25/23.75 µg/disc).

After 16 to 18 hours of incubation at 37°C, the diameters of the zones of inhibiton were measured to the nearest whole millimetre using Antibiotic zone scale. The sizes of the zones of inhibition were interpreted as per CLSI guidelines (CLSI, 2016) and the organisms were reported as susceptible, intermediate, or resistant to the antimicrobial drug tested.

RESULTS AND DISCUSSION

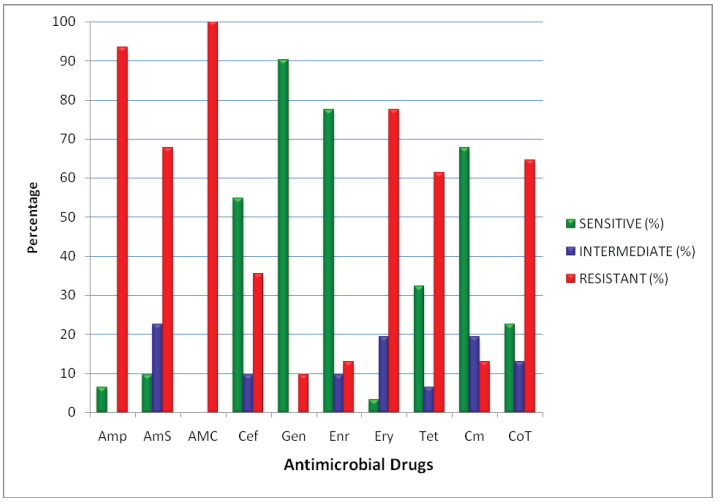
Thirty one isolates *of E. coli* were recovered from the chicken meat samples and the prevalence rate of *E. coli* in chicken meat was found to be 29.52%. The isolates were tested for their susceptibility towards 10 antimicrobial drugs representing different antimicrobial classes and their sensitivity and resistant profile of isolates were given in Table 1 and Fig 1.

Table 1 Antimicrobial susceptibility of *Escherichia coli* (n=31) isolated from chicken meat

ANTIMICROBIAL AGENT	Zone Diameter Interpretive Criteria (CLSI, 2012)	No. of isolates (%)		
		SENSITIVE (S) (%)	INTERMEDIATE (I) (%)	RESISTANT (R) (%)
Ampicillin	**S -** ≥ 17 **I -** 14-16 **R -** ≤ 13	2 (6.45)	0	29 (93.55)
Ampicillin + sulbactam	**S -** ≥ 15 **I -** 12-14 **R -** ≤ 11	3 (9.68)	7 (22.58)	21 (67.74)
Amoxycillin + Clavulanic acid	**S -** ≥ 18 **I -** 14-17 **R -** ≤ 13	0	0	31 (100)
Ceftriaxone	**S -** ≥ 23 **I -** 20-22 **R -** ≤ 19	17 (54.84)	3 (9.68)	11 (35.48)
Gentamicin	**S -** ≥ 15 **I -** 13-14 **R -** ≤ 12	28 (90.32)	0	3 (9.68)
Enrofloxacin	**S -** ≥ 20 **I -** 17-19 **R -** ≤ 16	24 (77.42)	3 (9.68)	4 (12.90)

Erythromycin	**S -** ≥ 18 **I -** 14-17 **R -** ≤ 13	1 (3.23)	6 (19.35)	24 (77.42)
Tetracyclines	**S -** ≥ 15 **I -** 12-14 **R -** ≤ 11	10 (32.26)	2 (6.45)	19 (61.29)
Chloramphenicol	**S -** ≥ 18 **I -** 13-17 **R -** ≤ 12	21 (67.74)	6 (19.35)	4 (12.90)
Co-Trimoxazole	**S -** ≥ 16 **I -** 11-15 **R -** ≤ 10	7 (22.58)	4 (12.90)	20 (64.52)

Fig 1. Susceptibility pattern of *E. coli* isolates



Amp – Ampicillin; AmS – Ampicillin-Sulbactam; AMC – Amoxycillin-Clavulanic acid; Cef – Ceftriazone; Gen – Gentamicin; Ery – Erythromycin; Enr – Enrofloxacin; Tet- Tetracycline; Cm – Chloramphenicol; CoT – Co-Trimoxazole

Among the antimicrobial agents tested, the isolates showed complete resistance (100%) to amoxicillin-clavulanic acid. Following amoxicillin-clavulanic acid, the isolates showed high level of resistance to ampicillin (93.55%). The ampicillin resistant isolates were also found to be resistant to amoxicillin-clavulanic acid.

In comparison to other antimicrobials, isolates were highly susceptible to gentamicin and more than 65% of the isolates were found to be sensitive to enrofloxacin (77.42%) and chloramphenicol (67.74%). The isolates exhibited high level of resistance to erythromycin, tetracyclines and co-trimoxazole.

The drug resistance pattern of the isolates was given in Table 2. It was observed that 31.03% of the isolates were resistant to 6 antimicrobials and 17.24% were resistant to 5 antimicrobials. Most of this isolates exhibited unique resistance pattern and multidrug resistance was observed in 29 (93.55%) isolates and they are resistant to at least 3 antimicrobials.

Table 2 Prevalence of multiple antimicrobial drug resistant pattern in *E. coli* isolates

No. of antimicrobials resistant	Resistance Pattern	Frequency
0	-	0
1	-	0
2	Amp-AMC	2
3	Amp-AMC-Cef, AMR-Ery-Tet	1:1
4	Amp-AmS-AMC-CoT; Amp-AmS-AMC-Ery; Amp-AMC-Cef-CoT; Ams-AMC-Ery-CoT	1:2:1:1
5	Amp-AmS-AMC-Tet-CoT; Amp-AMC-Ery-Tet-CoT; Amp-AmS-AMC-Cef-Ery; Amp-AMC-Ery-Tet-Cm; Amp-AmS-AMC-Ery-CoT	1:1:1:1:1
6	Amp-AMC-Cef-Ery-Tet-CoT; Amp-AmS-AMC-Ery-Tet-CoT; Amp-AmS-AMC-Cef-Tet-CoT; Amp-AmS-AMC-Ery-Tet-Cm; Amp-AmS-AMC-Cef-Ery-CoT; Amp-AMC-Cef-Ery-enr-CoT;	2:4:1:2:1:1
7	Amp-AmS-AMC-Cef-Ery-Tet-CoT; Amp-AMC-Cef-Ery-Enr-Tet-CoT; Amp-AmS-AMC-Gen-Ery-Tet-CoT	2:1:1
8	-	0
9	Amp-AmS-AMC-Cef-Gen-Ery-Enr-Tet-CoT Amp-AmS-AMC- Gen-Ery-Enr-Tet-Cm-CoT	1:1

Amp – Ampicillin; Ams – Ampicillin-Sulbactam; AMC – Amoxycillin-Clavulanic acid; Cef – Ceftriazone; Gen – Gentamicin; Ery – Erythromycin; Enr – Enrofloxacin; Tet-Tetracycline; Cm – Chloramphenicol; CoT – Co-Trimoxazole

The prevalence and frequency of drug resistance observed in our study agrees with the findings of Miles *et al.* (2006) and they have reported high level of resistance to tetracycline (82.4%) and low level of resistance to gentamicin and enrofloxacin. Similarly, Fofana *et al.* (2006) reported the prevalence of drug-resistant *E. coli* in chicken meat with high levels of resistance to ampicillin, tetracycline, trimethoprim-sulfametoxazole and low levels of resistance to gentamicin.

In poultry reared in flocks, antimicrobials are administered in flocks rather individually. Antimicrobials are also

fed continuously as growth promoters. Hence, the selection pressure for resistance development is high in poultry. At slaughter, bacterial strains from gut may contaminate poultry carcass and these may harbor pathogenic bacteria (Miles *et al*., 2006). Poultry farmers, slaughter house workers and Veterinarians who have direct contact with birds or poultry carcasses are at a greater risk of acquiring these resistant strains. The infections caused by these resistant strains are very difficult to treat and can have grave consequences.

