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Case Study | Open Access

Rare Cases of Primary Canine Extragenital Transmissible Venereal Tumours

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Abstract A 4½ months old female Boxer pup, 6 years old Dachshund bitch and 10 years old Labrador dog were presented with extragenital transmissible venereal tumors. Based on the anamnesis, clinical symptoms and cytological examinations the cases were diagnosed as primary extragenital transmissible venereal tumors and histopathological examination of the specimens confirmed the diagnosis. The animals were treated successfully using Vincristine Sulphate intravenously at the rate of 0.025 mg/kg body weight.
Keywords *Extragenital TVT; Dogs; Histopathology; Vincristine Sulphate*

1. Introduction

Canine Transmissible venereal tumor (TVT) also called *Sticker's sarcoma* is a reticuloendothelial tumor that affects mucosa of extra genital organs [1, 2]. It is a well characterized sexually transmitted neoplasm but can also be transmitted by other social behaviours such as licking, sniffing and biting of the tumor affected areas [3, 4]. Canine Transmissible Venereal Tumor is generally considered as a benign tumor [5] and it is rarely reported in extragenital locations [3, 6]. The present report records primary extragenital transmissible venereal tumors in a pup and two adult dogs and their successful treatment.

2. Case History and Observations

Case 1

A 4½ months old female Boxer pup was presented with the history of having tumor like masses on the neck for the past 25 days. Clinical examination revealed two subcutaneous round alopecic nodules of 1” diameter one on the anterior dorsal region and another one in the central area of the neck.

Case 2

A 6 years old Dachshund bitch was presented with the history of having non healing wounds scattered in various parts of the body for the past 2 months. Clinical examination revealed multiple nodular ulcerated lesions of varying sizes from 1 cm to 1.5 inches diameter over the nasal, gingival, neck, back, and flank and thigh regions. Bloody nasal discharge was also noticed.

Case 3

A 10 years old male, Labrador dog was presented with the history of having ulcerated swelling in the mammary gland for the past 1½ months. Clinical examination revealed a nodular, ulcerated tumor growth of 2 inches diameter in the left 2nd mammary gland.

Anamnesis revealed that the owners of case 2 and 3, allowed the dogs at times to go out of the house and roam. Cytology of the fine needle aspiration of the samples revealed a uniform population of round to oval cells, with lightly basophilic cytoplasm that contained multiple distinct vacuoles. Frequent mitotic figures and occasional lymphocytes were also observed. The cytologic diagnosis was TVT in a progressing growth phase. However the external genitalia had no signs of TVT in all the three cases. But vaginal TVT was diagnosed in the pup's mother in case 1. According to anamnesis, clinical and cytological examinations, the cases were diagnosed as primary extra genital TVT. Histopathological examination of the specimen excised from the thigh, neck and mammary gland confirmed the diagnosis (Figure 1).

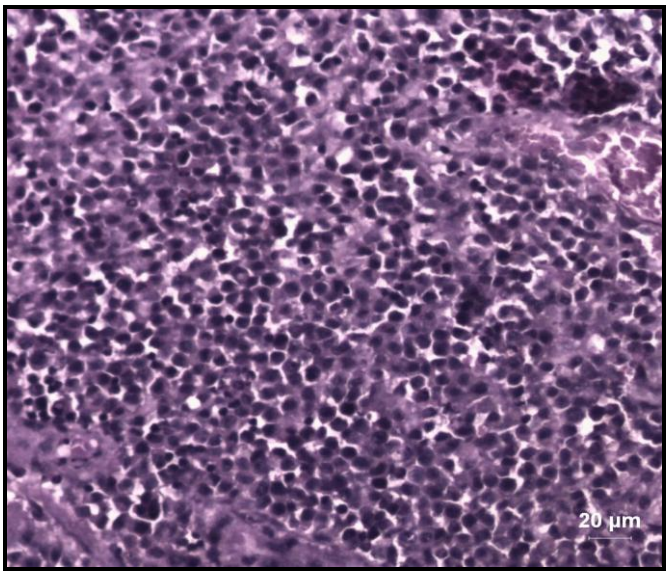

Figure 1: *Histopathology of Extragenital Transmissible Venereal Tumors in Dogs*

3. Treatment and Discussion

The pup and the dogs were administered with Vincristine Sulphate (Cytocristin, Cipla Ltd., Mumbai) intravenously at the rate of 0.025 mg/kg, at weekly intervals. Case 1, 2 and 3 required 3, 6 and 4 injections respectively. The nodules regressed completely by the end of 5, 8 and 6 weeks in case 1, 2 and 3 respectively and there was no recurrence even after 6 months.

