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Review article

Full length articles

Short Communication

Review article

CONTRAST ENHANCED ULTRASOUND IN SMALL ANIMAL PRACTICE

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Conventional grey scale ultrasonography are non-invasive techniques that,by virtue of their cross sectional nature and ability to detect blood flow ,have had a considerable impact on the imaging assessment of small animal patients. Despite these advantages, the broadly similar attenuation properties of normal and diseased tissues may, in certain instances, limit identification and characterisation of pathology. Furthermore, the weak echoes and physical characteristics of blood flow restrict the various Doppler techniques to the assessment of macrovascular structures.Ultrasound contrast agents (UCAs) overcome this limitation by substantially increasing the signal generated within the tissues in which they accumulate and contrast. Furthermore by acting as blood pool agents, UCAs demonstrate microvasculature –thus permitting perfusion studies and functional imaging (King 2006).

Contrast ultrasound: Contrast agents have been integral in all imaging modalities except US.The reasons for this limitation in ultrasound imaging is because of the cost of media and need for intravenous pool access .Unlike MR and CT contrast media ,ultrasound contrast agents, are pool agents .These contrast agents are microscopic bubbles made from an outer shell and central core of gas. There are limited numbers of manufacturers of ultrasound contrast media. Older first generation contrast agents use air in the core (Lenovist).Air is not a potent medium for emission of ultrasound signal and results in poorimages.Second generation contrast media utilize inert gases in the core, which provide more non-linear effects.The first second generation contrast media (Optison) used human albumin in the shell.This had obvious limitations in veterinary medicine for immunological concerns.

Second generation contrast agents utilize an immunologically inert lipoprotein shell and more efficient inert gas in the lumen. The range of bubble sizes are predominantly small (most less than 10 micro milimeter, pass through the pulmonary circulation unaffected and can imaged in the small vessels of all organs currently being imaged in the diagnostic veterinary imaging. Contarst media currently recommended for the veterinary clinical applications are definity (USA) and sonovue (Canada). Both are very safe in dogs and no side effects have been reported. The bubbles of definity are more concentrated and rigorous than sonovue providing and advantagefor clinical characterisation or detection of lesions, such as liver nodules .For experimental studies sonovue is advantageous.

New contrast agents are available like Targestar is a blood pool agent that demonstrates great promise because of high resonant frequency and improved stability of the reconstituted bubbles. Reconstituted Targestar is stable with refrigeration for weeks and months. This agent has the ability conjugate to an active coupling molecule. This is a very exciting new area of research called molecular imaging. Conjugation of ligands to a blood pool US contrast agent may provide an opportunity to couple monoclonal antibodies ,various mediator proteins or possible therapeutics (Correas *etal.,* 2009).

Practical advantage of using contrast enhanced ultrasound includes the relatively low cost of contrast ultrasound examinations: comparable sensitivity to computed tomography and magnetic resonance imaging for detecting the metastatic disease and the absence of ionising radiation.

Limitation is that the keeping quality is very short and should be used with in hours after its recon situation.

CLINICAL APPLICATION

Liver: UCA demonstrates affinity for certainorgans, such as liver in which they persist. The reason for the accumulation is not very clear, but is suspected to be associated with accumulation within the hepatic sinusoids or incorporation within Kupffer cells. As a result, three phases of liver enhancement –aterial, early and delayed portovenous phases-can be recognised. The clinical utility of the UCA is particularly related to the perfusion in the later two phases. A continuous low MI imaging technique is usually employed in contrast enhanced examinations of the liver .The use of contrast ultrasonography in dog and cat livers has been widely reported in the localisation and characterisation of nodular hepatic lesions-in particular the differentiation of benign primary nodular disease from metastatic or primary hepatic malignancy. In general benign lesions including lipid granulomas, tend to demonstrate variable enhancement during the aterial and early portovenous phases (hypo,iso or hyperechoeic) with similar enhancements to liver during normal liver during the late potal phase.

Malignant lesions characteristically show a rapid and complete contrast washout and appear hypoechoic, compared to the enhanced liver, during the late portovenous phase. These characteristics are related to tumoral neo angiogenesis from the hepatic artery and the absence of normal hepatic sinusoidal architecture. Hemangiosarcoma metastases, particularly relevant in veterinary patients,may not demonstrate enhancement at any stage. The imaging characteristics during the early phases of liver enhancement generally appear to be of little clinical significance in the assessment of metastatic phase. Although macrovascular patterns ,such as the basket pattern associated with hepatocellular carcinoma and the spoke wheel patter typical of nodular hyperplasia ,may be of significance in humans with primary hepatic masses , the validity of extrapolating these features to the dog and cat is uncertain. Diagnosis of large complex or lobar masses should be done with caution, as these may have areas of haemorrhage or

necrosis that confound thediagnosis.Careful correlation with fundamental images and anticipated histopathological findings is necessary, as predominantly cystic lesions may demonstrate rapid wash out and non enforcement of haematomas may mimic malignant disease.

Contrast ultrasonography of the liver should be employed primarily to assess lesion margination and extent and to improve lesion detection in veterinary clinical practice. Isoechoeic or poorly marginated solid components of masses with larger cystic or necrotic regions may not be recognised on fundamental imaging .Contrast –enhanced ultrasonography may be then be used to guide biopsy to avoid sampling non-representative regions of such mass lesions. Equally iso echoeic or poorly marginated metastatic nodules may be difficult to recognise on fundamental imaging and contrast enhanced ultrasound is reported to increase the number of metastases identified. These factors particularly relevant where exploratory surgery is being considered .The findings of the contrast enhanced examination and aspirate or biopsy results should not be interpreted in isolation ,but considered together to determine if they are representative .Two or three UCA boluses are usually required to ensure a satisfactory assessment of all liver segments. The quality of the later examinations is usually superior due to UCA persistence in the liver .The technique is dependant on operator experience and all studies should be acquired as cine loops for review.

Salwei*et al.*(2005) described the use of ultrasound in the diagnosis of portosystemic shunts (PSS) .Shunting vessels are not recognised as such, but the generation of time –intensity curves of hepatic perfusion demonstrate a shorter time to peak liver enhancement when compared to normal dogs, due to the marked increase in hepatic arterialisation associated with PSS.

Spleen: Spleen demonstrates an early arterial phase of enhancement with a patchy or mottled appearance. The late paranchymal phase results in more homogenous enhancement and should be used to asses any changes. Contrast ultrasonogarphy has been reported to be useful to discriminate between benign and malignant lesions. Benign lesions are characterised by enhancement at a rate and intensity similar to that of a normal spleen .Malignant tumors are characterised by a hypoechoeic appearance during the late paranchymal phase,with rapid enhancement (wash in) a feature of lymphoma and soft tissue sarcomas .Extensive non-perfused regions during all phases are a feature of haemangiosarcoma.The diagnosis of lymphoma is usually facilitated by identifying concurrent lymphadenopathy or organomegally and cytology is usually diagnostic .Therefore contrast enhanced ultrasound should reserved for those cases with disease limited to spleen (suspected to stage four disease) and or/ to support equivocal cytological findings. Differentiation between haematoma and haemangiosarcoma is not possible(Ivan *etal.,* 2009). UCAs may be useful to confirm and differentiate splenictorsion or infarction, from splenitis infiltrative disease, where the conventional B mode findings are not typical for the appearance of vascular compromise.

Pancreas: The use of contrast enhanced ultrasound has been reported in the characterisation of pancreatic disease in humans .The utility and relevance of the technique in canine pancreatitis has not been demonstrated,but may be of value in identifying extensive or irreversible pancreatic necrosis. Contrast ultrasound ofthe diseased pancreas in the demonstrated increased perfusion and vascularity comparedto normal cats according to one study (Rademacher *etal*.,2008).

Differentiation between inflammatory benign nodular changes and neoplasticdisease is possible using this technique.Insulinoma in common with other neuroendocrine tumors ,may demonstrate strong enhancement during the arterial phase, with rapid washout of contrast media.Contrast enhanced ultrasonography of the superficial lymphnodes has been reported to better define the distribution of vessels ,which may characterise lesion morphology(Salwei etal.2005) This may assist in staging of disease or demonstrate sentinel lymph node involvement.

CEUS has been used extensively in fields such as cardiology and hepatology. This technique is promising for studies for kidney structure and function because of its safety profile and its ability to provide information on single kidney as well as regional blood flow.It has been used to determine changes in renal function in response to physiologic and pharmacologic interventions and to study disease such as renal artery stenosis and post kidney transplantation (Kalantarinia and Okusa 2007)

Contrast agents can increase echogenicity within and around the altered parenchyma ,thus optimizing Doppler evaluation and consequently the diagnosis of prostate gland disease in dogs(Bigliardi and Ferrari, 2010)

Contrast enhanced ultrasound is a valuable,minimally invasive technique particularly suited to the identification and characterisation of liver and splenic nodular lesionsorgans and diseases. This technique may assist in identifying suitable areas for tissue sampling. The technique's value for the evaluation of other organs and diseases and more work is progressing in tis area. The requirements of specialised equipment, transducers and software, the dependence of operator experience and the cost of the contrast ultrasound media must be recognised as significant limitations.

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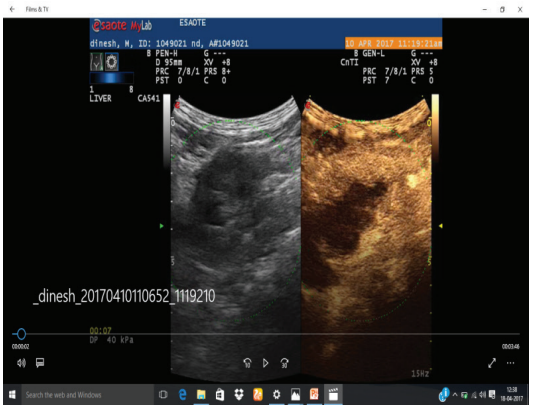
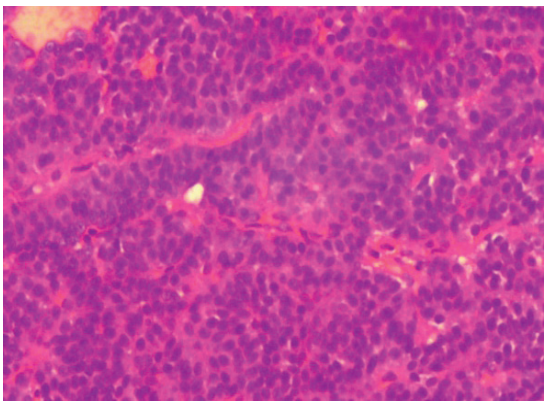
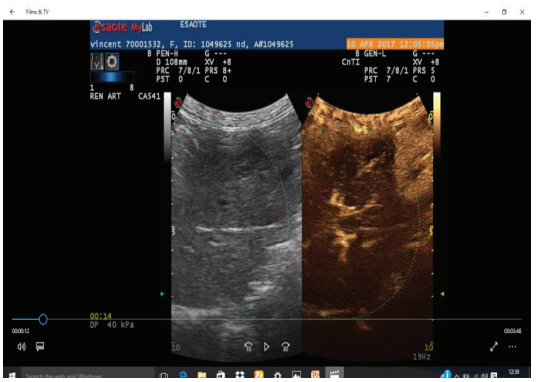


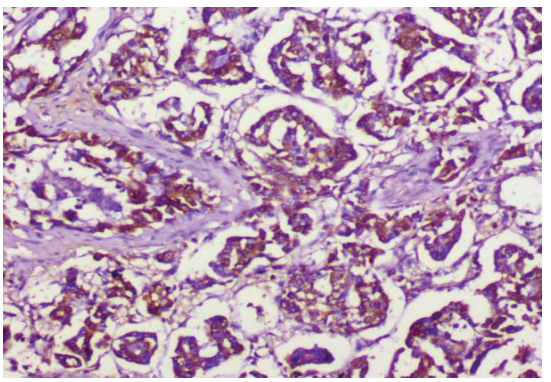
Peripheral enhancement Pattern – Ductal tubular adenocarcinoma of Liver



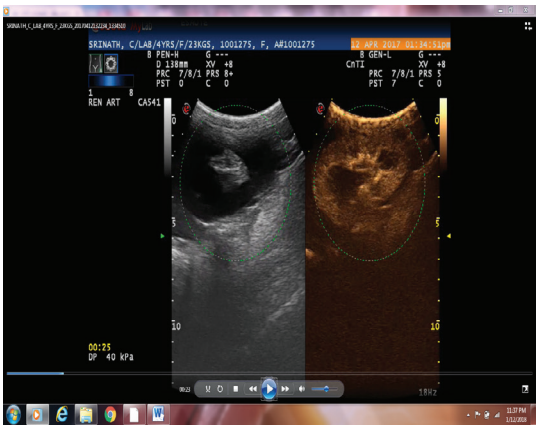
Liver-HP-Metastatic Ductal adeno carcinoma. H&E 200 x