In human medicine, amoxicillin-clavulanate, ciprofloxacin, gentamicin, ceftriaxone, trimethoprim sulfamethoxazole, are widely recommended for treatment of infections caused by *E. coli.* The high level of resistance observed in this study for amoxicillin-clavulanate and trimethoprim-sulfamethoxazole is of great concern. These drugs are considered as key access antibiotics (WHO, 2017a) and are first choice of drugs used in the treatment of *E. coli* infections and hence their usage in Veterinary medicine should be monitored and rationalized. Ceftriaxone is a critically important antibiotic in human medicine and is recommended for specific, limited indications (Collignon *et al.,* 2009). In the recent World Health Organization report on essential medicines, ceftriaxone is categorized as watch group antibiotics that are at high risk for selection of bacterial resistance (WHO, 2017a). In the WHO global priority pathogen list, third generation cephalosporin-resistant *E. coli* are reported as critical and high priority pathogen that pose health threat and warrants new drug alternative (WHO, 2017b). Development of resistance even at low levels for ceftriaxone is a cause for alarm and its usage in food producing animals should be limited. Gentamicin, enrofloxacin and trimethoprim-sulfamethoxazole have satisfactory activity against *E. coli* and caution must be exercised while using these drugs for maintaining long term utility in the field. Erythromycin is mainly active against gram-positive bacteria but the development of resistance in *E. coli* for erythromycin can confer cross resistance to other macrolide antibacterials like azithromycin and clarithromycin, which are used to treat Gram-negative infections in human beings (NCDC guidelines, 2016).

Considering the health risk due to emergence of bacteria resistant to antimicrobials has led various authorities worldwide to implement measures to decrease antimicrobial use in livestock production (Lhermie *et al*., 2016) and many countries have banned the use of growth promoters in food producing animals. (Lekshmi *et al*., 2017).

The development of resistance is an issue of great concern. It is recognized that there is an urgent need to emphasize their rational and judicious use of antimicrobials and to avoid the misuse of available antimicrobials. Non-therapeutic use of medically important antimicrobials in food animal production should be totally curbed. Furthermore, regular surveillance system should be strengthened. The data obtained from our study points to an emerging trend of resistance. Results from such studies will largely help in identifying the pattern of resistance which would help in formulation of suitable measures to combat this great menace.

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INFLUENCE OF SEASON ON PLASMA BIOCHEMISTRY OF EMU BIRDS

***G. Suganya and V. Leela[1]**
Department of Veterinary Physiology, Madras Veterinary College, Chennai -7
Tamil Nadu Veterinary and Animal Sciences University, Chennai

ABSTRACT

The emus are primarily monogamous and seasonal breeders. Blood biochemical parameters and hormones are known to influence production and reproduction in emus. There are no literatures available on the study of blood biochemical parameters during the different periods of breeding seasons. Hence, an attempt was made to evaluate the plasma biochemical profile of adult emu birds during different periods of seasons. The study was conducted in ninety apparently healthy emu birds (forty five of each sex) of 5 to 6 years of age, reared at a commercial emu farm in Tamil Nadu. Blood samples were collected in heparin coated tubes during the pre-breeding, breeding and post-breeding seasons. Plasma was separated by centrifugation and biochemical parameters such as total protein, albumin, total cholesterol, LDL cholesterol and HDL cholesterol were analyzed using the commercially available kits. The albumin: globulin ratio was then calculated. The experimental data were statistically analyzed by one – way analysis of variance (ANOVA) and post hoc analysis were carried out using Duncan's test for multiple comparisons using SPSS software version 20 for windows. The results revealed that during the pre-breeding season, the globulin and HDL cholesterol concentrations were significantly higher, whereas, during the breeding season, the total protein, albumin concentration and the albumin: globulin ratio was significantly higher and the total cholesterol and LDL cholesterol concentrations were found to be significantly higher during the post-breeding season. Analysis of plasma biochemistry helps in understanding the reproductive functions of the emu birds.

Keywords: Emu, breeding season, plasma, biochemistry

INTRODUCTION

Emus are ratites reared primarily for their tender, delicious, low cholesterol meat and the oil which has therapeutic and cosmetic properties. The emus are primarily monogamous and seasonal breeders. The annual cycle of reproductive functions and the biochemistry that regulates their reproductive patterns is essential for understanding and controlling the breeding activity of the captive emus. Emus are short day breeders and in India, the breeding season starts from October to March. Emus reduce their feed intake during the breeding season and lose a significant portion of their body weight during the process. Emus exhibit peculiar reproductive cycle that the

*Corresponding author, Email id: drsuganya_vet@rediff.com
*Assistant Professor, [2]Professor and Head,Part of Ph.D Thesis of First Author

male incubates the egg and the incubation period ranges from 52 to 56 days.

Blood biochemical parameters are considered as true indices of the health status of the subject. Blood biochemistry are known to be influenced by various factors such as diseases, nutritional status, body condition, sex, age, diet, circadian rhythms, captivity etc (Quintavalla *et al*., 2001). As clinical signs of diseases in poultry and ratites are very subtle and confusing (Black and Glatz, 2011), for more accurate diagnosis, it is important to establish standard values for the various blood parameters and interpret them according to age, sex, physiological state and stress level (Fudge, 2003). There are very little information available on the plasma biochemistry of emu birds during the pre-breeding, breeding and post-breeding seasons, an attempt was taken to study the biochemical parameters during the different seasons.

MATERIALS AND METHODS

The study was conducted in Emu birds maintained at commercial emu farms in Tamil Nadu. The birds were reared under intensive system with standard management practices. Blood samples were collected from ninety adult emu birds (45 male and 45 female) of 5 to 6 years of age to study the influence of pre-breeding, breeding and post-breeding seasons on biochemical parameters. From each bird, about 5 ml of blood was collected from the right jugular vein (Reddy *et al.,* 2003) in heparin coated tubes and was centrifuged at 3000 rpm for 15 minutes for the separation of plasma. The separated plasma samples were stored in aliquots at – 20 ^{o}C till further analysis. The biochemical parameters such as total protein, albumin, total cholesterol, LDL cholesterol and HDL cholesterol were analyzed using the commercially available kits. The albumin: globulin ratio was then calculated.

STATISTICAL ANALYSIS

The experimental data were statistically analyzed by one – way analysis of variance (ANOVA) and post hoc analysis were carried out using Duncan's test for multiple comparisons using SPSS software version 20 for windows.

RESULTS AND DISCUSSION

The influence of season on the plasma biochemical parameters in emu birds is presented in Table 1.

Table 1. Influence of seasons on plasma biochemical parameters in emu birds

Seasons	TP (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)	HDL cholesterol (mg/dl)
Pre-breeding (n = 30)	6.27 ± 0.10^{ab}*	2.78 ±0.06 ^{a}	3.49± 0.10^{b}*	0.82± 0.03 ^{a}	128.16± 3.45 ^{a}	53.29 ± 1.76 ^{a}	60.93± 1.53 ^{b}**
Breeding (n = 30)	6.37 ± 0.07^{b}*	3.23 ±0.08 ^{c}**	3.13± 0.11^{a}	1.11± 0.08 ^{b}	130.86± 4.17 ^{a}	50.01 ± 2.33 ^{a}	55.25± 2.28 ^{a}
Post-breeding (n =30)	6.10 ± 0.03 ^{a}	2.97 ±0.02 ^{b}	3.12± 0.04 ^{a}	0.95± 0.02 ^{a}	147.73±3.00 ^{b}**	81.08 ± 2.16 ^{b}**	51.19± 1.41 ^{a}
Pooled Mean ± SE (n = 90)	6.25 ± 0.04	2.99 ±0.04	3.25± 0.05	0.96± 0.03	135.58± 2.24	61.46 ± 1.90	55.79± 1.10

**- Highly significant (P < 0.01), *- Significant (P < 0.05)

Mean values having same superscript within a column do not differ significantly

Influence of season on total protein, albumin and globulin

The overall mean total protein concentration during the different seasons in the emu was 6.25 ± 0.04 g/dl. The total protein concentration was significantly higher ($P < 0.05$) during the breeding season (6.37 ± 0.07 g/dl) in comparison with pre-breeding and post-breeding season. The overall plasma albumin and globulin concentrations during different season were 2.99 ± 0.04 g/dl 3.25 ± 0.05 g/dl. The plasma albumin concentration was significantly higher ($P < 0.01$) during the breeding season (3.23 ± 0.08 g/dl) than that of post-breeding and pre-breeding seasons. Whereas, the globulin concentration was significantly increased ($P < 0.05$) with a value of 3.49 ± 0.10 g/dl during the pre-breeding season than that of the breeding and post-breeding seasons. The overall mean albumin: globulin ratio observed was 0.96 ± 0.03. The albumin: globulin ratio was found to be significantly increased ($P < 0.01$) during the breeding season when compared to the pre-breeding and post-breeding seasons.