Canine transmissible venereal tumor is most common in dogs of 2 to 5 years old and there is no breed or sex predisposition [7] but in the present report even 4½ months old pup also got affected. Canine TVT was a benign reticulo-endothelial tumor of the dog that mainly affected the genitalia and

the etiology appeared to be cell transplant from affected to unaffected dogs [8]. In the present study the occurrence of TVT in a pup suggested that TVT cells could be inoculated into puppy skin lesions by the mother during social interactions such as grooming and other mothering behavior [8]. The identification of individuals by smelling the genital areas was a common type of behaviour of canine species. The location of TVT on the nasal mucosa would be due to inspiration during the action of smelling [4]. Transmission of tumors to the various regions of the skin might be facilitated during the action of scratching [3] as observed in case 2. In case 3, the tumor cells might have got transferred to the mammary gland, when it got contacted with the genitalia of another affected dog. TVT which proliferate in genital organs had a cauliflower like appearance with ulceration but extra genital TVT were generally observed in nodular form, bearing ulcerations of variable sizes which might invade the mucosa and sub mucosa [3].

In animals that have an appropriate antitumor immunologic response, spontaneous tumor regression might occur after the tumor reached a certain size. However, in animals that were unable to mount an appropriate immunologic response, the tumor might continue to grow and metastasize. Therapy was generally recommended when a canine TVT is definitively diagnosed [9]. Several therapeutic modalities had been used with canine TVT including surgery, radiation therapy, biologic response modifiers and chemotherapy. Chemotherapy was the most effective way to treat canine TVT and among the various chemotherapeutic agents, Vincristine was considered one of the most effective agents [10]. In the present study also, the response to Vincristine was very effective and there was complete regression of extragenital TVT in all the three cases. Dogs recovered uneventfully and there was no recurrence during the follow up period of six months.

4. Summary

Transmissible Venereal Tumor (TVT) is a round cell tumor which affects primarily the external genitalia of dogs of both sexes. Rarely the tumour may be found in extragenital regions such as nasal cavity, oral cavity, skin, rectum, lips, oral mucosa and musculature. The present report records rare cases of primary non genital transmissible venereal tumor in a pup and two adult dogs and their successful treatment with Vincristine sulphate.

References

[1] Albanese, F., Poli, A., Millanta, F. and Abramo, F. *Primary Cutaneous Extragenital Canine Transmissible Venereal Tumour with Leishmania-Laden Neoplastic Cells: A Further Suggestion of Histiocytic Origin.* Vet. Dermat. 2002. 13; 243.

[2] Foster, R.A., 2007: *Female and Male Reproductive Systems.* In: Pathologic Basis of Veterinary Disease. McGavin, M.D. and James Zachary, J.F. (eds.). 4th Ed. St. Louis: Mosby. 1306.

[3] Gurel, A., Kuscu, B., Gulanber, E.G. and Arun, S.S. *Transmissible Venereal Tumors Detected in the Extragenital Organs of Dogs*. Israel J. Vet. Med. 2002. 57; 97.

[4] VonHoldt, B.M and Ostrander, E.A. *The Singular History of a Canine Transmissible Tumor.* Cell. 2006. 126; 445.

[5] Rogers, K.S., Walker, M.A. and Dillon, H.B. *Transmissible Venereal Tumor: A Retrospective Study of 29 Cases*. J. Am. Anim. Hosp. Assoc. 1998. 34; 463.

[6] Bastan, A., Duygu, B. and Mehmet, C. *Uterine and Ovarian Metastasis of Transmissible Venereal Tumor in a Bitch.* Turkish J. Vet. Anim. Sci. 2008. 32; 65.

[7] Das, U. and Das, K. *Review of Canine Transmissible Venereal Sarcoma.* Vet. Res. Communic. 2000. 24; 545.

[8] Marcos, R., Santos, M., Marrinhas, C. and Rocha, E. *Cutaneous Transmissible Venereal Tumor without Genital Involvement in a Prepubertal Female Dog.* Vet. Clin. Pathol. 2006. 35; 106.

[9] MacEwen, G., 2001: *Transmissible Venereal Tumor.* In: Small Animal Clinical Oncology. 3rd Ed. Philadelphia, Pa: WB Saunders Co. 651.

[10] Nak, D., Nak, Y. Cangul, I.T. and Tuna, B. *A Clinico-Pathological Study on the Effect of Vincristine on Transmissible Venereal Tumour in Dogs.* J. Vet. Med. A. Physiol. Pathol. Clin. Med. 2005. 52; 366.