Radial enhancement – Cholangiocellular carcinoma



Liver-IHC- Cytokeratin -Cholngio cellular carcinoma. Diffuse intense cytoplasmic signal. DAB 200x



CEUS of Kidneys: Filling of Renal cortex with contrast agent

CEUS of Kidneys: Filling of Renal cortex with contrast agent

Method of preparation of sonovue

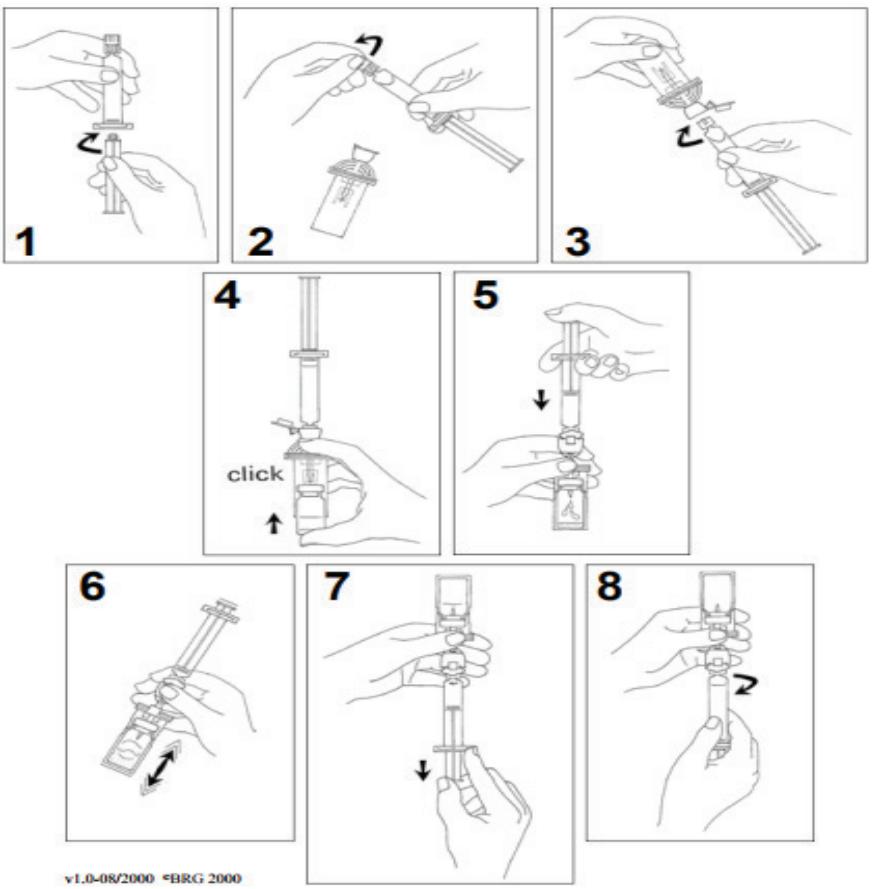


Image from product catalogue

1. Connect the plunger rod by screwing it clockwise into the syringe.
2. Open the MiniSpike transfer system blister and remove syringe tip cap.
3. Open the transfer system cap and connect the syringe to the transfer system by screwing it in clockwise.
4. Remove the protective disk from the vial. Slide the vial into the transparent sleeve of the transfer system and press firmly to lock the vial in place.
5. Empty the contents of the syringe into the vial by pushing on the plunger rod.
6. Shake vigorously for 20 seconds to mix all the contents in the vial to obtain a white milky homogeneous liquid.
7. Invert the system and carefully withdraw SonoVue into the syringe.
8. Unscrew the syringe from the transfer system.

- Do not use if the liquid obtained is clear and/or if solid parts of the lyophilisate are seen in the suspension.
- SonoVue should be administered immediately by injection into a peripheral vein for use in echocardiography and in vascular Doppler imaging
- If SonoVue is not used immediately after reconstitution the microbubble dispersion should be shaken again before being drawn up into a syringe.
- Chemical and physical stability of the microbubble dispersion has been demonstrated for 6 hours.

Full Length Research Articles

EVIDENCE OF SHARED GENOMIC SEGMENT 6 BETWEEN BLUETONGUE VIRUS SEROTYPES 1 AND 2

Y. Vishuvardhan Reddy[1], B. Susmitha[1], Y. Narsimha Reddy[2], D. Sreenivasulu[3], Kalyani Putty[2],K. S. R. Siva Sai[4], Pavuluri Panduranga Rao[1,*], Nagendra R Hegde[1,5]

ABSTRACT

Bluetongue virus (BTV) belongs to the genus *Orbivirus* of family *Reoviridae*. Twenty seven serotypes of BTV have so far been recognized. The genome of BTV consists of ten double-stranded segmented RNA which can reassort independently during mixed infections. The outer capsid proteins VP2 and VP5, coded by segments 2 and 6, are the major determinants of serotype, and serotype-dependent linkage between these segments has been observed. Natural and *in vitro*reassortants of segment 2 and 6 of different serotypes of BTV have been reported earlier. Here we report a BTV isolate which is a natural reassortant of segment 2 and 5 of BTV-1 and -2.

Key words: Bluetongue virus, Serotype, Australasian topotype, Segment reassortment, Shared segment 6

INTRODUCTION

Bluetongue (BT) is endemic in most of the tropical and some of the subtropical areas. The disease is caused by bluetongue virus (BTV) belonging to genus *Orbivirus,* family *Reoviridae*. The BTV structure is a triple layered icosahedron. The innermost subcore is a cage formed by the viral protein (VP) VP3. The subcore encases the viral RNA which is associated with VP1, VP4 and VP6, together forming the transcriptase complex (Mertens & Diprose, 2004). The middle, outercore layer is composed of VP7. Finally, the outermost, outer capsid is formed by VP2 and VP5 proteins (Roy, 2005). The genome of the virus consists of ten segments of double-stranded RNA designated as Seg-1 to -10 in the order of decreasing molecular size, and coding respectively for VP 1 (Seg-1), VP2 (Seg-2), VP3 (Seg-3), VP4 (seg-4), NS1 (Seg-5), VP5 (Seg-6), VP7 (Seg-7), NS2 (Seg-8), VP6 , NS4 (seg-9) and NS3, NS2a, NS5

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(Seg-10) proteins(Sung & Roy, 2014). The genomic RNA segments can reassort independently and can generate several variants of BTV during mixed infections. Genome segment reassortment is thought to be one of the important mechanisms of BTV evolution and fitness (Niedbalski, 2013; Nomikou et al.,, 2015; Shaw et al.,, 2013a).

BTV exists in 27 serotypes. Although, VP2 and VP5 are serotype determining proteins (Mertens et al.,, 1989), it has been shown by us and others that VP5 can be shared among closely related serotypes (Rao et al.,, 2017; Shaw et al.,, 2013a). Natural reassortants of segment 2 and 6, coding for VP2 and VP5, respectively, has been observed between BTV-1 and -8. Furthermore, *in vitro* reassortment of segment 2 and 6 between BTV-20 and -21 has also been reported earlier. In this study, we describe the investigation of a BT outbreak and characterization of the associated BTV-1 and -2 serotype isolates. We found and report possible reassortment and sharing of segment 6 between those serotypes.

MATERIALS AND METHODS

Isolation of the viruses

BTV from field samples was isolated by passaging in embryonated chicken eggs (ECE) followed by passaging in BHK-21 (Clavijo et al.,, 2000). Briefly, heparinized blood samples were collected aseptically from sheep showing BT-like symptoms, and used to inoculate ECE. Triturates of ECE showing hemorrhages were used to infect BHK-21 cells, and observed for cytopathic effect (CPE) characteristic of BTV infection. Details of viruses used during this study are given in Table 1. Culture supernatants from cells showing CPE were titrated on BHK-21, and the Reed and Muench method (Reed & Muench, 1938) was applied to calculate the 50% tissue-culture infectious dose ($TCID_{50}$).

RNA isolation and sequencing

BHK-21 cells were infected with different BTV isolates at a multiplicity of infection (MOI, i.e., $TCID_{50}$ / cell) of 0.01 and incubated at 37°C until CPE was observed. Total RNA was isolated from cell lysates using the MPGIT solution (Gauthami et al.,, 2015) and cDNA was synthesized. Sequencing library was prepared and cDNA was sequenced by "sequencing by synthesis"(SBS) on Illumina HiSeq (Rao et al.,, 2013). Briefly, RNA was fragmented into small pieces, reverse transcribed, multiplexing adapters were ligated to the cDNA ends and PCR was performed using primers complimentary to the adapters. PCR products were purified, diluted to obtain 100-1000x coverage of the genome, heat denatured and hybridized to oligonucleotides immobilized on flow cell. Paired end sequencing was carried out, adapter sequences were deleted and reads were assembled using Velvet 1.1.01 (Zerbino & Birney, 2008) or Bowtie1.0 (Langmead et al.,, 2009). BTV-specific reads were identified by aligning them to reference sequences and the sequences were submitted to GenBank (Table 1).

Sequence analysis

Segment 2 sequences were analysed to identify the serotype of the virus. For

phylogenetic analysis, coding sequences of segment 6of global isolates of BTV-1 and BTV-2 were aligned, and Maximum Likelihood (ML) tree was constructed with TN93+G+I as nucleotide substitution model with 1000 Bootstrap replications using MEGA 5.03 suite (Tamura et al.,, 2011).

RESULTS AND DISCUSSION

Three isolates of BTV-1 (BTV01IND2007, BTV01IND2010/01, BTV01IND2010/02) and two isolates of BTV-2 (BTV02IND1993, BTV02IND2010) were sequenced using SBS methodand Seg-2 and Seg-6 sequences coding for VP2 and VP5 were analysed. Sequence homology of Seg-2 indicated that all the BTV-1 isolates belonged to the Australasian topotype with sequence similarity of more than 87%. Similarly, Seg-2 of BTV-2 isolates also belonged to Australasian topotype with sequence similarity of more than 89%. Seg-6 sequences of all the Indian isolates of BTV-1 were similar (more than 90%) to that of otherAustralasianBTV-1 sequences. While Seg-6 of BTV02IND2010 had high sequence similarity (>99%) with one of the Indian isolates of BTV-2 isolated in 1982 and Australasian viruses (>91%), Seg-6 of BTV02IND1993 had very high sequence similarity with Indian isolates of BTV-1 (>99%) and Australasian viruses (>90%).

Phylogenetic analysis of Seg-6 of BTV-1 and -2 from different parts of the world indicate that this segment did not cluster monophyletically according to serotype. BTV-1 and 2 of African and American viruses formed monophyletic groups and are more related to each other than their relation with Seg-6 of BTV-1 and -2 serotype viruses of Australasian origin. The available data indicate that Seg-6 of Australasian BTV-1 and -2 also clustered as per their serotype, except in the case of BTV02IND1993.

BTV is a segmented RNA virus and all the genomic segments of BTV can reassort independently during mixed infections, leading to the generation of huge number of variants(Niedbalski, 2013; Nomikou et al.,, 2015; Shaw et al.,, 2013a). Widespread genomic reassortment followed by selection may be one of the important evolutionary mechanisms of BTV. Although all the segments can reassert independently, there seems to be some level of linkage between Seg-2 and -6 coding for VP2 and VP5 the outer coat proteins(Rao et al.,, 2017; Shaw et al.,, 2013a). Cursory analysis of hundreds of BTV isolates from different parts of the world indicates that VP5 is also a serotype determining protein, and with some exceptions, there is a linkage between Seg-2 and -6 and serotypes. Isolation of a natural reassortant Seg-2 and -6 of BTV-8 and -1 has been reported and Seg-2 and -6 reassortants of these serotypes could be developed using reversegenetic approaches(Shaw et al.,, 2013a). Similarly, generation of reassortants between BTV-20 and -21 has been reported (Cowley & Gorman, 1989). Here, we report a natural reassortant of BTV-1 and -2 with Seg-2 of BTV-2 and Seg-6 of BTV-1 isolated in 1993. On the other hand, the BTV-2 isolates of 1982 and 2010 are different from this isolate and contain natural BTV-2 specific Seg-2 and -6. Isolation of a natural reassortant of Seg-2 and -6 of BTV-2 and -1 suggests that Seg-6/ VP5 can be shared between serotypes 1 and 2, and supports the

hypothesis that VP5 can be shared among closely related serotypes.