An increase in the total protein and albumin concentrations were observed during the pre-breeding season and higher values were found during the breeding season, which decreased during the post-breeding season. The high plasma proteins are closely related to higher protein synthesis, which is a prerequisite to high egg production. Females of oviparous species demonstrate a marked increase in plasma total protein concentration just before egg production. This is due to estrogen induced hyperproteinemia, which is associated with an increase in vitellogenin and lipoproteins necessary for yolk production. These proteins are produced in the liver, transported in the blood and incorporated into the oocytes of the ovary (Hrabcakova *et al*., 2014).

The globulin concentration was found to be significantly higher during pre-breeding season and decreased during the breeding season and post-breeding season. Similar observation was reported by Menon *et al*. (2013) in the emu birds and Hrabcakova *et al*. (2014) in pheasant hens. The higher globulin values in the female emus could be due to the developing follicles (Lumeij, 1987) or enhanced investment in humoral immune defense in anticipation of the forth coming breeding season (Horak *et al*., 1998). In oviparous females, vitellogenin and other proteins used in egg formation increases dramatically during reproductive activity. Therefore, globulin fraction increases more than the albumin fraction, causing the albumin: globulin ratio to decrease physiologically in female emu birds in addition to egg formation causing an increased globulin concentration.

The albumin concentration and albumin: globulin ratio in female birds was found to be increased during the breeding season. The higher values could have also been due to less water intake at the onset of breeding season. The albumin: globulin ratio was found to be increased during the post-breeding season may be due to sustained increase in the albumin concentration.

Influence of season on total cholesterol

The overall mean total cholesterol concentration during different periods of

season was 135.58 ± 2.24 mg/dl. The total cholesterol concentration was significantly higher ($P < 0.01$) during the post-breeding season with a value of 147.73 ± 3.00 mg/dl when compared to pre-breeding and breeding seasons.

A decrease in the total cholesterol concentration was noticed during the pre-breeding and breeding seasons and a higher value during the post-breeding season, but the values were within the normal range by Otokie - Eboh *et al*. (1992) in the emus. Suchy *et al*. (2010) and Nazifi *et al*. (2012) observed the cholesterol values near the lower limit of normal cholesterol range for domestic fowl and common pheasants. Menon *et al*. (2013) in the emus and Hrabcakova *et al*. (2014) in pheasant hens did not observe any change in the total cholesterol levels prior to breeding.

Cholesterol is the precursor of steroid hormones and a lower cholesterol value prior to and during the breeding seasons may be due to increased utilization of cholesterol for the synthesis of hormones involved in reproduction. Cholesterol is also important for vitellogenesis and egg formation in birds and a decrease in its concentration indicate that more of cholesterol is being utilized for egg formation. An increase in the total cholesterol concentration may indicate a decrease in the estrogen and testosterone production at the termination of breeding season. A wide variation of cholesterol levels among avian species due to circadian rhythm and the effect of diet had been reported by Harr (2002) and Palomeque *et al*. (1991) in the ostriches.

Influence of seasons on LDL and HDL cholesterol

The overall mean concentration of LDL cholesterol during different seasons observed in the emu birds was 61.46 ± 1.90 mg/dl. The LDL cholesterol concentration of 81.08 ± 2.16 mg/dl was significantly higher ($P < 0.01$) in the emu birds during the post-breeding season than during the pre-breeding and breeding seasons. The overall mean HDL cholesterol concentration was 55.79 ± 1.10 mg/dl. The HDL cholesterol concentration was significantly higher ($P < 0.01$) with a concentration of 60.93 ± 1.53 mg/dl in the emu birds during pre-breeding season which gradually decreased during the breeding and post-breeding seasons.

The plasma LDL cholesterol concentration was decreased during the pre-breeding and breeding seasons and increased during the post-breeding season, whereas plasma HDL cholesterol was found to be significantly decreased during the different seasons and the lowest value was found during the post-breeding season. The cholesterol is the precursor for steroid hormone synthesis and the lower levels of cholesterol during the breeding season could be attributed to its utilization in steroid hormone synthesis.

CONCLUSION

The results of this study serve as a complementary diagnostic tool and provide physiological baseline values of biochemical parameters in the adult emu birds during different pre-breeding, breeding and post-breeding seasons.

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Short Communications

A PILOT STUDY ON CRYOPRESERVATION OF CANINE PLATELETS

Ranjith Kumar.M[1], P.Selvaraj[2], S.Prathaban, G.Dhinakar Raj and V.Maroudam

Referral Centre for Veterinary Transfusion Medicine,
Veterinary University Peripheral Hospital, Madhavaram,
Department of Veterinary Clinical Medicine, Madras Veterinary College &
TANUVAS-DBT Translational Research Platform for Veterinary Biologicals,
Tamil Nadu Veterinary and Animal Sciences University, Chennai - 600 051.

In veterinary medicine, though the need for platelet transfusions are very high, given to the thrombocytopenic crisis occurring in many blood parasitic diseases and immune mediated diseases of dogs. However, platelet transfusion is rarely used because platelet products are not readily available, compared to the red cell and plasma products.

Platelet harvesting is currently done by various centrifugation methods. Centrifugation of whole blood yields Platelet Rich Plasma (PRP) and further centrifugation of PRP can yield Platelet Concentrate (PC). The harvested platelets should have to be used immediately or within 24 hours of collection or it may be stored in a Platelet Agitator System at 22˚C. However they have to be used within 5 days and cannot be stored further with the current methods. Recently platelet cryopreservation methods are being used to provide long-term storage and to facilitate immediate availability of platelet products for transfusion without bacterial contamination during

Client owned healthy dogs, which are serving as volunteered canine blood donors and presented to the Referral Centre for Veterinary Transfusion Medicine (RCVTM),Veterinary University Peripheral Hospital and, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai-51, for periodical health assessment and for blood donation purposes were utilized for this study. Platelets were collected from healthy donors, using an advanced technique called Plateletpheresis, as per the protocols used by Selvaraj (2016). This procedure yielded high volume singe donor platelets and they served as the platelet concentrate for usage in this preservation studies. From the fresh platelet concentrate collected, 5ml of aliquot was removed from the platelet collection bag for aerobic bacterial culture and the evaluation of fresh platelet.

1: MVSc Student; Part of the MVSc Research Work
2: Corresponding Author; E-mail: chennaivet@hotmail.com

The PC was then divided into three categories: fresh, cryopreserved in 6% Dimethyl Sulfoxide (DMSO) and cryopreserved in 6 percent Hydroxy Ethyl Starch (HES). The fresh PC was stored in the original platelet collection bag itself and was stored in a Platelet Agitator System at 22˚C with periodical agitations. For 6 percent DMSO cryopreservation, 6.5 ml of 24 percent DMSO (1.5 ml 100 percent medical grade DMSO diluted with 5 ml autologous fresh plasma) was added to the 20 ml PC gradually over 20 minutes with gentle, continuous mechanical rotation at room temperature. For 6 percent HES cryopreservation, 2 volumes of 6 percent HES was added slowly to 1 volume of PC and mixed with gentle rotation at room temperature. Immediately after mixing, the cryoprotectant added PC was transferred to cryovials which was then placed in a cryocyte container with controlled cooling rate of about -1˚C per minute. These cryocyte containers were placed in a -80˚C freezer and stored for 14 days before evaluation on 7th and 14th days. The protocols followed by Callan *et al*, (2008) were used in this study.

Assessment of the platelet count and recovery was carried out. Platelet count was taken up by using an automated hematology analyzer as per the protocols of Dijkstra-Tiekstra *et al*., (2007) Cryopreserved platelets were thawed in a 37˚C water bath for 5 minutes and manually mixed gently. Before in vitro evaluation the cryopreserved platelets were washed with PBS and albumin. A 5 ml aliquot was removed from each thawed unit for *in vitro* evaluation. Within 1 hour of fresh PC collection or post-thawing, platelet counts were measured using an automated hematology analyser. PC samples were diluted (1:2 to 1:5) with Phosphate-buffered saline, as needed, to obtain a platelet count within the measurement range of the analyser. Platelet recovery was calculated with the following formula, as per the protocols of Appleman (2009):

Total Platelet Number = Platelet concentration of PC (per µl) × Volume of PC (ml) × 1000

$$\textit{in vitro}\ \text{platelet recovery (\%)} = \frac{\text{Total platelet number of thawed PC}}{\text{Total platelet number of PC prefreezing}}$$

Platelet morphology was assessed. Fresh and cryopreserved PC (50µl) was pipette onto a glass slide and cover-slipped. Platelets were then examined using a microscope with a 40X objective. Around 100 cells were counted and categorized as spherical, discoid or unclassified (not clearly discoid or spherical) Appleman (2009).