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Research Article | Open Access

Electron Microscopic Studies of Spleen in Chicken (*Gallus domesticus*)

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Abstract Electron microscopic studies on spleen of layer chicken were done in various age groups ranging from day-old to forty weeks. The spleen was encapsulated by a connective tissue capsule and the trabeculae were poorly developed in all the age groups studied. The major cellular population of the white pulp included lymphoblasts, lymphocytes of various sizes, follicular dendritic cells and reticulum cells. The splenic red pulp was composed of pulp cords consisted of erythrocytes, reticular cells and lymphocytes of various sizes, macrophages, granulocytes, plasma cells and mast cells. The arterioles that continued into the red pulp formed sheathed capillaries or ellipsoids.
Keywords *Electron Microscopy; Spleen; Chicken*

1. Introduction

Spleen is the principal organ of systemic immunity and its importance in disease resistance is accentuated by the scarcity of avian lymph nodes. The avian spleen functions as a major blood filtering organ and is the major source of antibody production. It does not function as a reservoir of blood as in mammals and its function is not oriented towards supply of oxygen (Jeurissen, 1991). The spleen also plays an important role in erythrocyte destruction, phagocytosis and antigen-antibody interactions (Burke and Simon, 1970). Though there is extensive work done on the light microscopic details, a little work was done about the ultrastructural studies of the spleen in Chicken. Hence, the present study was designed to explore the details of spleen in the layer chicken of different age groups.

2. Materials and Methods

Spleen for transmission electron microscopic studies were collected from six different age groups such as day-old, four, eight, twelve, twenty and forty weeks. Six birds were used in each age group.

For electron microscopic study, small pieces of spleenic tissue (1-2 mm thickness) were collected and prefixed at 3 per cent glutaraldehyde and stored at 4^0C. Subsequently, the tissues were washed, three changes (each 30 minutes) in cold sodium cacodylate buffer solution (pH 7.4) and post fixed in 1 per cent osmium tetroxide for two hours at 4^oC. The tissues were then dehydrated in ascending grades of alcohol (50, 70, 80, 90, 95 per cent and absolute ethyl alcohol), propylene oxide: epoxy resin mixture and embedded in Epon-araldite mixture. Semi thin (1 micron) sections were stained by toluidine blue. Ultra-thin sections (600 A^o to 900A^o) were prepared on Leica ultracut microtome, mounted on uncoated copper grids and stained with saturated solution of uranyl acetate and lead citrate. The ultra-thin sections were examined under Phillips (Teknai-10) computer augmented transmission electron microscope operated at 60-kilowatt ampere (KVA).

3. Results and Discussion

3.1. Capsule

In all the age groups, the spleenic capsule was composed of collagen bundles and a few elastic fibres. The capsule was also observed with smooth muscle cells and stellate shaped fibroblasts with fibrillar cytoplasm and mitochondria (Burke and Simon, 1970). External surface of the capsule was observed to be lined by a single layer of mesothelial cells with small microvillous projections at their free border. The inner surface of the capsule was found to have subcapsular sinus lined by endothelial cells, filled with erythrocytes. The presence of collagen fibres increased as age advanced in the present study (Moore, et al., 1964).

3.2. Parenchyma

White Pulp

In all the age groups studied, white pulp of the spleen was observed with predominant lymphocytes of various sizes and reticulum cells. These cells were arranged in the form of clumps (Figure 1). These clumps were separated by a meshwork composed of collagen, fibroblasts and reticulum cells (Olah and Glick, 1982).

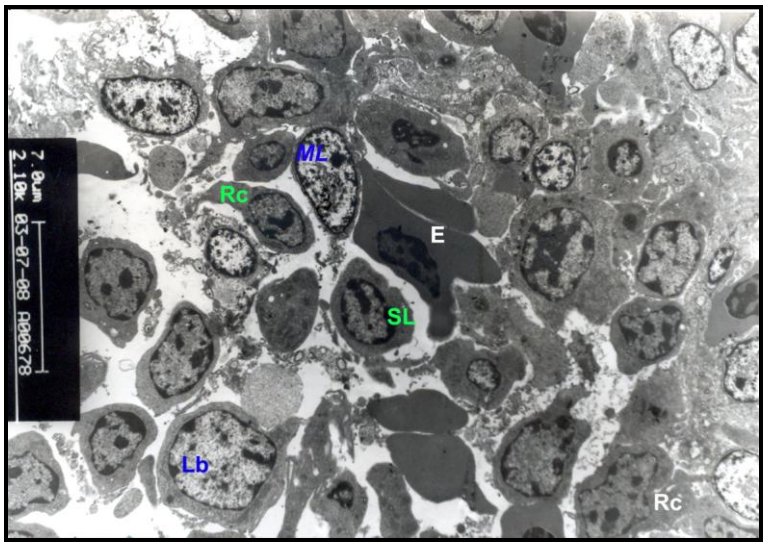

Figure 1: *Transmission Electron Micrograph of Spleen of a Four Week-Old Chicken Showing the Cellular Components of the White Pulp X 2100*