Phylogenetic analysis of Seg-6 of BTV-1 and -2 indicate thatSeg-6 of western topotypes (African, American) cluster monophyletically, whereas Seg-6 of eastern BTV-1 and -2 probably evolved independently. The isolation of natural reassortant of Seg-2 and -6 of these serotypes also supports the possibility of independent evolution of Seg-6 of BTV-1 and -2 in Australasia. Earlier work indicates that the reassortants are immunologically different from their parent strains(Cowley & Gorman, 1989; Shaw et al.,, 2013a),and may play a role in escaping host immunity to some extent, in a manner similar to antigenic shift observed with influenza viruses.

To conclude, a novel BTV with reassorted serotype determining segments (Seg-2 and -6) from two different serotypes of BTV i.e., BTV-1 and -2, was identified. Immunological characterization of these viruses may illuminate the role of antigenic shift in epidemiology of BT.

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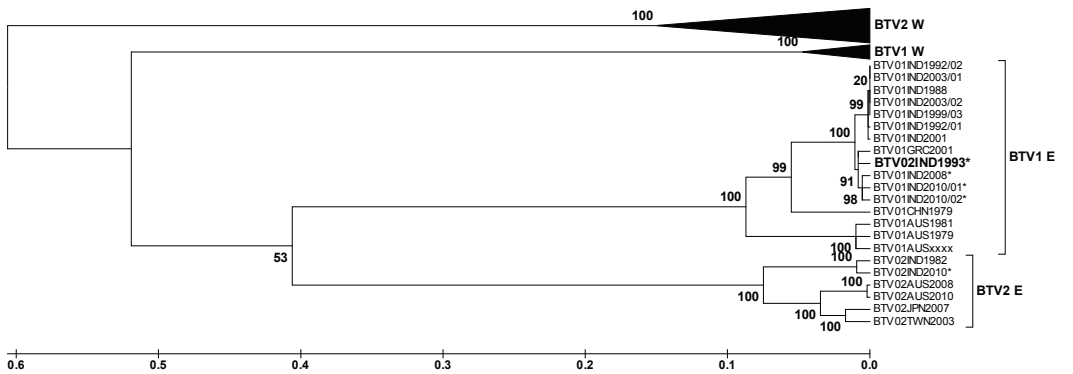

Fig 1: Phylogenetic analysis of segment 6 BTV serotypes 1 and 2.

Maximum Likelihood tree of Seg-6 of BTV serotype 1 and 2 using MEGA5.03 The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Sequenced during this study

Table 1: Details of BTV sequences analysed

Virus ID	Serotype	Place of Isolation	Year of isolation	Acc. No	Reference
BTV01ZAF1958	BTV-1	South Africa	1958	JX272384	
BTV01AUS1979	BTV-1	Australia	1979	JN881990	(Boyle et al.,, 2012)
BTV01CHN1979	BTV-1	China	1979	KC879620	(Zhu et al.,, 2013)
BTV01AUS1981	BTV-1	Australia	1981	AJ631214	
BTV01NGA1982	BTV-1	Nigeria	1982	AJ586657	(Singh et al.,, 2004)
BTV01SDN1987	BTV-1	Sudan	1987	AJ586658	(Singh et al.,, 2004)
BTV01IND1988	BTV-1	India	1988	AJ586661	(Singh et al.,, 2004)
BTV01IND1992/01	BTV-1	India	1992	AJ586659	(Shaw et al.,, 2013b)
BTV01IND1992/02	BTV-1	India	1992	AJ586660	(Singh et al.,, 2004)
BTV01IND1999	BTV-1	India	1999	AJ586662	(Singh et al.,, 2004)
BTV01GRC2001	BTV-1	Greece	2001	AJ586664	(Singh et al.,, 2004)
BTV01IND2001	BTV-1	India	2001	AJ586663	(Singh et al.,, 2004)
BTV01IND2003/01	BTV-1	India	2003	AJ783903	
BTV01IND2003/02	BTV-1	India	2003	AJ783902	
BTV01DZA2006	BTV-1	Algeria	2006	EU422952	(Maan et al.,, 2008)
BTV01MAR2006	BTV-1	Morocco	2006	EU422953	(Maan et al.,, 2008)
BTV01FRA2007	BTV-1	France	2007	JX861493	(Shaw et al.,, 2013b)

BTV01IND2008*	BTV-1	Khammam	2008	JX399153	(Rao et al.,, 2013)
BTV01IND2010/1*	BTV-1	Nellore	2010	KP339149	(Reddy et al.,, 2016)
BTV01IND2010/2*	BTV-1	Karimnagar	2010	KP339139	(Reddy et al.,, 2016)
BTV01ITA2013	BTV-1	Italy	2013	KJ019210	(Lorusso et al.,, 2014)
BTV01AUSxxxx	BTV-1	Australia	xxxx	M21845	(Gould & Pritchard, 1988)
BTV02ZAF1959	BTV-2	South Africa	1959	JX272604	
BTV02IND1982	BTV-2	India	1982	AJ586675	(Singh et al.,, 2004)
BTV02NGA1982	BTV-2	Nigeria	1982	AJ586667	(Singh et al.,, 2004)
BTV02USA1982/01	BTV-2	USA	1982	AY855277	(Mecham & Johnson, 2005)
BTV02USA1982/02	BTV-2	USA	1982	AY855278	(Mecham & Johnson, 2005)
BTV02SDN1985	BTV-2	Sudan	1985	AJ586666	(Singh et al.,, 2004)
BTV02IND1993*	BTV-2	Chittor	1993	KP339159	(Sreenivasulu et al.,, 1999)
BTV02USA1999	BTV-2	USA	1999	AY855279	(Mecham & Johnson, 2005)
BTV02ITA2000/01	BTV-2	Italy	2000	JN255867	(Caporale et al.,, 2011)
BTV02ITA2000/02	BTV-2	Italy	2000	JN255877	(Caporale et al.,, 2011)
BTV02TUN2000	BTV-2	Tunisia	2000	AJ586668	(Singh et al.,, 2004)
BTV02FRA2001	BTV-2	France : Corsica	2001	AJ586674	(Singh et al.,, 2004)
BTV02ITA2001	BTV-2	Italy	2001	AJ586672	(Singh et al.,, 2004)
BTV02ITA2002	BTV-2	Italy	2002	AJ586670	(Singh et al.,, 2004)
BTV02TWN2003	BTV-2	Taiwan	2003	AY493690	(Ting et al.,, 2005)
BTV02JPN2007	BTV-2	Japan	2007	AB686238	(Shirafuji et al.,, 2012)
BTV02AUS2008	BTV-2	Australia	2008	JQ086246	(Boyle et al.,, 2012)
BTV02IND2010*	BTV-2	Karimnagar	2010	KP339169	(Reddy et al.,, 2016)
BTV02AUS2010	BTV-2	Australia	2010	JQ240326	(Boyle et al.,, 2012)
BTV02USA2010	BTV-2	USA	2010	JQ822253	(Maclachlan et al.,, 2013)
BTV02FRAxxxx	BTV-2	France :Corsica	xxxx	AY129083	
BTV02ITAxxxx	BTV-2	Italy	xxxx	AY189951	

* Sequenced during the current study

DEVELOPMENT OF GHEE RESIDUE CANDY INCORPORATED WITH ORANGE PEEL

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ABSTRACT

This study was carried out to develop a candy utilizing the by-products of juice and dairy industry viz. orange peel and the ghee residue. Ghee residue candy with incorporation of dried orange peel powder and aqueous extract at various levels of 5,10,15 per cent (T_1,T_2,T_3) were prepared. The per cent inhibition of Di-phenyl-picryl-hydrazyl free radical antioxidant activity of fresh orange peel, orange peel powder and aqueous extract were found to be 76.91± 0.13, 68.84± 0.22, 72.45± 0.21 respectively. Nutritional, textural and sensory qualities of the ghee residue candy incorporated with various levels of orange peel was carried out. On sensory evaluation by using 9-point hedonic scale T_2 was found to be the best among the various levels tried. Texture analysis of ghee residue candy T_1,T_2 and T_3 were carried out for the parameters viz. hardness and stickiness and it was found that there was a significant difference between various inclusion level of orange peel powder and aqueous extract. The nutritional quality of the ghee residue candy (T_1, T_2 and T_3) were analyzed and it was found that, as the percentage of inclusion of orange peel increased there was a corresponding increase in the nutrients except nitrogen free extract. The ghee residue candy incorporated with orange peel T_2 was found to possess a higher antioxidant activity 70.44± 0.10 compared to control 10.50+ 0.21.

Key words: Ghee residue candy, Orange peel, Antioxidant activity, Vitamin C, Aqueous extract.

INTRODUCTION

Candy is a confectionary sweet food prepared mainly from sugar and liquid glucose. Candy is consumed globally by all age group people and is popular, especially among children. There is a high demand in global market for value added candies of functional importance. A vast variety of candies are available in international market and is accepted by consumers based on its flavor and color. Developing an innovative candy with enhanced nutrients may add a new variety of the candy to the existing array of candies available in global market.

Candy is a sweet food prepared from fruits or vegetables by impregnating them with sugar syrup followed by draining of excessive syrup and then drying the product

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to a shelf stable state. Fruits and vegetables like apples, ginger, mangoes, guava, carrot and citrus peels have been used to prepare candies (Mehta and Bajaj, 1984).

The ghee residue, a by-product of ghee is rich in phospholipids, sulphydryl compounds, poly unsaturated fatty acids (PUFA), lipid, non-lipid constituents, flavor concentrates like FFA, carbonyls, and lactones (Serunjogi *et al.,* 1998). Antioxidant property of ghee residue is due to the mixture of its constituents. Ghee residue can be used as a source of natural antioxidant for improving the shelf life of food products including dairy products.

Orange constitutes about 60 per cent of the total citrus production (Lucia and Calogera, 2008). Peel represents between 50 to 65 per cent of total weight of the fruits and is the primary by-product (Ashbell and Donahaye, 1984). Orange peels are rich in flavonones, powerful antioxidants that help to reduce oxidative damage and fight free radicals.

Orange peels are also loaded with natural histamine suppressing compounds. The vitamin C in orange peel is about 136 mg/100 g whereas in the pulp it is about 50 mg/100 g (Zamantha *et al.,* 2014). It also contains considerable amounts of calcium, copper, magnesium, vitamin A, folate, other B complex vitamins and dietary fiber. Citrus peels contain principal dietary fiber sources such as cellulose, hemicelluloses, lignin, pectin, gums and bioactive compounds.

The present study was undertaken to develop ghee residue candy by incorporating orange peel, to effectively utilize the nutrients in the orange peel.

MATERIALS AND METHODS

Ingredients for ghee residue candy

Ghee residue, Skim milk powder, Orange peel, Liquid glucose, Sugar, Cocoa powder and dark chocolate were used for this study.

Standardization and selection of ingredients for preparation of ghee residue candy incorporated with orange peel

The ghee residue candy incorporated with orange peel was standardized using three different level of orange peel.

S.No.	Ingredients	Control	T_1	T_2	T_3
1.	Skim milk powder	100g	100g	100g	100g
2.	Liquid glucose	80g	80 g	80g	80g
3.	Cocoa powder	32g	32g	32g	32g
4.	Dark chocolate (Morde 45 per cent cocoa)	48g	48g	48g	48g
5.	Ghee residue (processed)	---	500g	500g	500g
6.	Sugar	240g	240g	240g	240g
7.	Orange peel powder and aqueous extract	---	50g (5 per cent)	100g (10 per cent)	150g (15 per cent)

T_1 – Ghee residue candy incorporated with 5 per cent orange peel and extract.

T_2 - Ghee residue candy incorporated with 10 per cent orange peel and extract.

T_3 - Ghee residue candy incorporated with 15 per cent orange peel and extract.

Flow chart for the preparation of ghee residue candy incorporated with orange peel (modified technique of Wadhwa, 1997)

Skim milk powder (125 g)

↓

Sugar (300 g)

↓

Liquid glucose (semi solid) (100 g)

↓

The contents were heated and mixed well until desired consistency was reached

↓

Cocoa powder (40 g)

↓

Dark chocolate (solid) (60 g)

↓

Heated at 80° C (1 min)

↓

Ghee residue was added and heating was stopped

↓

Aqueous extract of orange peel & powder each at 5, 10, 15 per cent (T_1, T_2, T_3 respectively) were added and contents were mixed well (55° C)

↓

The contents were cooled for 15 min at room temperature

↓

Filled in molds and set in refrigerator for 1 h

↓

Candy was packed in aluminium foil for individual serving

↓

Optimum inclusion level of orange peel powder and orange peel extract each at 10 per cent (T_2) was chosen best by sensory panel for further studies.