Cellular viability was assessed using the Trypan blue assay, as per the protocols of Mascotti *et al*., (2000). Trypan blue staining was used to differentiate the live and dead cells upon microscopic examination. The live cells with intact cell membrane could not be traversed by the dye but the dead cells allowed the dye into the cell membrane. For this method, 50µl PC diluted in PBS was pipetted out and added with Trypan blue dye 1:10 to 1:20 ratio. After incubation of the sample for 1-2 min, the sample was pipetted into a glass slide and cover-slipped and examined under microscope with 40X objective.

Platelet number, yield, recovery and viability

The in vitro platelet recovery, total platelet number and platelet count (per µl) of fresh and cryopreserved PC in both 6 percent DMSO and 6 percent HES are summarized in the Table-1.

Parameter	Fresh PC	6 percent DMSO PC	6 percent HES PC
Platelet count (cells per µl)			
Pre-freezing	1.78×10^6	1.42×10^6	1.60×10^6
Post thawing (7th day)	1.424×10^6	0.99×10^6	Nil
Post thawing (14th day)	Nil	0.76×10^6	Nil
Total Platelet number			
Pre-freezing	0.356×10^{11}	0.284×10^{11}	0.32×10^{11}
Post thawing (7th day)	0.285×10^{11}	0.198×10^{11}	Nil
Post thawing (14th day)	Nil	0.152×10^{11}	Nil
In vitro platelet recovery (%)			
7th day	80.0	69.71	Nil
14th day	Nil	53.52	Nil
Platelet morphology (%) 7th day			
Discoid			
Spherical	56	40	Nil
Unclassified or dead	39	49	12
14th day	5	11	88
Discoid			
Spherical	Nil	36	Nil
Unclassified or dead	Nil	50	Nil
	100	14	100
Platelet viability (%)			
7th day	Approx. 80	Approx. 70	Nil
14th day	Nil	Approx. 50	Nil

The in vitro platelet recovery was higher for 6 percent DMSO units versus 6 percent HES units. Aerobic bacterial cultures from all tested platelet units (fresh and cryopreserved units) yielded no growth.

Microscopic evaluation of fresh and PC cryopreserved in 6 percent DMSO showed no major differences in the discoid or spherical platelets. But both of them were compared with the PC cryopreserved in 6 percent HES, in which major cellular differences i.e. unclassified platelets or platelets with storage lesions had been observed.

Cellular viability studies after 7 and 14 days of storage, performed with trypan blue assay revealed that 80 percent and 50 percent of cells were viable in the PC

cryopreserved in 6 percent DMSO, and 80 percent and 0 percent of cells were viable in fresh PC stored at 22˚C with agitation. No viable cells were observed in PC cryopreserved in 6 percent HES.

Many of the emergency cases and critically ill animals in small animal practice invariably require blood transfusion at some stage of their clinical care. In terms of availability of canine blood, especially in cities like Chennai Metropolitan, which has very high pet populations, the canine blood or blood product availability is abysmally minimal. To overcome such challenges, blood component therapy can help in a better way. Appleman *et al.* (2009) observed that in most veterinary facilities, canine platelet products were not readily available and were being prepared on an "as-needed basis", and it included fresh whole blood, platelet rich plasma and platelet concentrate. They concluded that regardless of the collection method, fresh platelet products remained a limited resource in veterinary blood banks.

Indications for platelet transfusion in canine clinical practice included many conditions with severe thrombocytopenia or thrombocytopathia, which cause life-threatening hemorrhage. It is also used in anticancer therapy, in combination with chemotherapy. Hence the need for making available canine platelets, for round the year usage is high and it requires preservation. Cryopreservation of canine platelets may offer a practical and much-needed alternative. However it has many challenges. It was well established in human medicine that all cryopreservation techniques caused platelet damage known as the "storage lesions", and they included a wide array of biochemical, structural and functional alterations (Kahn, 1978).

In the current study, the PC cryopreserved in 6 percent DMSO showed 80 percent and 50 percent viability during storage for 7 and 14 days respectively. Similar findings were reported by Alving *et al.* (1997). They observed that there was a considerable loss of platelets during the freeze/thaw/wash process with all methods of platelet cryopreservation and that the same was documented as quantified by *in vitro* platelet recovery. Studies of 6 percent DMSO cryopreserved human platelets reported an *in vitro* recovery ranging from 50-80 percent (Valeri *et al.* 2005 and Balint *et al.* 2006). The recovery of canine platelets in this study was similar to these findings. Preservation using 6 percent HES did not provide any appreciable advantage for improving *in vitro* function when compared to that of PC preserved with 6 percent DMSO.

Fresh platelet concentrates stored at 22˚C with agitation, had demonstrated low platelet recovery rate and platelet viability. This was in accordance with Brecher (2005) and Vassallo and Murphy (2006), who observed that the potential of contaminating bacteria increased at such temperatures and a progressive decrease occurred in the platelet viability and function over time. Most of the frozen platelets may be unable to withstand the events that occur during freezing and thawing and the same was attributed to the osmotic fragility and poor permeability of the membrane.

The present study the use of 6 percent DMSO as a cryo-protectant was found to be better for improved platelet recoveries without causing much of damages or storage lesions. But this approach did not fully prevent the freezing-thawing injury. This study is of interest that the canine

platelets in this study were stored up to 7 days at 22˚C with gentle agitation. However, in a previous study, Klein *et al.* (1999) reported that the canine platelets can be stored up to 4 days at 22˚C. Valeri *et al.* (1986) demonstrated that the storage of canine platelets cryopreserved in 6 percent DMSO were stored at -80˚C for up to 1 year and had an in vitro recovery of 70 percent. The recovery in the present study was 53.52 percent. This was in accordance to some extent, with the findings of Valeri *et al*. (1986). However this study lacked such a long storage and post storage assessments. The DMSO method gave a higher platelet recovery than HES method in this study. One of the important factors in using the DMSO preserved platelets is that, several washing steps would be required to remove DMSO before transfusion to recipients, because of the potentially harmful nature of DMSO to the recipients.

This study indicated that the use of DMSO as a cryo-protectant with a controlled cooling rate is a simple, rapid and efficient method for canine platelet cryopreservation. But further studies are needed so to assess and reduce the cellular damages that occur during thawing and washing, in order to improve the post thaw platelet recovery and reduced transfusion reactions.

The authors are thankful to the Dean, Madras Veterinary College and the Director of Clinics, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai for the facilities provided.

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INCIDENCE OF CANINE PERIANAL TUMOURS : A RETROSPECTIVE STUDY

Chandini.G.Ashok*, Ravi Sundar George[1], Mohd. Shafiuzama[1] and Hemalatha, S.[2]

Madras Veterinary College, Chennai- 600 007

Tamil Nadu Veterinary and Animal Sciences University

The perianal region of dogs contain multiple glands, some of which are unique to the area, namely the perianal or circum-anal (hepatoid) glands, the anal sac glands and the anal glands (Moulton, 2008). Perianal (circumanal or hepatoid) glands are modified sebaceous glands present in the perianal skin (Gross *et. al*.,2008). These glands are unique non-secretory abortive sebaceous glands in dogs and marsupials, situated around the anus in a uniform circle up to 2 cm from the anal orifice and scattered areas on the prepuce, tail, hind legs and trunk.

Tumors of perianal glands are the third most common tumours among intact aged male dogs (Turek and Withrow, 2013). Perianal adenomas comprise more than 80 per cent of the perianal gland tumours and are slow growing and develop under the influence of androgens, therefore occur in older intact male dogs and occasionally in spayed females. Perianal adenocarcinomas occur in castrated or intact males, as well as in females. Apocrine gland adenocarcinomas occur more commonly in females. Management of this condition is essential to prevent patient discomfort, distress and other related complications.

Surgical excision of the tumour with appropriate margination based on the tumour type is the more prudent approach for the management of perianal tumours in canines (Morris and Dobson, 2008). In case of perianal adenomas, castration must also be performed. Electro-chemotherapy has been reported to be effective for the treatment of perianal tumours but due to the long therapeutic period, its use as an adjunct for cyto-reduction before or after surgical excision is preferred (Torzon*et al*. 2005).