E - Erythrocyte *Lb - Lymphoblast*
ML - Medium sized lymphocyte *SL - Small lymphocyte*
Rc - Reticular cell

In day-old and four week-old birds, the lymphoblasts and small lymphocytes were observed more. In eight week-old birds, all the types of lymphocytes such as small, medium and large lymphocytes were noticed. Whereas in twenty week-old birds, the existence of large lymphocytes predominated and in forty week-old birds, the depletion of lymphocytes and the amount of collagen was maximum in the stroma.

The lymphoblasts were characterized by large euchromatic nuclei and predominance of free poly-ribosomes. The small and medium sized lymphocytes were found to be round to oval shaped and were seen with high nuclear-cytoplasmic ratio (Figure 2). The cytoplasm was sparsely seen with occasional little perinuclear rough endoplasmic reticulum, a few ribosomes and mitochondria. The smooth endoplasmic reticulum was seen in the form of small vesicles dispersed in the cytoplasm and the Golgi zone was not prominent. Nucleus was observed mostly round or slightly indented with heterochromatin (Cross and Mercer, 1993).

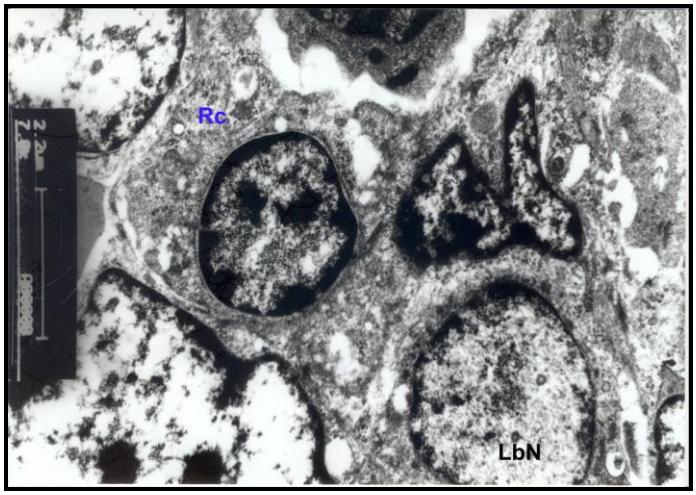

Figure 2: *Transmission Electron Micrograph of Spleen of a Eight Week-Old Chicken Showing the Details of Lymphoblast and Reticulum Cell in White Pulp x 7000*
LbN - Nucleus of Lymphoblast *Rc - Reticular cell*

The reticulum cells were stellate shaped; they had smaller nuclear-cytoplasmic ratio and more organelles in the cytoplasm (Figure 3). Rough endoplasmic reticulum was found to be more. Well-developed mitochondria, ribosomes and a prominent Golgi zone were also observed. The presence of reticulum cells were commonly observed in all the age groups studied (Burke and Simon, 1970).

The fibroblasts appeared flat and stellate with long cytoplasmic prolongations. They had rough endoplasmic reticulum seen filled with a fine flocculent material. The smooth endoplasmic reticulum also had similar material and some of them appeared fibrillar (Galindo and Freeman, 1963).

The follicular dendritic cells were seen in all the age groups and confirmed by their large and irregular shaped nucleus. It had a little heterochromatin. The cytoplasm was found to be ramified as very thin processes in many directions. Some of the processes were observed long and straight. Mitochondria, rough and smooth endoplasmic reticulum, vesicles were rarely seen within the cytoplasm (Banchereau and Steinman, 1998).