Estimation of antioxidant activity

The antioxidant activity was assessed by DPPH free radical scavenging assay (Nur Alam *et al.,* 2013) to study the influence of added orange peel in the candy prepared using ghee residue.

Proximate analysis of ghee residue candy incorporated with orange peel (T_2)

The developed product was analyzed for moisture, crude protein, crude fiber, ether extract, nitrogen free extract and total ash as per the procedure described in AOAC (1997).

Sensory evaluation

Sensory evaluation was conducted by using the 9 point hedonic scale for the sensory attributes developed by Larmond (1997).

Texture analysis

The ghee residue candy incorporated with orange peel was observed for textural properties, by using texture analyzer as per Afoakwa *et al.* (2007) with a penetration probe P/5 attached to a 50 kg load cell (Stable Micro Systems; Model : TA XT Plus), connected to a computer, programmed with texture analysis software, assembled for candy analysis.

RESULTS AND DISCUSSION

Table 1. Sensory evaluation of ghee residue candies incorporated with orange peel using 9-point hedonic scale (Mean + SE)@

Sensory attributes	Treatments				
	Control	T_1	T_2	T_3	F-value
Colour	7.50±0.22	7.10±0.16	7.60± 0.30	7.33±0.21	2.312^{NS}
Flavour	7.15^{d}±0.21	7.23^{c}±0.30	8.00^{a}± 0.22	7.30^{b}±0.16	18.932**
Texture	6.90^{c}±0.19	7.30^{a}±0.22	7.30^{a}± 0.22	7.00^{b}±0.21	5.098**
Sweetness	6.67^{d}±0.18	7.50^{a}±0.22	7.00^{b}±0.20	6.92^{c}± 0.22	4.615**
Overall acceptability	7.05^{d}±0.25	7.28^{b}±0.22	7.47^{a}± 0.25	7.13^{c}±0.16	5.942**

@Average of six trials

Mean with different superscripts within a same row differ significantly from each other (P<0.01)

NS – Non significant (P > 0.05)

** Highly significant (P < 0.01)

T_1 – Ghee residue candy incorporated with 5 per cent orange peel and extract.

T_2 - Ghee residue candy incorporated with 10 per cent orange peel and extract.

T_3 - Ghee residue candy incorporated with 15 per cent orange peel and extract.

The sensory evaluation of ghee residue candy incorporated with orange peel at 5 per cent (T_1), 10 per cent (T_2) and 15 per cent (T_3) were conducted by using 9-point hedonic scale along with the control, revealed a highly significant difference ($P < 0.01$) for all sensory attributes viz. flavor, texture, sweetness and overall acceptability except for color. T_2 was found to be the best followed by T_1, T_3 and control. Highly significant difference was noticed for the sensory attributes viz. texture, sweetness, overall acceptability between control and treatments. Negligible difference was noticed for texture between T_1 and T_2 whereas for sweetness T_1 scored higher scores. The decrease in sweetness might be due to the increase in addition of orange peel contributed by the higher fiber content in the orange peel as per Gorinstein *et al.* (2001). As per the scores for overall acceptability T2 was found to be the best

followed by T_1, T_3 and control and hence T_2 was considered to be the optimum level of addition of orange peel in preparation of ghee residue candy.

Table 2. Nutritional composition of ghee residue candy incorporated with orange peel in per cent (Mean + SE) @

S.No.	Constituents	Treatments				
		Control	T_1	T_2	T_3	F-value
1.	Moisture	$5.26^{c}\pm 0.22$	$10.46^{b}\pm 0.12$	$10.60^{b}\pm 0.11$	$11.45^{a}\pm 0.11$	343.388**
2.	Crude Protein	$5.60^{d}\pm 0.11$	$6.19^{c}\pm 0.02$	$6.75^{b}\pm 0.02$	$7.32^{a}\pm 0.13$	70.892**
3.	Crude Fibre	$0.15^{d}\pm 0.01$	$2.81^{c}\pm 0.02$	$3.53^{b}\pm 0.01$	$3.97^{a}\pm 0.05$	476.179**
4.	Ether extract	$9.47^{c}\pm 0.13$	$10.53^{b}\pm 0.16$	$10.88^{b}\pm 0.17$	$10.94^{a}\pm 0.21$	89.960**
5.	Total ash	$0.99^{d}\pm 0.04$	$1.09^{c}\pm 0.15$	$1.49^{b}\pm 0.04$	$1.72^{a}\pm 0.04$	50.764**
6.	Nitrogen free extract	$78.53^{a}\pm 0.32$	$68.92^{b}\pm 0.17$	$66.75^{c}\pm 0.17$	$64.60^{d}\pm 0.34$	516.647**

@Average of six trials

Mean with different superscripts within a same row differ significantly from each other (P<0.01)

** Highly significant (P < 0.01)

T_1 – Ghee residue candy incorporated with 5 per cent orange peel and extract.

T_2 - Ghee residue candy incorporated with 10 per cent orange peel and extract.

T_3 - Ghee residue candy incorporated with 15 per cent orange peel and extract.

The nutritional composition of control, 5 per cent (T_1), 10 per cent (T_2) and 15 per cent (T_3) ghee residue candy incorporated with orange peel revealed a highly significant difference (P < 0.01) for moisture, crude protein, crude fiber, ether extract, total ash and nitrogen free extract. All the nutrients were found to increase as level of inclusion of orange peel increased. Moisture content in ghee residue candy incorporated with orange peel when compared to control was found to increase when compared to control which might be due to the addition of aqueous extract of orange peel. Increase in crude protein per cent in treated sample might be due to the inclusion of ghee residue, cocoa powder and orange peel in the candy as per Santha and Narayanan (1978) and Grohmann *et al.* (1995).

All the other nutrients like crude fiber, ether extract, total ash and NFE increased. This finding almost correlates with the findings of Nasser *et al.* (2008) and Magda *et al.* (2008) who stated that crude fiber, carbohydrates increased as the inclusion level of orange peel increased. These findings also correlate the findings of Hanan *et al.* (2012) who found that 10 per cent incorporation of citrus peel powders in wheat biscuits increased crude protein, crude fat contents as well as crude fiber and moisture content.

Table 3. Texture Analysis of ghee residue candy incorporated with orange peel (Mean + SE)@

Constituents	Control	Treatments			
		T_1	T_2	T_3	F – value
Hardness (g)	$100.60^{d}\pm 0.36$	$102.58^{a}\pm 0.31$	$101.38^{b}\pm 0.34$	$101.36^{b}\pm 0.20$	9.433**
Stickiness (g)	$-102.31^{a}\pm 0.36$	$-102.04^{b}\pm 0.38$	$-101.93^{c}\pm 0.39$	$-101.44^{d}\pm 0.40$	4.616**

@Average of six trials

Mean with different superscripts within a same row differ significantly from each other (P<0.01)

** Highly significant (P < 0.01)

T_1 – Ghee residue candy incorporated with 5 per cent orange peel and extract.

T_2 - Ghee residue candy incorporated with 10 per cent orange peel and extract.

T_3 - Ghee residue candy incorporated with 15 per cent orange peel and extract.

The texture analysis of control and ghee residue candy incorporated with orange peel at 5 per cent (T_1), 10 per cent (T_2) and 15 per cent (T_3) were analyzed for the parameters hardness and stickiness revealed a highly significant difference (P < 0.01) between treatments.

Increase in hardness and decrease in stickiness in treated samples compared to control might be due to the hydrophilicity of orange peel in the ghee residue candy as per Nassar *et al.* (2008) and Kohajdova *et al.* (2011).

Table 4. Antioxidant activity of fresh orange peel, powdered dried orange peel and aqueous extract (Mean + SE) (per cent inhibition of DPPH free radical)@

Control (Ascorbic acid)	Fresh orange peel	Powdered dried orange peel	Aqueous extract	F – value
$83.99^{a}\pm 0.21$	$76.91^{b}\pm 0.13$	$68.84^{d}\pm 0.22$	$72.45^{c}\pm 0.21$	2346.9**

The antioxidant activity of fresh orange peel, powdered dried orange peel and aqueous extract of orange peel were 76.91+0.13, 68.84+0.22 and 72.45+ 0.21 per cent inhibition of DPPH free radical respectively and a highly significant difference (P < 0.01) was noticed between them.

The decreased antioxidant activity exhibited in the powdered dried orange peel might be due to the partial destruction of vitamin C which also possesses antioxidant property as per Lario *et al.* (2004). The higher antioxidant activity of aqueous extract compared to powdered dried orange peel might be due to the higher concentration of flavonoides in the aqueous extract which possess antioxidant activity as per Chakraborthy and Shah (2011).

Table 5. Antioxidant activity of ghee residue candy incorporated with orange peel (T_2) (Mean + SE) (per cent inhibition of DPPH free radical)@

Antioxidant activity per cent inhibition of DPPH free radical		t - value
Control candy with Ghee residue	Ghee residue candy incorporated with orange peel (T_2)	
$10.50^{b}\pm 0.21$	$70.44^{a}\pm 0.10$	13435.9**

@Average of six trials

Mean with different superscripts within a same row differ significantly from each other (P<0.01)

** Highly significant (P < 0.01)

T_2 - Ghee residue candy incorporated with 10 per cent orange peel and extract.

Ghee residue candy incorporated with orange peel (T_2) and control candy were 10.50+0.21 and 70.44+0.10 per cent inhibition of DPPH free radical and revealed a highly significant difference (P < 0.01) between control and ghee residue candy.

The increase in antioxidant activity ghee residue candy incorporated with orange peel compared to control is due to the incorporation of orange peel which contains vitamin C and phenolic compounds which have the ability to scavenge the reactive oxygen capable of causing oxidative effect as per Gil- Izquierdo *et al.* (2001).

CONCLUSION

Candy, also known as sweet or lolly, is a confection that features sugar as a principal ingredient. Vegetables, fruit, or nuts which have been glazed and coated with sugar are said to be candied. Physically, candy is characterized by the use of a significant amount of sugar or sugar substitutes. Sensory analysis revealed that ghee residue candy incorporated with orange peel (T_2) was preferred than other levels of inclusion. Increase in crude protein per cent in treated sample is due to the inclusion of ghee residue in the candy. Increase in hardness and decrease in stickiness in treated samples compared to control might be due to the hydrophilicity of orange peel in the ghee residue candy.

The antioxidant activity of the candy was carried out as ghee residue was used as one of the ingredients in the preparation of candy. The higher antioxidant activity of aqueous extract compared to powdered dried orange peel might be due to the higher concentration of flavonoides in the aqueous extract which possess antioxidant activity. The increase in antioxidant activity ghee residue candy incorporated with orange peel compared to control is due to the incorporation of orange peel which contains vitamin C and phenolic compounds which have the ability to scavenge the reactive oxygen capable of causing oxidative

effect. Hence the present has explored the possibilities to develop an innovative candy with enhanced nutrients to suite the palate of the consumers utilizing the by-products of dairy and juice industry.

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INFLUENCE OF HOUSING SYSTEM ON CUMULATIVE FEED CONSUMPTION AND FEED CONVERSION RATIO OF BROILERS FED WITH DIFFERENT LEVELS OF ENERGY AND PROTEIN

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ABSTRACT

Commercial broilers were fed with different levels of energy (2850, 2950 and 3050 kcal/kg in pre-starter diet, 2950, 3050 and 3150 kcal/kg in starter diet and 3050, 3150 and 3250 kcal/kg in finisher diet) and protein (21.5, 22.5 and 23.5% in pre-starter diet, 20.5, 21.5 and 22.5% in starter diet and 19, 20 and 21% in finisher diet) for a period of five weeks to assess the production performance in both environmentally controlled and open curtained housing systems. All the chicks were reared up to five weeks under standard managemental condition. During this experimental period, data on body weight, feed consumption and mortality if any were recorded at weekly interval. The result of the study revealed lower feed intake in group T_7 (2640.16 g) in environmentally controlled housing system and group T_6 (2667.19 g) in open curtained housing system. The broilers reared in environmentally controlled housing system had significantly better feed conversion ratio (1.31 to 1.42) than broilers reared in open curtained housing system (1.45 to 1.52).

Key words: Housing system, Cumulative feed consumption, Feed conversion ratio, Broilers.