A survey of all cases of canine tumours presented from May 2016 to May 2017 was included in the study. The research was carried out on clinical cases of canine perianal tumours presented to the Small Animal Surgery Out-patient Unit, Madras Veterinary College Teaching Hospital, Chennai -7. A total of n=590 dogs were screened for the presence of tumours. Among this 46 dogs had perineal tumours. Fine needle aspiration cytology was performed on all cases to confirm the type of tumour. The incidence of perianal tumour with respect to age, breed, sex and type of tumour was recorded in this study.

*Corresponding author

[1]Department of Surgery and Radiology, [2]Department of Veterinary Pathology

Email id: drchandiniashok@gmail.com

Contact number: 9176850506

A total of 590 dogs were presented to Small Animal Surgery Out-patient unit at Madras Veterinary College Teaching Hospital, with presence of tumours during the period of 13 months (May 2016 to May 2017); out of which 46 dogs (7.60%) were diagnosed to have perineal tumours and out of which 41 cases had perianal tumours (91.11%). Perianal tumours had an overall incidence of 6.93 per cent. The incidence rate was in accordance with the findings of Sostaric *et al.* (2013) who reported hepatoid tumours to account for 6.19 per cent (97 cases of 1568 cases) and Lakatos *et al.* (2009) who reported 7 per cent of perianal tumours out of 121 cases of cutaneous tumours.

In this study, the occurance of perianal adenomas was 43.90 per cent (18 cases) and perianal adenocarcinomas was 56.10 per cent (23 cases). These findings were antithetical to the findings of Turek and Withrow *et al.* (2013) who reported the occurrence perianal adenocarcinomas to be approximately 20 per cent. However, Sostaric *et al.* (2013) reported a higher incidence of perianal adenocarcinomas in his study.

In this study, the age group of dogs greater than ten years had a higher incidence (75.61 %) when compared to dogs less than 10 years of age (24.39%). The mean age of occurance of perianal tumour was found to be 11.98 ± 0.55 years. The findings in this study could be attributed to the fact that older intact male dogs had long term exposure to the male hormone testosterone. The findings of the present study were in accordance with that of Tozon *et al.* (2005), Moulton (2008), Withrow *et al.* (2013) and Yumusak *et al*. (2016) who reported that the affected dogs were mostly older in age and intact, the average being 8 to 12 years of age.

Of the 41 clinical cases of perianal tumours presented to the Small Animal Surgery Unit of Madras Veterinary College Teaching Hospital, the breed-wise distribution was as follows: Dachshund (2.43 %), Doberman Pinscher (2.43 %), German Shepherd (2.43 %), Golden Retriever (2.43 %), Rajapalayam (2.43 %), Lhasa Apso (2.43 %), Labrador Retriever (14.63 %), Spitz (26.84 %) and Non-descript canines (43 95 %). Tozon *et al.* (2005) and Yumusak *et al*. (2016) also reported high breed susceptibility in mixed breeds, Labrador Retriever, English Cocker Spaniel, Terriers, Charles Spaniel, Springer Spaniel and Border collie. This could be attributed to possible underlying individual genetic predisposition and that no specific breed susceptibility can be attributed.

With regard to the 41 cases of perianal tumours reported, 38 were males and 3 were females depicting a distribution of 92.68 per cent in males and 7.32 per cent in females. All males were intact and the females were spayed. The sex distribution of the cases concurred with the findings of Moulton (2008), Sostaric *et al.* (2013), Withrow *et al.* (2013) and Yumusak *et al*. (2016) who reported a significantly higher percentage; 5 to 6 fold increased incidence of perianal tumours in male intact dogs than in bitches. This could be attributed to androgen dependency in male dogs and the protection offered by endogenous estrogens in the females.

In the present study, a higher incidence of perianal tumour in older male dogs, showed the need for early neutering of pet dogs to prevent perianal tumour occurrence.

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EFFECT OF SUPPLEMENTATION OF FEED ADDITIVES ALONE AND IN COMBINATION ON LIVER FUNCTION TEST PROFILE IN CROSSBRED LAMBS FED OATS STRAW BASED COMPLETE DIET

Yasir Afzal Beigh[*1], Abdul Majeed Ganai[1], Haidar Ali Ahmad[1], Ishfaq Ahmad[1], Umar Amin[2], Masood Saleem Mir[2], Danish Masood[1]

Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, Jammu & Kashmir, India

In developing countries like India, small ruminants are the only source of income for small and landless farmers, roughages often form the major part of the diet for these animals, and in most cases, are their only feeding source. The majority of the world's ruminants (cattle, sheep, and goats) depend on these feeds throughout their lifetime that are often poor in quality characterized by low nutritive value. Thus, feeding of these poor quality feed resources is required to be integrated with the techniques of improving their efficiency of utilization in animal system by using various feed additives so as to derive maximum nutrients for animal body. Supplementation of feed additives in the ration of animals improves efficiency of utilization of such poor quality roughages for maximizing livestock productivity. In this context, the role of exogenous fibrolytic enzymes (EFEs) and medicinal plants as feed additives in the ration of animals is of vital significance in the countries like India. EFEs have a great potential to increase fibre digestion in the rumen, because even under ideal conditions, total tract digestibility of neutral detergent fibre in ruminants is usually less than 50% (Beauchemin *et al*., 2001), thus can enhance feed utilization and animal performance. Herbal feed additives could either influence feeding pattern or influence the growth of favorable microorganisms in the rumen or stimulate the secretion of various digestive enzymes, which in turn may improve the efficiency of utilization of nutrients, resulting in improved productive and reproductive performances (Bakshi and Wadhwa, 2000). Use of herbs as feed additives is experiencing resurgence in animal health and production.

With the demand for organic food and ban on the use of antibiotics/hormones/ arsenicals etc. in animal diets, the search for alternative feed additives has been increasing. Among non-antibiotic feed additives, the use of EFEs and herbs have ability to augment livestock production performance; but prior to advocation, information regarding their effect on

[1]Division of Animal Nutrition, 'F'VSc & AH, Shuhama, Alusteng, Srinagar-190 006

[2]Division of Veterinary Pathology, 'F'VSc & AH, Shuhama, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, Jammu & Kashmir, India

*Corresponding author: Dr. Yasir Afzal Beigh, Assistant Professor/Jr. Scientist, Division of Animal Nutrition, Faculty of Veterinary Sciences & Animal Husbandry, Shuhama, Alusteng, Srinagar-190 006, SKUAST-Kashmir, Jammu & Kashmir, India. Tel: 0194-2262217; Mob: 09419024721; E-mail: vetyasir1@gmail.com

physiological health status need to be investigated. In this endeavor, the present study was carried out to study the effect of feeding oats straw based complete feed supplemented with EFEs cocktail and Afsanteen (*Artemisia absinthium* L.) herb as feed additives alone and in combination on the liver function test profile in crossbred lambs.

Animal management and experimental feeding

Twenty growing crossbred ram lambs (4-6 months age and 11.58±0.01 kg mean BW) of uniform conformation procured from sheep unit of Mountain Research Centre for Sheep and Goat (MRCSG), Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-Kashmir, were randomly allotted to four groups having five animals in each. The lambs were stall fed individually for a period of 90 days. Animals in all the groups were offered complete feeds to meet their nutrient requirements as per ICAR (2013). The complete feeds were based on oats straw- 40 parts, mixed grass hay- 20 parts and concentrate mixture- 40 parts without (T_0) or with feed additives alone as EFEs cocktail (T_1) @ 6 g/kg DM or Afsanteen herb (T_2) @ 4.5% of DM and in combination (T_3), where the animals of group T_0 served as control maintained on the complete ration consisting of oats straw, mixed grass hay and concentrate mixture without any supplementation. The concentrate mixture consisted of crushed maize (27.5%), wheat bran (12.5%), deoiled rice bran (10%), mustard oilcake (15%), soybean (28.5%), molasses (2.5%), mineral mixture (1.5%), salt (1%) and urea (1.5%).

All lambs were kept under uniform management conditions, housed in well ventilated, hygienic and protected sheds with batten floor, and appropriate facilities for individual feeding and watering. All the animals were dosed for ecto- and endo-parasites, and vaccinated against prevalent contagious diseases before the start of study. Proper ethical care and management procedures were adopted during the entire period of the study.