INTRODUCTION

Indian broiler industry has gone through tremendous development and expansion during the last two decades. Increased genetic potential of broilers coupled with higher placements helped in rapid development of broiler production in hot climates and requires greater emphasis on finding solutions to alleviate growth depression due to heat stress. Ambient temperature is an important determinant of bird performance. The main consequence of heat exposure is reduction in feed intake in order to reduce metabolic heat production. In broilers this reduction is approximately 1.5 to 2.5 per cent per ^{0}C increase in ambient temperature above 20^{0}C. Reduction in growth is often greater than the reduction in feed intake, resulting in lower feed efficiency.

Prevailing conditions of increasing ambient temperature and system of rearing in tropical countries are a major concern in broiler production. The future of broiler

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industry may increasingly depend on environmentally controlled poultry house rather than open curtained poultry house, due to conditions like global warming. In India, studies are being focused primarily on the requirement of energy and protein and their proportion for augmentation of broiler growth only for open sided houses.

MATERIALS AND METHODS

The biological experiments were designed and conducted during January to February 2014 in environmentally controlled broiler house as well as in open sided broiler house located at the Poultry Farm Complex, Department of Poultry Science, Veterinary College and Research Institute, Namakkal, Tamil Nadu. Namakkal is situated in northwestern agro-climatic zone of Tamil Nadu at 11º2' N latitude and 78º2' E longitude at an altitude of 192 m above the mean sea level. The minimum and maximum temperature and relative humidity during the study period were 19.82 ºC and 30.43 ºC and 52.12 and 77.45 per cent, respectively.

The biological experiment was carried out with five hundred and seventy six (each 288 in environmentally controlled deep litter house and open sided deep litter house), sex separated, day-old, commercial (Vencobb 400) broiler chicks belonging to single hatch purchased from local hatchery.

Chicks were wing banded, weighed and randomly allotted into nine treatment groups with four replicates of eight chicks in each under both system of rearing.

Throughout the study period of five weeks, data on body weight, feed intake, mortality if any were recorded at weekly interval. The data collected on feed intake, body weight and calculated data on feed conversion ratio were subjected to statistical analysis as per the method suggested by Snedacor and Cochran (1989). Angular transformation was applied to percentages wherever needed before carrying out statistical analysis. The treatments applied to the nine groups of the experiment are provided in Table I.

Table-I

Treatment groups for each system of rearing	Particulars			Number of replicates per treatment	Number of birds per replicate	Total number of birds per treatment
	Type of feed	CP (%)	ME (kcal/kg)			
T_1	Pre-starter	21.5	2850	4	8	32
	Starter	20.5	2950			
	Finisher	19.0	3050			
T_2	Pre-starter	22.5	2850	4	8	32
	Starter	21.5	2950			
	Finisher	20.0	3050			

T_3	Pre-starter	23.5	2850	4	8	32
	Starter	22.5	2950			
	Finisher	21.0	3050			
T_4	Pre-starter	21.5	2950	4	8	32
	Starter	20.5	3050			
	Finisher	19.0	3150			
T_5	Pre-starter	22.5	2950	4	8	32
	Starter	21.5	3050			
	Finisher	20.0	3150			
T_6	Pre-starter	23.5	2950	4	8	32
	Starter	22.5	3050			
	Finisher	21.0	3150			
T_7	Pre-starter	21.5	3050	4	8	32
	Starter	20.5	3150			
	Finisher	19.0	3250			
T_8	Pre-starter	22.5	3050	4	8	32
	Starter	21.5	3150			
	Finisher	20.0	3250			
T_9	Pre-starter	23.5	3050	4	8	32
	Starter	22.5	3150			
	Finisher	21.0	3250			
					Total	288

RESULTS AND DISCUSSION

Effect of housing system on mean (± S.E.) feed consumption (g/bird) of broilers from first to fifth week of age as influenced by different levels of energy and protein is given in Table III.

The results revealed that at first week of age, broilers reared in environmentally controlled housing system recorded higher feed consumption than the broilers reared in open sided housing system. At the end of fifth week, the feed consumption of broilers reared in environmentally controlled housing system was significantly higher than broilers reared in open sided housing system in all treatment groups indicating that housing system influenced cumulative feed consumption in broilers, irrespective of various combinations of dietary energy and protein.

The result of the study revealed a lower feed intake was noticed in group T_7 (2640.16 g) in environmentally controlled housing system and group T_6 (2667.19 g) in open sided housing system.

The group T_7 was fed with broiler pre-starter diet with 3050 kcal/kg of ME and 21.5% CP, broiler starter diet with 3150 kcal/kg of ME and 20.5% CP and broiler finisher diet with 3250 kcal/kg of ME and 19% CP. The group T_6 was fed with broiler pre-starter diet with 2950 kcal/kg of ME and 23.5% CP, broiler starter diet with 3050 kcal/kg of ME and 22.5% CP and broiler finisher diet with 3150 kcal/kg of ME and 21% CP whereas highest feed intake was recorded in group T_3 reared in both the housing system. The group T_3 was fed with broiler pre-starter diet with 2850 kcal/kg of ME and 23.5% CP, broiler starter diet with 2950 kcal/kg of ME and 22.5% CP and broiler finisher diet with 3050 kcal/kg of ME and 21% CP.

Effect of housing system on feed conversion ratio of broilers from first to fifth week of age as influenced by different levels of energy and protein is given in Table IV.

The broilers reared in environmentally controlled housing system had significantly better feed conversion ratio (1.38 to 1.52) than the broilers reared in open sided housing system (1.47 to 1.68). Interaction of dietary treatments and housing system recorded significant and highly significant difference between treatment groups at fifth week feed conversion ratio in broilers. This indicated that the housing system influenced the feed conversion ratio irrespective of various combinations of dietary energy and protein in broilers. Within the same dietary protein and energy level, environmentally controlled housing system recorded approximately 10 per cent better feed conversion ratio than open sided housing system.

The result of the study indicated that the cumulative feed conversion ratio was best in group T_7 for broilers in environmentally controlled housing system. The group T_7 was fed with broiler pre-starter diet with 3050 kcal/kg of ME and 21.5% CP, broiler starter diet with 3150 kcal/kg of ME and 20.5% CP and broiler finisher diet with 3250 kcal/kg of ME and 19% CP. In case of open sided housing system group T_6 showed better result which was fed with broiler pre-starter diet with 2950 kcal/kg of ME and 23.5% CP, broiler starter diet with 3050 kcal/kg of ME and 22.5% CP and broiler finisher diet with 3150 kcal/kg of ME and 21% CP.

The results are in accordance with the earlier works of Al-Batshan and Hussein (1999), Ghaffari *et al.* (2007b), Kamran *et al.* (2008a), Kabir *et al.* (2010), Moosavi *et al.* (2011) and Elagib and Elzubeir (2012) who also reported that broilers fed with diet containing high energy had less feed consumption.

Al-Batshan and Hussein (1999), Maiorka *et al.* (2005), Ghaffari *et al.* (2007a), Kabir *et al.* (2010), Jafarnejad and Sadegh (2011), Moosavi *et al.* (2011), Malomo *et al.* (2013) also reported similar results, that broilers fed with diet containing high energy showed better feed conversion.

Table II. Effect of housing system on mean (± S. E.) body weight (g) of broilers from 1 to 5 weeks of age as influenced by different levels of energy and protein

Treatment groups	House	I Week	II Week	III Week	IV Week	V Week
T_1	OPHS	157.47±2.2	377.69±7.37	713.66±20.40	1212.22±18.96	1696.13±26.01
	EC	165.63±2.47	415.91±8.45	801.41±20.95	1317.13±22.87	1878.28±36.30
	"t" value	**2.46***	**3.40***	**3.00***	**3.53***	**4.07****
T_2	OPHS	158.46±2.69	390.70±6.77	788.75±10.03	1271.63±32.34	1772.82±18.91
	EC	158.00±1.73	413.41±6.34	815.66±11.51	1316.97±11.59	1856.22±36.00
	"t" value	**1.37^{NS}**	**2.44^{NS}**	**3.71***	**1.31***	**2.05***
T_3	OPHS	161.37±3.72	401.06±6.66	752.85±9.37	1289.15±23.76	1730.47±9.49
	EC	161.62±1.35	432.41±5.66	859.22±10.35	1382.06±18.37	1930.44±30.74
	"t" value	**0.63^{NS}**	**3.58***	**7.61***	**3.07***	**6.21****
T_4	OPHS	164.03±1.16	389.37±10.57	762.75±13.64	1242.68±25.60	1720.59±28.45
	EC	162.09±1.73	428.91±5.97	840.68±6.11	1376.69±17.83	1951.44±20.54
	"t" value	**9.28^{NS}**	**3.26***	**5.21****	**4.29****	**6.57****
T_5	OPHS	165.06±2.53	399.03±6.72	787.41±12.53	1300.35±10.04	1803.82±23.03
	EC	157.94±0.77	428.28±3.72	839.82±5.56	1369.66±17.85	1908.16±13.43
	"t" value	**2.69***	**3.86***	**3.82***	**3.38***	**3.91***
T_6	OPHS	168.00±2.78	400.60±8.93	790.63±15.76	1308.60±35.43	1819.25±29.16
	EC	159.75±3.99	426.16±8.94	855.25±13.07	1368.01±24.07	1937.79±57.06
	"t" value	**1.69^{NS}**	**2.02***	**3.15***	**1.38***	**1.85***
T_7	OPHS	162.58±2.36	390.84±13.10	760.10±25.80	1261.45±27.78	1724.84±42.45
	EC	162.63±2.76	431.02±10.43	852.56±13.06	1377.74±13.56	1963.13±50.13
	"t" value	**0.01^{NS}**	**2.40^{NS}**	**3.19***	**3.76***	**3.62***
T_8	OPHS	162.63±3.61	395.13±8.82	773.19±14.90	1286.13±16.96	1757.63±44.48
	EC	160.57±1.61	426.97±6.00	852.35±19.90	1411.38±21.63	1989.60±47.68
	"t" value	**0.52^{NS}**	**2.98***	**3.18***	**4.55****	**3.57***
T_9	OPHS	165.41±3.10	406.19±12.22	811.35±17.10	1372.10±25.45	1884.38±50.73
	EC	158.19±3.72	426.60±8.35	875.07±11.80	1448.47±17.60	2022.19±42.75
	"t" value	**1.49^{NS}**	**1.37^{NS}**	**3.06***	**2.46***	**2.07***

OPHS - Open sided housing system, EC- Environmentally controlled housing system.
NS- Non significant, * - Significant (P<0.05) and ** - Highly significant (P<0.01)

Table-III. Effect of housing system on mean (± S. E.) cumulative feed consumption (g/ bird) of broilers from 1 to 5 weeks of age as influenced by different levels of energy and protein

Treatment groups	House	I Week	II Week	III Week	IV Week	V Week
T_1	OPHS	152.19±2.21	484.85±5.84	1009.85±25.65	1852.35±32.58	2846.25±12.95
	EC	145.60±1.54	348.32±7.12	1018.91±23.58	1861.41±31.54	2855.32±14.01
	"t" value	**2.28^{NS}**	**7.12****	**11.24****	**7.12****	**3.37****
T_2	OPHS	150.38±2.95	481.03±6.25	990.10±12.05	1818.25±14.22	2802.28±28.22
	EC	138.13±2.56	348.13±5.01	995.31±13.51	1824.07±9.85	2807.50±30.14
	"t" value	**2.31***	**1.12^{NS}**	**4.66****	**5.17****	**2.95***
T_3	OPHS	151.41±1.45	485.32±4.86	1021.88±14.57	1908.91±25.21	2860.00±35.21
	EC	145.16±2.01	346.88±3.54	1028.60±13.22	1815.63±23.35	2866.77±34.66
	"t" value	**1.73 ^{NS}**	**4.76****	**6.06****	**6.54****	**3.06****
T_4	OPHS	146.88±1.89	470.00±11.26	959.69±25.25	1802.04±20.55	2703.60±18.96
	EC	140.63±1.02	333.13±12.54	963.44±28.69	1830.78±18.82	2771.41±12.45
	"t" value	**2.98***	**0.73 ^{NS}**	**4.48****	**3.86****	**2.47***
T_5	OPHS	143.38±1.87	481.63±5.20	1027.25±9.20	1854.13±21.20	2759.60±35.21
	EC	137.13±1.20	350.69±4.56	1033.44±8.55	1797.82±18.98	2703.28±30.28
	"t" value	**3.16***	**1.83^{NS}**	**6.18****	**1.78^{NS}**	**1.18^{NS}**
T_6	OPHS	149.53±1.54	466.72±6.87	946.25±19.65	1745.63±25.65	2667.19±40.12
	EC	143.28±1.46	332.03±6.01	954.85±17.21	1754.22±24.55	2675.88±36.58
	"t" value	**4.71****	**2.05^{NS}**	**1.61^{NS}**	**1.17^{NS}**	**0.74^{NS}**
T_7	OPHS	152.66±1.21	475.32±7.85	986.57±13.41	1785.94±18.54	2728.91±30.55
	EC	143.13±1.89	326.10±9.84	980.47±14.84	1726.72±21.70	2640.16±17.84
	"t" value	**4.16****	**2.30***	**4.86****	**4.06****	**4.49****
T_8	OPHS	148.60±8.54	481.57±12.88	981.88±34.58	1824.07±38.54	2732.82±48.86
	EC	142.35±5.86	352.66±9.54	995.32±33.85	1837.50±39.56	2746.25±45.62
	"t" value	**3.83****	**0.92^{NS}**	**5.60****	**2.79***	**2.38***
T_9	OPHS	165.63±8.54	506.41±22.14	1008.28±20.65	1867.35±34.21	2808.91±42.10
	EC	137.50±2.10	350.63±10.35	990.00±16.32	1874.06±33.22	2780.31±40.65
	"t" value	**2.88***	**1.09^{NS}**	**2.81***	**1.95^{NS}**	**0.91^{NS}**

OPHS - Open sided housing system, EC- Environmentally controlled housing system.

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

Table-IV. Effect of housing system on mean (± S. E.) cumulative feed conversion ratio of broilers from 1 to 5 weeks of age as influenced by different levels of energy and protein

Treatment groups	House	I Week	II Week	III Week	IV Week	V Week
T_1	OPHS	0.97±0.03	1.29±0.04	1.42±0.07	1.53±0.03	1.68±0.03
	EC	0.88±0.01	1.19±0.01	1.27±0.01	1.41±0.01	1.52±0.02
	"t" value	**3.34***	**2.44***	**2.17***	**3.26***	**4.29****
T_2	OPHS	0.95±0.02	1.23±0.03	1.31±0.03	1.43±0.04	1.58±0.02
	EC	0.87±0.02	1.18±0.01	1.22±0.01	1.39±0.02	1.51±0.03
	"t" value	**3.50***	**1.93 NS**	**2.89***	**1.10 NS**	**1.84 NS**
T_3	OPHS	0.94±0.02	1.21±0.03	1.36±0.03	1.48±0.03	1.65±0.02
	EC	0.90±0.01	1.14±0.02	1.20±0.01	1.39±0.01	1.49±0.01
	"t" value	**1.92NS**	**2.19***	**5.55****	**2.98***	**7.77 ****
T_4	OPHS	0.90±0.01	1.21±0.02	1.26±0.03	1.46±0.03	1.57±0.03
	EC	0.87±0.01	1.11±0.03	1.15±0.04	1.33±0.02	1.42±0.02
	"t" value	**2.32***	**2.82***	**2.40***	**3.33***	**4.69****
T_5	OPHS	0.87±0.01	1.21±0.01	1.31±0.01	1.43±0.02	1.53±0.01
	EC	0.87±0.01	1.14±0.01	1.23±0.01	1.32±0.02	1.42±0.02
	"t" value	**0.18NS**	**4.70***	**4.39***	**4.76****	**5.81****
T_6	OPHS	0.89±0.01	1.17±0.03	1.20±0.03	1.34±0.06	1.47±0.05
	EC	0.90±0.02	1.12±0.03	1.12±0.03	1.28±0.02	1.38±0.03
	"t" value	**0.34 NS**	**1.17 NS**	**1.97***	**0.85 ***	**1.58 ***
T_7	OPHS	0.94±0.01	1.22±0.06	1.30±0.06	1.42±0.04	1.59±0.05
	EC	0.88±0.02	1.09±0.04	1.15±0.03	1.26±0.01	1.37±0.03
	"t" value	**2.20***	**1.90NS**	**2.38***	**3.74***	**3.65***
T_8	OPHS	0.92±0.03	1.22±0.04	1.27±0.04	1.42±0.02	1.56±0.04
	EC	0.89±0.02	1.16±0.03	1.17±0.03	1.30±0.03	1.38±0.02
	"t" value	**0.82 NS**	**1.24 NS**	**2.01***	**3.72***	**4.29***
T_9	OPHS	1.00±0.06	1.25±0.05	1.25±0.04	1.36±0.04	1.50±0.05
	EC	0.87±0.02	1.15±0.02	1.13±0.01	1.30±0.01	1.38±0.01
	"t" value	**2.02 NS**	**1.96 NS**	**2.56***	**1.59 NS**	**2.24 ***

OPHS - Open sided housing system, EC- Environmentally controlled housing system.

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

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Short Communications

ANTIMICROBIAL EFFECT OF *PSIDIUM GUAJAVA*, *MANGIFERA INDICA* AND *PUNICA GRANATUM* EXTRACTS AGAINST BOVINE MASTITIS PATHOGEN

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ABSTRACT

The present study was carried out to assess the antibacterial activity of aqueous extracts of *Psidium guajava* leaves, *Mangifera indica* kernel and *Punica granatum* pericarp. Antimicrobial activity against *Escherchia coli*, *Staphylococcus aureus* and *Streptococcus* spp. isolated from bovine mastitis cases were tested by measuring MIC by Resazurin dye broth microdilution method. All the three plant extracts exhibited antibacterial activity against organism tested. Of the three extracts, *Punica granatum* extracts was found to have lower MIC of 2.5 mg/ml against *E. coli* and *Streptococcus* sp compared to other two extracts. However, *Mangifera indica* kernel extract was found to have good antibacterial activity against *Staphylococcus aureus* with a lower MIC of 0.625 mg/ml compared to other two extracts. The results of the present study confirmed the antibacterial potential of plant extracts. Further studies are required to explore the bioactive molecules which would probably become an alternative source of new and natural antibacterial agents.

Key Words: Mastitis - Antibacterial activity - *Psidium guajava- Mangifera indica – Punica granatum.*

INTRODUCTION

Bovine mastitis continues to be one of the most problematic diseases of the dairy cattle and it is usually treated with antimicrobial agents. There has been increasing incidence of resistance development against antimicrobial drugs commonly employed in the treatment of mastitis. Development of multidrug resistance in bacterial pathogens has led to treatment failure and it necessitates search for new alternative drugs. Plants have been used for medicinal purposes since time immemorial and the role of medicinal plants in the discovery of new drugs is overwhelming. In India, large numbers of plants have been used traditionally for treatment of various ailments. In the present study, we have selected three medicinal plants viz., *Psidium guajava* (Common name: Guava), *Mangifera indica* (Common name: Mango) and *Punica granatum* (Common name: Pomegranate), to evaluate

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their antimicrobial activity against bovine mastitis pathogens.

MATERIALS AND METHODS

Plant Collection

Guava leaves (*Psidium guajava*) were collected from Villupuram district of Tamilnadu, India. Pomegranate fruits (*Punica granatum*) and mango (*Mangifera indica*) were purchased from local market in Chennai, Tamilnadu, India.

Extract preparation

Pomegranate pericarp, mango seed kernel and guava leaves were shade dried and powdered using blender. The aqueous extract of each plant material was prepared by dissolving 20 g of powdered plant material in 100 ml of triple distilled water. This solution was thoroughly mixed using magnetic stirrer for 6 hrs and kept overnight without any disturbance. Following day, the solution was strained using muslin cloth and then filtered twice using Whatman filter paper No.1. The filtrate thus obtained was transferred to sterile petri dish and dried in hot air oven at 48–50ºC overnight. Dried extract was scraped using sterile surgical blade, weighed, transferred to sterile glass vials and stored at -20ºC.

Bacterial strains

A total of 20 bacterial isolates from bovine clinical/subclinical mastitis cases were studied. These isolates were collected, characterized and maintained by Department of Veterinary Pharmacology and Toxicology, MVC, Chennai. Isolates tested included *Staphylococcus aureus* (8 isolates), *Escherichia coli* (10 isolates) and *Streptococcus* spp (2 isolates).

Antimicrobial susceptibility testing

The MICs of plant extracts were determined by Resazurin dye broth microdilution method (Sarker *et al*., 2007). Serial two fold dilutions of drugs in nutrient broth (50 µl) were prepared in 96 well ELISA plates. 10 µl of Resazurin dye, 30 µl of nutrient broth and 10 µl of bacterial suspension (adjusted to 5 x 10^6 CFU/ml) were added to the wells. Sterility control without bacteria and growth control without drug were also included. Plates were wrapped and incubated at 37°C for overnight. The lowest concentration that inhibited visible growth (persistence of blue color) of bacteria was recorded as MIC. The MICs at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited were calculated. All the experiments were performed in triplicate

RESULTS AND DISCUSSION

The MICs of the plant extracts against bacterial isolates are presented in Table 1. The aqueous extract of all the three plants showed antibacterial activity against both Gram positive (Streptococci spp. and *Staphylococcus aureus*) and Gram negative (*Escherichia coli*) organisms tested.

In the present study, it was observed that the extracts of mango seed kernel showed antibacterial activity against organisms tested. Similarly, Sowmiya *et al* (2009) reported that aqueous and ethanol extracts of mango kernel showed good antibacterial activity against *E. coli, S. aureus* and *S. pyogens*. The extract of mango seed kernel

showed excellent activity against *S. aureus* with MIC of 0.3125-5 mg/l compared to other two extracts. Mirghani *et al.* (2009) reported that extracts of mango seed kernel are more effective against Gram-positive than the Gram-negative bacteria. In our study, similar result was found in which MIC of mango seed kernel against *E. coli* (MIC 5 mg/l) was higher than *S. aureus* (MIC_{50} 0.625 mg/l). Antibacterial activity of mango seed kernel may be due to the presence of considerable amount of total phenolic compounds (Mirghani *et al.,* 2009). The presence of phellandrene, α-pinene, ambolic acid, ascorbic acid, β-carotene, gallic acid, gallotannic acid, mangifelic acid, mangiferol peroxidase, phenyl alanine and proline (Kabuki *et al*, 2000) and gallotannins (Engels *et al.,* 2009) in mango seed kernel extracts showed antimicrobial activity to both Gram positive and Gram negative bacteria.

The extract of *Punica granatum* was equally effective in inhibiting both Gram positive and Gram negative organisms tested. These results are in agreement with those reported by Kadi *et al* (2011). They reported that extracts of *Punica granatum* bark showed broad spectrum of activity against both Gram positive and Gram negative organisms. We found that extract of *Punica granatum* was more effective than the other two extracts against *E. coli* and *Streptococcus* spp. Silva *et al*. (2008) reported that extract of *Punica granatum* was effective against *S. aureus* isolated from bovine mastitis and can be used as an alternative therapy in veterinary medicine. The broad-spectrum antibacterial activity of this plant extract might be due to the presence of secondary metabolites such as tannins, alkaloids, phenolic compounds or saponins (Machado *et al*, 2002).

We observed that guava leaf extract inhibited both Gram positive and Gram negative organisms with an MIC of 5.0 mg/ml. Ajariyakhajorn and Samnagamnim (2005) also reported that guava leaf extracts were effective against *S. aureus* isolates from bovine mastitis with a MIC of 0.2-51.2 mg/l. The active ingredients present in guava leaves include alkaloids, saponins, tannins, phenols, flavonoids, triterpenoids and steroids. (Arima and Danno, 2002)

This study reconfirmed the effectiveness of the extracts of *Punica granatum, Psidium guajava* and *Mangifera indica* as potential antibacterial agents against mastitis pathogens. Further studies are needed to elucidate the horizon of antibacterial activity and the structural information of the biomolecules.

Table 1 MIC results of plant extracts against bacterial isolates from bovine mastitis

Organism	Extract	MIC	MIC_{50}
Escherchia coli	*Punica granatum* (mg/ml)	2.5	2.5
	Mangifera indica (mg/ml)	5.0	5.0
	Psidium guajava (mg/ml)	5.0	5.0
	Enrofloxacin (µg/ml)	2.44 – 156.25	2.44
Staphylococcus aureus	*Punica granatum* (mg/ml)	2.5	2.5
	Mangifera indica (mg/ml)	0.3125-5.0	0.625
	Psidium guajava (mg/ml)	5.0	5.0
	Enrofloxacin (µg/Ml)	0.305 – 2.44	1.22
Streptococcus sp	*Punica granatum* (mg/ml)	2.5	2.5
	Mangifera indica (mg/ml)	2.5	2.5
	Psidium guajava (mg/ml)	5.0	5.0
	Ceftriaxone (µg/ml)	0.625	0.625

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EVALUATION OF POSTERIOR CAPSULAR OPACIFICATION IN DOGS – A REVIEW OF 12 CASES

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ABSTRACT

Posterior capsule opacification (PCO) is the most common long-term postoperative complication following cataract surgery. It causes impaired visual acuity by directly blocking the visual axis. A total of 12 cases were included in the study. The cases were divided into two groups, group I (cyclosporine A) and group II (mitomycin C) consisting of six eyes each. The concomitant preoperative conditions (stage of cataract, age of the patient, etc.) and intra operative conditions (Phacoemulsification parameters: power, irrigation aspiration fluid volume, time) were related with the development of post-operative complications. Evaluation and grading of PCO by using indirect ophthalmoscopy and EPCO 2000 software and the mean average of PCO scores in visual assessment by using indirect ophthalmoscopy was 0.29 ± 0.18 and mean PCO score in EPCO software score was 0.37 ± 0.1. A higher PCO score were found on EPCO 2000 software than visual function assessment by using indirect ophthalmoscopy. This study was concluded that the software method of evaluation of PCO was easy, applicable and more appropriate method to evaluate in clinical studies.