To assess the effect of supplementation, periodic monitoring of blood parameters (LFT profile) was carried out. Blood samples were collected in the morning before feeding from jugular vein at the start and subsequently at monthly intervals of the experimental period from each animal. Around 8mL of whole blood was collected in well cleaned, dry test tubes and allowed for clotting, serum was separated and stored at –20°C. Total serum proteins and serum albumins were estimated using standard kits by Beacon Diagnostic Pvt. Ltd. Serum globulin was obtained by subtracting the serum albumin content from serum total proteins. Serum albumin: globulin ration was calculated by division of the two. Hepatic enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated using standard kits by Transasia Bio-medicals Ltd. The data generated were subjected to statistical analysis using Duncan's multiple range test (Snedecor and Cochran, 1994) in a completely randomized design.

The results pertaining to the hepatic function attributes of crossbred lambs *viz.* serum total protein, albumin, globulin and their ratios, and hepatic enzymes have been presented in Table 1. Supplementation of

feed additives had significant (P<0.05) effect on overall mean ALT levels and highly significant (P<0.01) effect on overall mean serum globulin concentration and A:G ratio among the various attributes studied under LFT profile, while significant effect of period on overall means for all the LFT parameters except serum globulin, A:G ratio and ALT were recorded.

Table 1: Liver function test profile of crossbred lambs at monthly intervals in different groups

Attribute	Period (days)	Treatment groups				Mean±SE	Sig.
		T_0	T_1	T_2	T_3		
Serum total proteins (g/dL)	0	6.57±0.21	6.34±0.09	6.50^{A}±0.09	6.52^{A}±0.10	6.48^{A}±0.05	P*
	30	6.50±0.24	6.40±0.17	6.68^{AB}±0.15	6.66^{AB}±0.14	6.56^{A}±0.07	
	60	6.50±0.29	6.68±0.19	6.80^{AB}±0.16	6.96^{AB}±0.17	6.73^{AB}±0.10	
	90	6.55±0.27	6.80±0.19	6.94^{B}±0.16	7.12^{B}±0.18	6.85^{B}±0.12	
	Mean±SE	6.53±0.02	6.55±0.09	6.73±0.07	6.81±0.09	6.66±0.05	
Serum albumin (g/dL)	0	3.43^{A}±0.09	3.42±0.11	3.46±0.07	3.48±0.07	3.45^{A}±0.01	P**
	30	3.65^{B}±0.05	3.48±0.17	3.44±0.05	3.48±0.11	3.51^{AB}±0.05	
	60	3.65^{B}±0.03	3.64±0.15	3.50±0.07	3.50±0.13	3.57^{AB}±0.04	
	90	3.67^{B}±0.10	3.76±0.20	3.52±0.12	3.58±0.07	3.63^{B}±0.05	
	Mean±SE	3.60±0.06	3.57±0.08	3.48±0.04	3.51±0.04	3.54±0.03	
Serum globulin (g/dL)	0	3.15±0.21	2.92±0.11	3.04±0.10	3.04^{A}±0.07	3.04±0.05	T**
	30	2.85±0.24	2.92±0.12	3.24±0.15	3.18^{AB}±0.12	3.05±0.10	
	60*	2.85^{a}±0.26	3.04^{ab}±0.07	3.30^{ab}±0.15	3.46^{bB}±0.14	3.16±0.14	
	90**	2.87^{a}±0.18	3.04^{ab}±0.07	3.42^{ab}±0.11	3.54^{bB}±0.18	3.22±0.16	
	Mean**±SE	2.93^{a}±0.07	2.98^{ab}±0.05	3.25^{ab}±0.07	3.31^{b}±0.08	3.12±0.06	
Serum A:G ratio	0	1.10±0.08	1.18±0.07	1.15±0.05	1.15±0.03	1.14±0.02	T**
	30	1.31±0.11	1.21±0.09	1.07±0.05	1.10±0.06	1.17±0.05	
	60**	1.31^{b}±0.10	1.20^{ab}±0.05	1.07^{ab}±0.06	1.02^{a}±0.06	1.15±0.06	
	90**	1.29^{b}±0.05	1.24^{ab}±0.08	1.03^{ab}±0.05	1.02^{a}±0.06	1.14±0.07	
	Mean**±SE	1.25^{b}±0.05	1.21^{ab}±0.03	1.08^{a}±0.03	1.07^{a}±0.03	1.15±0.03	
ALT (IU/L)	0	24.03±0.75	24.06±0.54	24.60^{B}±0.62	25.32^{B}±0.90	24.50±0.30	T*
	30	23.65±0.86	23.46±0.59	23.30^{AB}±0.76	21.22^{A}±1.06	22.91±0.57	
	60*	24.70^{b}±1.73	23.06^{ab}±0.58	20.98^{aA}±1.04	20.44^{aA}±1.02	22.29±0.98	
	90*	25.00^{b}±1.66	24.26^{b}±1.33	22.54^{abAB}±1.14	20.14^{aA}±0.87	22.98±1.07	
	Mean*±SE	24.34^{b}±0.31	23.71^{ab}±0.40	22.85^{ab}±0.52	21.78^{a}±0.65	23.17±0.41	

AST (IU/L)	0*	73.85^{ab}±1.93	72.48^{aB}±1.13	75.06^{abD}±0.88	75.94^{bD}±0.70	74.33^{C}±0.75	P**
	30	72.02±2.53	71.06^{AB}±1.21	71.16^{C}±0.68	70.78^{C}±1.04	71.26^{BC}±0.27	
	60	70.90±2.76	69.38^{AB}±1.58	67.22^{B}±1.13	67.16^{B}±1.33	68.66^{AB}±0.91	
	90*	69.67^{b}±3.06	67.58^{abA}±1.96	63.42^{aA}±1.40	61.90^{aA}±1.41	65.64^{A}±1.80	
	Mean±SE	71.61±0.89	70.12±0.81	69.21±1.11	68.94±1.29	69.98±0.96	
ALP (IU/L)	0*	212.40^{aA}±4.19	214.66^{abA}±3.95	219.98^{ab}±2.44	223.42^{bAB}±2.64	217.61^{A}±2.50	P*
	30	232.65^{AB}±7.88	233.26^{B}±5.60	232.56±6.57	229.18^{B}±2.02	231.91^{AB}±0.92	
	60*	250.32^{bB}±11.42	239.40^{abB}±7.15	228.78^{ab}±6.48	224.78^{aAB}±4.18	235.82^{B}±5.74	
	90**	252.10^{bB}±12.88	236.74^{abB}±6.84	218.08^{ab}±5.60	212.12^{aA}±8.24	229.76^{AB}±9.11	
	Mean±SE	236.87±9.26	231.01±3.55	224.85±2.89	222.37±2.68	228.78±3.04	

The means across the rows with different lower case superscript differ significantly (*P≤0.05; **P≤0.01)
The means across the columns with different uppercase superscripts differ significantly (P≤0.05)

The mean values of serum total protein, albumin, globulin and A:G ratio obtained in the present study were within normal physiological ranges (Kaneko *et al*., 1997), with higher values for total protein and globulin while lower values for albumin and A:G ratio in feed additive supplemented groups compared to un-supplemented group, although statistical significance could be observed only for serum globulin and A:G ratio which could be attributed to either higher dietary protein intake due to more feed consumption in respective groups resulting in higher nutritional plane or higher immune response causing reduction in gastro-intestinal parasitic burdens, since parasitism is known to cause fall in serum protein levels due to haemodilution, a compensatory mechanism for intestinal haemorrhage caused by migrating larvae and later on due to loss of large quantities of serum proteins in the gut through exudation. However, there were significant variations in the serum total protein (P<0.05) and albumin (P<0.01) levels at different periods with an increasing trend probably due to increased feed and thus protein intake with age. Non-significant increase in serum total protein and its fractions were also reported by Gupta *et al*. (2006) in heifers fed mixture of different herb; however, Mohammed *et al.* (2013) and Lee *et al*. (2015) have reported significant improvements in serum total proteins and its fraction concentrations in animals supplemented with different herbs as feed additives. Likewise, El-Kady *et al*. (2006) in buffalo calves and Peters *et al*. (2015) in lactating dairy cows also reported non-significant increase in serum total proteins by feeding diets supplemented with exogenous enzymes.

Perusal of the results revealed that dietary supplementation of the feed additives alone and in combination had no significant (P>0.05) influence on the serum levels of hepatic enzymes except ALT, although the values in all the treatment groups were within normal physiological ranges (Kaneko *et al*., 1997). The values of all the hepatic enzymes were lower for the feed additives supplemented groups in comparison to un-supplemented group with the lowest values for herb-enzyme

supplemented animals. These favorable changes in the levels of hepatic enzymes, especially in group supplemented with the feed additives in combination suggests functional property of the essential oils present in Afsanteen herb which exhibited hepato-protective activity. The hepato-protective action of Afsanteen herb has been already documented by Gilani and Janbaz (1995) and Romero *et al*. (2005). The results of the present study are comparable with the findings of El-Kady *et al*. (2006), and Rivero and Salem (2015) for fibrolytic enzymes, and Niwas *et al*. (2012) for herb, who also reported lower ALT activity by feed additive supplementation. However, Omidi *et al.* (2014) reported no significant differences in AST, ALT and ALP levels in lambs fed diets supplemented with hydro-alcoholic extract of saffron petal. Likewise, Peters *et al*. (2015) in lactating dairy cows and Rivero *et al*. (2016) in sheep and goat reported no significant differences in the levels of hepatic enzymes by feeding diets supplemented with exogenous enzymes.

Results of the present study suggested that EFEs cocktail as well as Afsanteen herb could be supplemented alone and in combination as feed additives in complete diet for raising lambs intensively without any adverse effect on hepatic functioning.

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SONOGRAPHIC DIAGNOSIS OF OSTEOMYELITIS IN A DOG

Sindhu G. Nair[2], Mohamed Shafiuzama[1], Ramani C.[3], Hemalatha S.[4]

Department of Veterinary Surgery and Radiology

Madras Veterinary College, Chennai - 7, Tamil Nadu Veterinary and Animal Sciences University

The Association of the Study of Internal Fixation (ASIF) made revolutionary changes in the development of instruments and implants for operative fracture treatment both in human as well as veterinary orthopaedics. Through the process of internal quality control (AO documentation) the clinical success of those new techniques and implants became evident and operative fracture treatment gained acceptance worldwide. Though, the chances of orthopaedic infections secondary to internal skeletal fixation remains as a flaw. Managing infections in fractures treated with open reduction and internal fixation is an ongoing issue.

Early diagnosis allows timely treatment, improves the prognosis and reduces the need for further invasive procedures in patients with osteomyelitis. Ultrasound being cheap and a portable modality has the best potential for evaluating the complicationsin fracture healing. The better axial and lateral resolution of ultrasonography owes an anticipation that even minute destructive, reparative or hypertrophic changes on the bone surface may be seen before they are apparent on plain radiographs (Backhaus, et al., 2001).

Doppler ultrasonography has been used to assess neovascularisation at the fracture site during both direct and indirect fracture healing. It permits non-invasive, repeatable and nearly real-time monitoring of bone fracture healing, which suggests the feasibility of this technique for the assessment of vascular changes associated with complications in fracture healing(Caruso *et al.,* 2000).

Previous reports regarding ultrasonographic studies had shown the diagnostic capability of evaluating the inflammation of soft tissues by detecting the colour Doppler flow in soft tissues. The present paper reports the sonographic changes in the early stages in a case of osteomyelitis.

A 2-year-old male mongrel dog was presented to the Small Animal Orthopaedics Unit of Madras Veterinary College Teaching Veterinary Hospital with a history of non-weight bearing lameness 28 days after open reduction and internal fixation for a simple transverse fracture of distal diaphysis of the left tibia.On general clinical examination, the patient was found to be apparently healthy and physical and haemato-biochemical parameters were

2: Corresponding author: sindhugnair13@gmail.com, (+91)9400511612

1,3: Professors, Department of Veterinary Surgery and Radiology

2: M.V.Sc. Scholar, Department of Veterinary Surgery and Radiology

4: Professor, Department of Veterinary Pathology

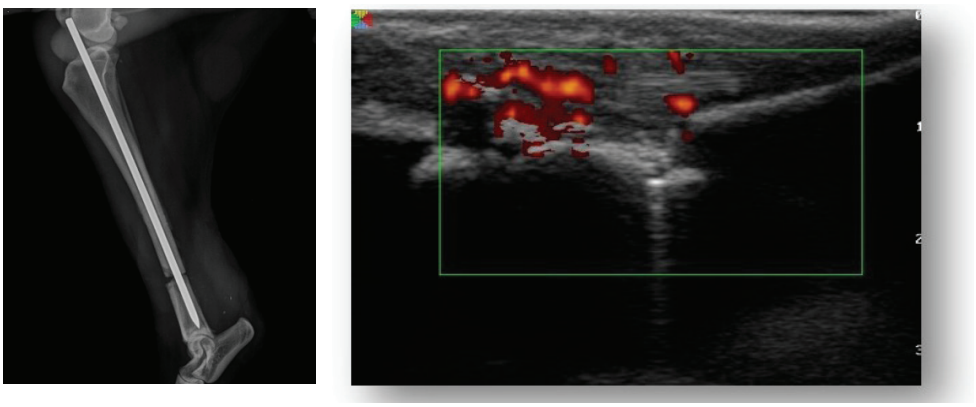
within the normal range. Bandaging was not intact and the surgical site was noticed to be undisturbed. No swelling could be appreciated at the fracture.

Radiographic and ultrasonographic evaluation of the fracture site was performed. In the light of ultrasonographic changes noticed in the first observations, repeat evaluation was conducted after 14 days and was diagnosed as a case of osteomyelitis.

Orthogonal radiographs obtained on the day of presentation revealed no signs of periosteal reaction or soft tissue changes. Fracture fragments were in alignment and the implant was noticed to be undisturbed.

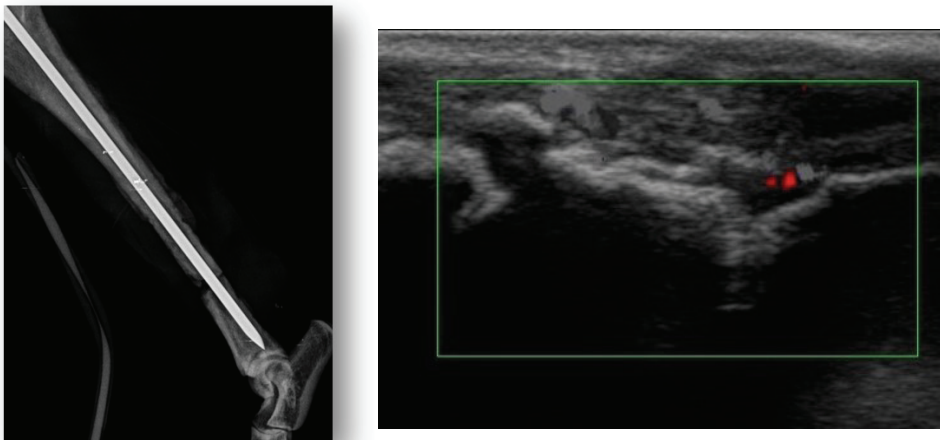
Ultrasonographic evaluation of the fracture site was performed using a 7.5 MHz linear array transducer, positioned in a longitudinal plain, approached through a craniolateral window (Risseladaet *al.* 2003). Ultrasound images of the fracture site revealed a disorganised hyperechoic echogenicity with intense vascularity. Appearance of small anechoic pockets at the fracture site and near the bone cortex was suggestive of inflammatory fluid collection.On power Doppler study, intense vascularity could be appreciable at the fracture site, soft tissue surrounding the fracture site and the surface of healthy bone cortex proximal to the fracture site (Plate 1).

Plate. 1. Radiographic and ultrasonographic appearance of fracture site on 28th post-operative day



Follow-up evaluation after 14 days revealed non- weight bearing lameness with soft swelling at the fracture site and mobility between fracture ends. Radiographs revealed severe osteolysis and non-union of fracture, indicative of osteomyelitis. Periosteal osteolytic changes were found to be extending to the proximal parts of tibia. Ultrasonographic examinations on the same day revealed larger anechoic areaswithdisorganised hyperechogenic areas.Compression of the fluid collection with the transducer could demonstrate the motion of fluid into the marrow cavity. Power doppler images showed a decline in vascular supply to the fracture site and surrounding tissues (Plate 2).

Plate. 2. Radiographic and ultrasonographic appearance of fracture site on 42nd post-operative day



In the light of radiographic and ultrasonographic findings, confirmative diagnosis was made as a case of Osteomyelitis.