Key words: PCO, Cyclosporine A, Mitomycin C

INTRODUCTION

Posterior capsule opacification (PCO) is the most common long-term postoperative complication following cataract surgery. It causes impaired visual acuity by directly blocking the visual axis.

Posterior capsule opacification (PCO) is the most common long-term postoperative complication following cataract surgery, also called as after cataract or secondary cataract. PCO results from the growth and abnormal proliferation of Lens Epithelial Cells (LECs) on the capsule at the time of cataract surgery. These cells migrate to the posterior capsule where they approach the central visual axis and cause visual axis obstruction, resulting in dimness of vision [(2)] The severity of PCO was greater in canine patients which might be related to the differences in inflammatory and fibrous response. While it was more difficult to quantify, it was likely that PCO represents a significant cause of reduced visual acuity in dogs after cataract surgery [(1)]

MATERIALS AND METHODS

The present study was conducted at Small Animal Ophthalmology Unit of the Madras Veterinary College Teaching Hospital, over a period of 12 months. Clinical cases which were presented with a history of visual deficit and clinical signs suggestive of cataract, were included in this study. The patients were further screened for routine hematological (hemoglobin,

packed cell volume, RBC count) and serum biochemical (blood glucose level) parameters as an assessment of preoperative health status. Twelve cases were randomly selected after phacoemulsification surgery. In all 12 cases, the pupil dilatation was achieved using 0.8% tropicamide eye drops.

Two methods were used for the evaluation of PCO post operatively after phacoemulsification surgery. After pupil dilatation both indirect ophthalmoscopy and EPCO 2000 software [(3)] evaluation were done in all the 12 cases.

RESULTS AND DISCUSSION

On EPCO software analysis, PCO could be well observed in 9 out of 12 cases. PCO after 50 days postoperative examination period. On visual assessment by using indirect ophthalmoscopy method PCO could be observed only in 5 out of 12 cases on 50th day postoperatively.

More number of PCO could be well observed and viewed in EPCO 2000 software method than visual assessment using indirect ophthalmoscopy method.

The mean of PCO score in visual assessment by using indirect ophthalmoscopy was **0.29 ± 0.18** and mean PCO score in EPCO software score was **0.37 ± 0.1.** A higher PCO score were found on EPCO 2000 software than visual function assessment by using indirect ophthalmoscopy.

CONCLUSION

This study was concluded that the software method of evaluation of PCO was easy, applicable and more appropriate method to evaluate in clinical studies than any other methods.

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EFFECT OF SILK FIBROIN LOADED SILVER NANOPARTICLES GEL ON WOUND HEALING IN THREE LABRADOR DOGS

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ABSTRACT

Three Labrador retriever dogs (5 months, 1 and 2 years of age) werepresented to Madras Veterinary College Teaching Hospital with the history of chronic non-healing open wounds on the left cranial elbow, right dorsolateral abdomen and right ventrolateral abdomen. Physical examination revealed mild yellowish coloured wound with putrid odour and purulent discharge. The Etiology of the wounds was laceration in all the three cases. The subcutaneous tissues were exposed; necrotic tissues and foreign debris were noticed.Silk fibroin loaded silver nanoparticle gel was prepared and applied on days 0, 3, 7, 14, 21 and 28 respectively on the open wound and bandaging done. Silk fibroin is a protein polymer which does not interfere with the host immune system and it can be used in chronic non-healing wounds accelerating wound healing, aids in spreading of keratinocytes and fibroblasts and thereby promoting the growth and development of skin tissue. Clinical examination revealed bright red colour granulation tissue with neovascularisation. On bacteriological examination, Staphylococcus aureus and Pseudococcusaeroginosa were isolated. Mean per cent epithelialization, wound contraction and wound healing were significantly high.

Keywords: Dog, Chronic non-healing wound, Silk fibroin loaded silver nanoparticle

INTRODUCTION

Wound repair was a complex process requiring coordination of cascade of cellular responses to injury including inflammation, epithelialization, proliferation, angiogenesis and remodelling (Barrientos*et al.* 2008). A major drawback in conventional dressing materials, mainly composed of gauze, is the adherence between the fibers of the gauze materials and the tissue. Separation between the dressing material and the tissue becomes very difficult (Lin *et al.,* 2000). In large wound cases if required donor site is not available, biomaterials like synthetic or natural polymers and their composites can be used for temporary closure of wounds (Babu*et al.,* 1996). Fibroin protein was known for wound dressing by regulating exudates of wound providing moist environment facilitated re-epithelialization, re-modelling of connective tissues and collagenisation (Teramoto*et al.* 2008). This study is done to assess the clinical outcome of silk fibroin loaded silver nanoparticle gel on wound healing in threeLabrador retriever dogs.

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MATERIALS AND METHODS

Three Labrador retriever dogs (5 months, 1 and 2 years of age) werepresented to Madras Veterinary College Teaching Hospital with the history of chronic non-healing open wounds on the left cranial elbow(Fig1.A), right dorsolateral abdomen(Fig1.B)and right ventrolateral abdomen(Fig1.C).Physical examination revealed mild yellowish coloured wound with putrid odour and purulent discharge. The parameters studied were clinical observation, wound planimetry, bacteriological evaluationand histopathological analysis on days 0, 3, 7, 14, 21 and 28. Clinical observationswere done based on colour, odour and exudate. ABST had taken for the bacteriological evaluation. The silk fibroin loaded silver nanoparticle gel was prepared in the Department of Animal Biotechnology by using silk cocoon and neem leaf extract (Rockwood *et al*. 2011 and Prusty*and Parida,* 2015). The cases were subjected to the application of Silk fibroin loaded silver nanoparticle gel on subsequent days.

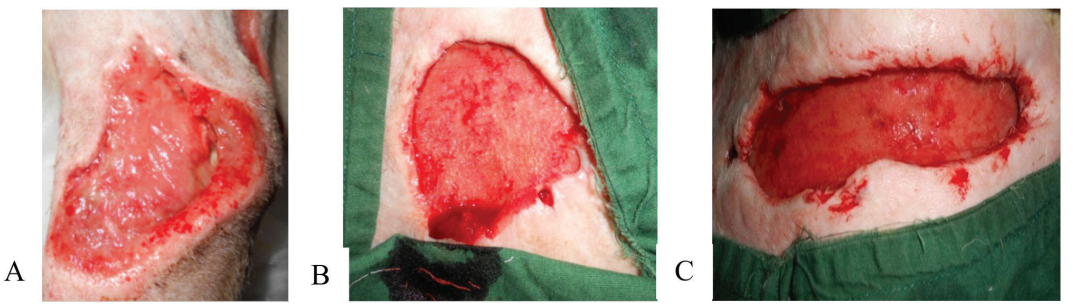
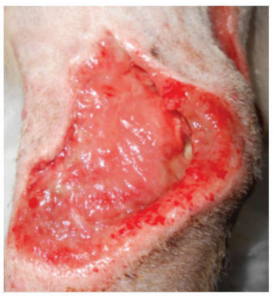


Figure 1. A: Case1- Wound on theleft cranial elbow, B: Case2- Wound on right dorsolateral abdomen andC: Case3- Wound on right ventrolateral abdomen.

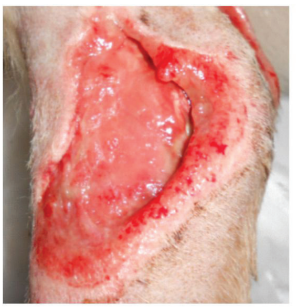
RESULT AND DISCUSSION

In this study, the colour of the wounds were black with offensive odour and serosanguineous discharge. For bacteriological examination, a sterile cotton swab was rolled over the open wound on days 0, 3, 7, 14, 21 and 28. On days 0 and 3, *Staphylococcus aureus* and *Pseudomonas aerogenosa* were isolated in all the cases. Under sedation, the wounds were debrided and lavaged with sterile normal saline solution. Punch biopsy of 3.5mm sample was taken for histopathological analysis. Wound planimetry was performed to assess the wound healing on days 0, 3, 7, 14, 21 and 28 by tracing of the wound margins onto a transparent sheet. The percentage of wound epithelialization, wound contraction and total healing were calculated in all the cases.The woundswere applied with silk fibroin loaded silver nanoparticle gel and bandaging was done to immobilise prevent self-mutilation. The bandaging was changed on every 2 days till the complete closure of wound was noticed. The clinical observation of the open wound in case 1 on days 0, 3, 7, 14, 21 and 28 were photographed (Plate 1).

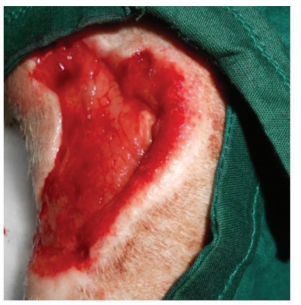
Plate 1: Clinical observation of the open wound – Case 1



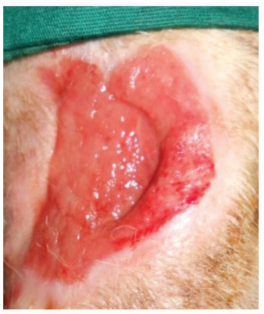
Yellow colour appearance of laceration wound - Day 0



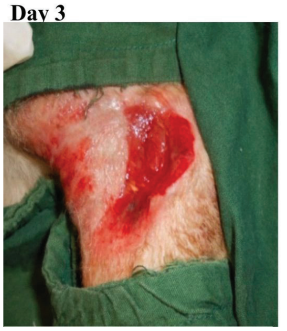
Yellow colour with Serosanguineous discharge - Day 3



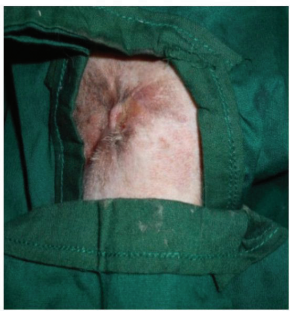
Presence of Serosanguineous discharge - Day 7



Reduction of wound size - Day 14



Red colour granulation tissue - Day 21



Complete wound healing - Day 28

In case 2 and 3, the clinical observation of open woundswere observed with thefoul smelling,necrotic tissues and purulent discharge on day 0. Manual debridement was done under sedation. Other parameters were performed in both the cases and subjected to the application of silk fibroin loaded silver nanoparticles gel on days 0, 3, 7, 14, 21 and 28. Bandaging was done to prevent self-mutilation and movement of the affected region. On days 3, 7 and 14, absence of wound discharge, appearance of granulation tissue and progression of wound contraction around the edges in both the cases. On days 21 and 28, complete epithelialization with granulation tissue formation and complete wound healing were observed in both the cases.

Histopathological Examination

A 3.5 mm punch biopsy instrument was used to take skin specimens from the open wounds on days 0, 3, 7, 14, 21 and 28with the dogs under general anaesthesia. Punch biopsy used in dogs as a method for skin healing investigation by histological analysis (Hamamoto*et al.,* 2009). The biopsy sampleswere sent for the histopathological analysis. The specimens were fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. Five micrometer sections were stained with haematoxylin and eosin(H&E) and the progressive decrease in macrophages, fibrosis, and progressive increase in

angiogenesis, epithelialization and collagen level were studied.