Kramer *et al.* (1997) opined that ultrasonography offered additional information in the evaluation of soft tissue changes associated with bone diseases, which was in accordance with the findings of the present case. The authors reported that the appearance of irregular separate growth with distal acoustic shadowing in ultrasonographic study of canine and feline long bones was suggestive of a gradually developing fibrocartilaginous callus.

In the present case, the appearance of bizarre and haphazard irregular bright echoes in ultrasonography gave a fake impression of mineralized fracture callus. Similar findings were reported by Maffulli and Thornton (1995) in a case of non-union.

Cardinal *et al*., (2001) reported that subperiosteal fluid collection was not necessarily a sensitive or specific sign for osteomyelitis; but once a fluid collection next to a bone is identified, the finding should be considered highly suspicious of osteomyelitis. The present study was in agreement with this findings. The authors further suggested that an immediate ultrasound guided aspiration of the fluid collection should be performed to identify the organism involved, for further treatment.

Chao *et al.* (1999) investigated the proficiency of color Doppler sonography in evaluating acute osteomyelitis in children. The authors reported that the color Doppler vascular flow within or around the infected periosteum correlated with advanced acute osteomyelitis. According to the authors, there were no doppler signals in the early stages of inflammation, a gradual increase in vascularity which persisted through out

the inflammatory process. In contrary to that, the present study revealed an initial high vascularity which reduced during the course of inflammation.

The present case report signifies the relevance of B-mode and power doppler ultrasonography in the early diagnosis of osteomyelitis when compared to radiography. Ultrasound guided aspiration of sub-periosteal fluid collection can be performed for bacterial culture and thereby for the selection of appropriate treatment protocol.

The authors acknowledge their gratitude to the Dean and Director of Clinics, Madras Veterinary College, for providing necessary facilities.

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SERUM BIOCHEMICAL INDICES IN CAPTIVE RHESUS MACAQUES (*Macaca mulatta*)

M.Palanivelrajan[1*], M.G.Jayathangaraj[2], R.Sridhar[3], M.Parthiban[4] and R.Thirumurugan[5]

Madras Veterinary College, Chennai - 7, Tamil Nadu Veterinary and Animal Sciences University

In India, Non-human primates are widely spread and more popular among the captive wild animals, due to their activities. However, the information related to health and diseases of non-human primates is still in a stage of infancy. Researches related to biology of non-human primates is being carried out, whereas research on health or disease of non-human primates is rare. Hence, this study was carried out to establish a base-line biochemical data. Serum biochemistry values provide information that is essential for the assessment of health, diagnosis of pathology and understanding of disease processes. The clinical biochemistry measures were commonly utilized as an indicator of the health status of non-human primates (Champoux *et al.*, 1996). The present study describes the biochemical values of captive Rhesus macaques in India.

Blood samples were collected from saphenous vein of six captive (3 males and 3 females) adult Rhesus macaques at Arignar Anna Zoological Park, Vandalur by the process as suggested by Wallach and Boever (1983); Fowler (1986) and Venkatesan *et al.* (2006). The animals were chemically restrained with combination of ketamine hydrochloride and xylazine hydrochloride at the dose rate of 10 mg/kg and 1 mg/kg body weight respectively. Blood samples were subsequently drawn to plain vacutainer tubes. Serum was separated by centrifugation at 2000 rpm for 10 minutes and was stored at – 20°C, until further processing.

*part of M.V.Sc., Research Work submitted by the first author to Tamilnadu Veterinary and Animal Sciences University, Chennai – 600 051 and corresponding author E-mail: palanivelrajan.m@tanuvas.ac.in

1- Assistant Professor, Department of Wildlife Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 007 palanivet@gmail.com +91-9442211887

2- Professor & Head, Department of Wildlife Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 007

3- Professor and Head, Department of Veterinary Pathology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 007

4- Associate Professor - Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 007

5 - Zoo Veterinary Assistant Surgeon, Arignar Anna Zoological Park, Vandalur, Chennai – 600 048

Subsequently, serum samples were analyzed for the following bio-chemical parameters;

PARAMETERS	METHOD	UNITS
• Blood urea nitrogen (BUN)	Glutamate dehydrogenase (GLDH) method	mg/dl
• Creatinine	Modified Jaffe's method	mg/dl
• Total protein	Direct Biuret method	g/dl
• Albumin	Bromocresol green method	g/dl
• Total and direct bilirubin	Modified dimethyl sulfoxide (DMSO) method	mg/dl
• Total cholesterol	CHOD- PAP method	mg/dl
• Calcium	Modified ortho-cresolphtalein complex (OCPC) method	mg/dl
• Phosphorous	Phosphomolybdate method	mg/dl
• Glucose	Test stripe II using the Glucocard (Accurex Biomedical Ltd.)	mg/dl
• Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT)	IFCC recommended procedure	U/L
• Alkaline phosphatase (ALP)	DGKC – SCE recommended procedure	U/L

The mean values of serum total protein, albumin, globulin, globulin ratio, glucose, cholesterol, calcium and phosphorus are given in Table 1. The mean values for organ function profile and serum enzyme profile like BUN, creatinine, total bilirubin, direct bilirubin, indirect bilirubin, AST/SGOT, ALT/SGPT and ALP are given in Table 1.

Table 1. Biochemical Values (Mean ± Standard Error) of Captive Rhesus Macaques (Macaca mulatta)

Group	BLOOD METABOLIC PROFILE							
Rhesus macaques (n = 6)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio	Serum glucose (mg/dl)	Serum cholesterol (mg/dl)	Calcium (mg/dl)	Phosphorus (mg/dl)
	7.21 ± 0.21	3.43 ± 0.27	3.78 ± 0.44	1.01 ± 0.20	71.33 ± 7.36	95.69 ± 6.00	9.65 ± 1.16	7.28 ± 1.91
	ORGAN FUNCTION PROFILE AND SERUM ENZYME PROFILE							
	BUN (mg/dl)	Creatinine (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Indirect bilirubin (mg/dl)	AST/SGOT (IU/l)	ALT/ SGPT (IU/l)	ALP (IU/l)
	31.42 ± 1.57	0.68 ± 0.09	0.19 ± 0.06	0.06 ± 0.02	0.13 ± 0.06	26.96 ± 5.11	16.32 ± 3.18	366.79 ± 112.33

The values of serum total protein were less than the values reported by Fowler (1986). The serum glucose level was 71.33 ± 7.36 mg/dl and was in accordance with the findings of Ramachandra *et al*. (1998). It is noted that fasting serum glucose concentration in macaques was normally lower (40-80 mg/dl) than the other common laboratory animals, as reported by Hall and Everds (2003).

The level of phosphorus was 7.28 ± 1.91 mg per cent, which was higher than the one reported by Kessler and Rawlins

(1983) and Ramachandra *et al*. (1998). The differences in plane of nutrition and metabolism might be attributed to such variations seen our study. The value of BUN was 31.42 ± 1.57 and was higher than the values reported by Wallach and Boever (1983) and lower than the one reported by Fowler (1986) who quoted BUN value as 16.9 ± 2.7 mg/dl. The total bilirubin values were closer to the values reported by Fowler (1986).

The mean level of alanine aminotransferase is presented in Table 1 and was lower than the values reported by Ramachandra *et al*. (1998). ALT is one of the useful parameters in detecting liver diseases in primates, the present values were closer to the values reported by Wallach and Boever (1983). Age wise comparison could not be drawn in the present study, it becomes significant to mention the documentation by Kessler and Rawlins (1983) who quoted that glutamic pyruvic transaminase activities were significantly lower in adult and aged monkeys, when compared to the juveniles.

Hence, the serum biochemical study was made with the samples obtained after chemical restraint. In case of non-human primates, blood samples were obtained by zoo veterinarians only after the immobilization using xylazine and ketamine. Ketamine associated immobilization seems to have caused reduction in serum concentrations of glucose, total protein, ALP, calcium, sodium and potassium, in case of animals like Bonnet macaques, as quoted by Venkatesan *et al*. (2006). However, it becomes impossible to collect blood samples without chemical immobilization in non-human primates and hence, the values obtained may be considered as the normal ones in the concerned species.

Non-human Primates need to be subjected to blood collection techniques in a periodical manner in Zoological Parks/ Zoos/Zoological Gardens. Further, random investigation may also be carried out especially if the zoo veterinarian becomes suspicious of the probable existence of a clinical problem in the concerned non-human primate reared under captivity.

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