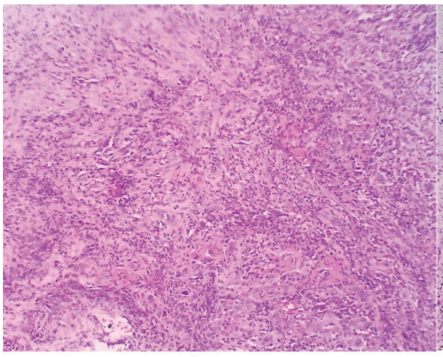
The dermis was devoid of epidermal covering and the wound consisted of large defects at the 0th and 3rdday. The wound area was filled with necrotic debris and crust made up of fibrin.The dermis of the wound area in the present study animals showed edema, vascular congestion and showed partial infiltration of neutrophils and macrophages (Plate 2 and 3).On days 7, 14 and 21, the wound area was covered by granulation tissue and fibrin crust, the defect was diminished due to partial contraction of the wound (Plate 4, 5 and 6).There wascontraction of fibrin clot, partial re-epithelialization, immature collagenisation and angiogenesis was present.

In the present study on 28th day, increased fibrovascular tissue with complete collagenisation, marked angiogenesis and re-epithelialization was observed in all the cases (Plate 7).This treatment promoted healing of chronic wounds by stimulating cellular activities and encompassed good epithelization with keratinization, matured fibroblast and neovascularisation as opined by Okabayashi*et al*. (2009).Silk protein promoted angiogenesis in all the three cases. The increased vessel growth could be facilitating both the extent and direction of fibroplasia. Improved angiogenesis, therefore, would be contributing significantly to wound healing activity of silk proteinswere reported by Padol*et al*. (2012).Silk fibroin supported adhesion and growth of anchorage dependent cells such as fibroblasts as opined by Minoura andAiba (1995). Min *et al.* (2004) reported greater collagen regeneration, less inflammation and less lymphocyte infiltration found in the wound treated with silk film.

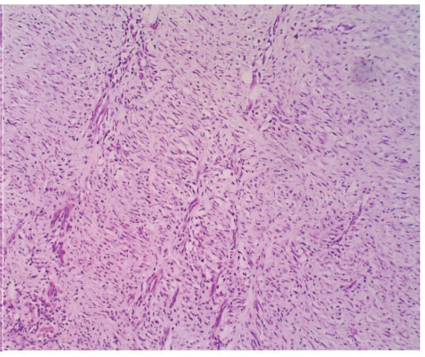
Bacterial contamination in a wound significantly impaired healing. The bacterial examination on days 7, 14, 21 and 28 revealed no bacterial organisms in all the cases after the application of silk fibroin loaded silver nanoparticles gel over the wounds. Ong*et al.* (2008) who reported complete destruction of both *Pseudomonas aeruginosa* cultures and *Staphylococcus aureus* cultures with >99.99% efficiency by incorporating silver-enhanced wound dressings*in vitro.*

In wound treatment, topical antiseptic application was toxic to fibroblasts and epithelial cells. Conventional dressings, which composed of gauze materials adhered to the tissue and separation of the dressing material became very difficult and interfered with wound granulation and healing process. No such problems were observed in this study for the management of open wounds in the above cases. Hydrogels used in the present study were suitable for cleansing of dry, sloughy or necrotic wounds. They were non-irritant, non-reactive with biological tissue, permeable to metabolites, didn't have residues and able to improve re-epithelialization of wounds. Similar findings were reported by Boateng*et al*. (2008) and Jayakumar*et al*. (2011).

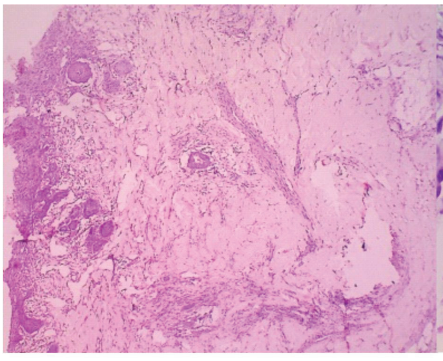
Histopathological examination of open wounds



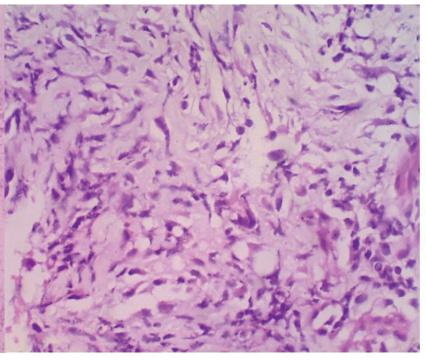
Lack of epithelium, presence of scab and neutrophilic exudate. H&E Stain = 100X (Day 0) - Plate 2



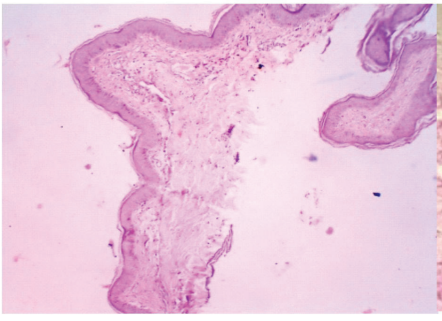
Lack of epithelium, presence of neutrophils with macrophages and mast cells andimmature collagen. H&E Stain = 100X (Day3)-Plate 3



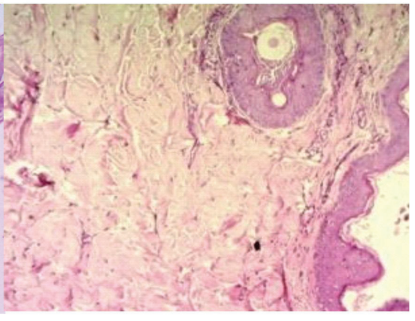
Partial re-epithelialization, mild collagenisation and neutrophilic exudate. H&E Stain = 40X(Day 7) - Plate 4



Partial immature collagenisation and angiogenesis.H&E Stain = 400X (Day 14)- Plate 5



Partial epithelialization and moderate collagenisation.H&E Stain = 100X (Day 21)- Plate 6



Complete re-epithelialization with organised collagenisation. H&E stain = 100 X (Day 28)- Plate 7

CONCLUSION

Silk fibroin loaded silver nanoparticle gel can be a better wound healing biomaterial in dogs. Silk fibroin acted as epidermal cell growth promoter and silver nanoparticles acted as antimicrobial agent leading to faster per cent wound closure. It is non-antigenic, non-toxic, non-irritating with good applicability and adhered well to fibroblasts and epidermal cells. It did notelicit any adverse reaction to the dog's skin in all the three cases.Based on the above observations, it was concluded that, silk fibroin loaded silver nanoparticles treated wounds showed early wound healing and the biomaterial can be safely be applied for cutaneous wound healing in dogs.

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GRANULOSA CELL TUMOUR ALONG WITH PYOMETRA IN A GERMAN SHEPHERD DOG

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ABSTRACT

A four year old, nulliparous German Shepherd dog was presented with the history of foul smelling vaginal discharge for the past one month. Abdominal palpation revealed a moderately distended uterus and a hard fluctuating mass in the anterior abdomen. Ultrasonography revealed an oval mass with numerous hypoechoic microcysts and non-uniform echotexture. Anechoic sacculations anterior to the urinary bladder suggestive of pyometra were visible. Ovariohysterectomy was performed as per standard surgical procedures. The left ovary was unusually large, irregular and hard with large number of greyish to brown nodules. Both uterine horns were distended with thickened uterine walls and uneven sacculations. Cut surface of the left ovary showed predominantly cystic parenchyma with fibrin network, filled with serous and blood tinged fluid. Histopathology revealed granulosa cell tumour of the left ovary.

Key words: Granulosa cell tumour, pyometra, dog

INTRODUCTION

Ovarian tumours are uncommon in dogs, accounting for 0.5 to 1.2 per cent of all canine tumours. Granulosa cell tumours (GCT) are sex cord stromal tumours, which accounts for 50 per cent of ovarian tumours and occur in elderly dogs with a median age of 12 years (Klein, 1996). This paper reports a case of unilateral GCT of ovary along with pyometra in a four year old German Shepherd dog.

Case History and Observations

A four year old nulliparous German Shepherd dog was presented to the clinic with the history of foul smelling vaginal discharge for the past one month. The animal was obese and lethargic. The animal had exhibited oestrus signs three months ago and was not bred in the previous cycles. The owner had presented the animal for neutering.

Abdominal palpation revealed a moderately distended uterus and a hard fluctuating mass in the abdomen. Trans-abdominal ultrasonography revealed a fairly oval structure of 5.4×8 cm in size, having a parenchyma of varying echogenicity with numerous hypoechoic microcysts

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(5–15 mm) and non-uniform echotexture. Anechoic sacculations of 2.5 to 3.0 cm diameter anterior to the urinary bladder, suggestive of pyometra was observed. Haematology indicated leukocytosis with thrombocytopenia in the animal.

Based on owner's request for neutering and clinical examination findings, it was decided to perform ovariohysterectomy.

Treatment and Discussion

Ovariohysterectomy was performed as per standard surgical procedures through a midventral abdominal approach under general anaesthesia. The left ovary was unusually large in size with irregular contour (5×8×6 cm) and hard with large number of grey and brown blunt projecting structures. Right ovary was small without any appreciable structures. Both uterine horns were distended (approx. 3 cm in diameter), with thickened uterine walls and uneven sacculations (Fig. 1). Separated left ovary from the bursa was capsulated with small cysts and areas of haemorrhages. Cut surface of the left ovary showed predominantly cystic parenchyma with serous and blood tinged fluids and a few uniform hard structures indicating functional corpora lutea (Fig. 2). Representative tissue samples were collected from left ovary, preserved in 10 per cent formol saline and sent for histopathology.

Histopathology revealed various sized macrofollicular cyst-like structures lined by loosely arranged, multiple layers of granulose cells. Cysts contained eosinophilic fluid or blood. Masses of granulosa cells were separated by fibrovascular stroma having lipid containing polygonal theca cells. Neoplastic cells were round or oval, moderately pleomorphic with round hyperchromatic nuclei and scanty eosinophilic cytoplasm. Histopathological features confirmed GCT of left ovary.

GCT originates from ovarian sex cords and may be unilateral or bilateral measuring 4 to 16 cm diameter. The major clinical findings of ovarian tumours are due to the space occupying nature of the intra-abdominal mass, metastatic spread and/or hormonal disturbances. Rarely, GCT may be non-functional and clinical manifestations were absent (Koivisto *et al.*, 2012). Incidence of GCT increases with age, and approximately 20 per cent cases show metastasis. In this case foul smelling vaginal discharge was noticed due to presence of pyometra, which might have produced due to increased production of progesterone from tumour tissues. Occurrence of cystic endometrial hyperplasia or pyometra along with GCT has also been reported (Oliveira *et al.*, 2016).

In the present study, the parenchyma of enlarged ovary was found to be heteroechoic with numerous cysts and had irregular margin, which can be considered as a feature of tumour as observed by Diez-Bru *et al.* (1998) and Kim *et al.* (2012).

Histopathologically, GCT in dogs has various microscopic appearances, such as follicular, cystic or poly cystic, and solid. In this case tumour was macrofollicular, characterized by various sized cystic structures lined with multiple layers of granulosa cells as observed by other researchers (Tavasoli and Solati, 2011).

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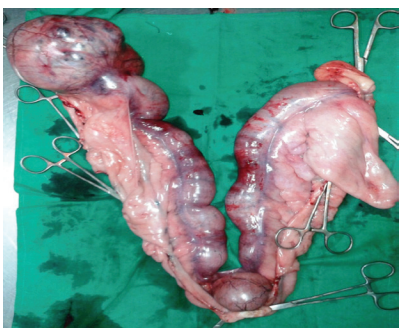

Fig. 1 Gross appearance of pyometra and tumourous left ovary

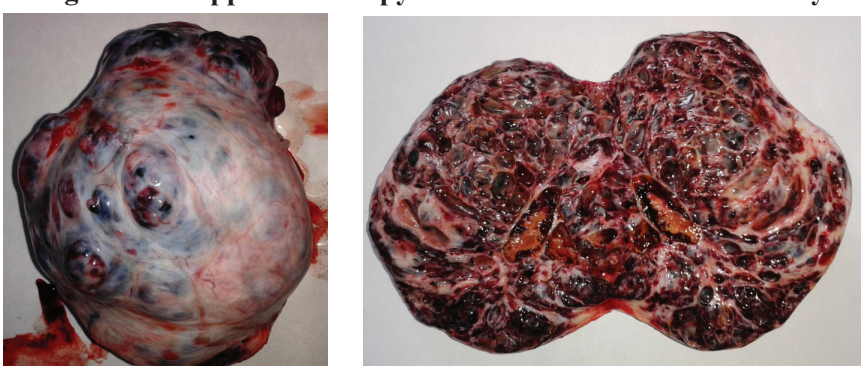

Fig. 2 Gross appearance of tumourous left ovary and its appearance in cut surface

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