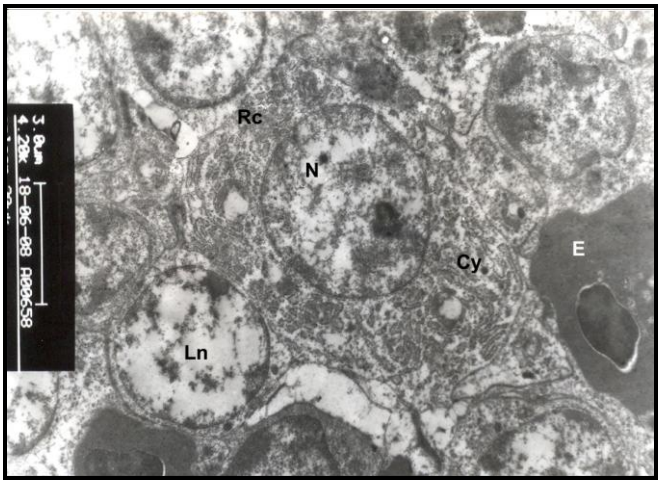

Figure 3: *Transmission Electron Micrograph of Spleen of a Twenty Week-Old Chicken Showing the Details of Reticulum Cell in White Pulp x 4200*

CY - Cytoplasm *E - Erythrocyte*

Rc - Reticular cell *N - Nucleus of reticulum cell*

Ln - Nucleus of lymphocyte

The plasma cells of the spleen were observed with well-developed rough endoplasmic reticulum. There were larger and denser mitochondria and numerous ribosomes. The heterochromatic nucleus was eccentrically placed (Ogata, et al., 1977). These plasma cells synthesize and secrete antibodies that bind specifically to the antigen that initially activated the precursor B lymphocyte. Antigen-antibody binding is a major means of immune defense.

The macrophages were ovoid or stellate shaped; the cytoplasm contained vacuoles and phagocytosed materials in the cytoplasm. The nucleus was heterochroamtic (Burke and Simon, 1970). Antibodies synthesized within the rough endoplasmic reticulum are processed and packaged within the Golgi prior to secretion. These macrophages in the spleen were the important site of erythrocyte destruction which was evident by the presence of several partially digested fragments of old erythrocytes. According to Weiss (1964 and 1990), it also played a role in antigen presentation and secretion of mediators of the immune response.

Red Pulp

The splenic red pulp was composed of anastomosing sinuses lined by endothelial cells were noticed. These sinuses were found to be separated with each other by the pulp cords. These pulp cords consisted of erythrocytes, reticular cells, lymphocytes of various sizes, macrophages, granulocytes, plasma cells and mast cells (Figure 4).

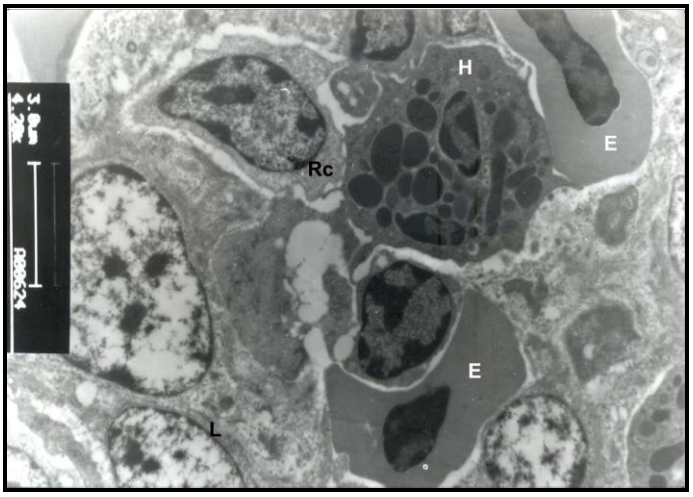

Figure 4: *Transmission Electron Micrograph of Spleen of a Day-Old Chick Showing the Cellular Components of the Red Pulp x 4200*

E - Erythrocytes *H - Heterophil*
L - Lymphocytes *Rc - Reticular cell*

The erythrocytes were present both in the sinuses and in the pulp cords which had different shapes. Macrophages were seen with phagocytic vacuoles which contained the degraded erythrocytes or leukocytes. The structure of reticulum cell and lymphocytes were similar to that present in the white pulp (Abe, et al., 1989).

The arterioles from the periphery of the white pulp were found to enter into the red pulp. In these arterioles, the lumen was surrounded by muscle cell and these arterioles continued into the red pulp and formed sheathed capillaries or ellipsoids (Olah and Glick, 1982). These ellipsoids were found to have a meshwork of polymorphic reticular cells, reticular fibres and a few macrophages.

4. Summary

The splenic capsule was composed of collagen bundles with a few elastic and smooth muscle fibres, smooth muscle cells and fibroblast in all the age groups. As age advanced the thickness of the capsule increased. The trabeculae were poorly developed in all the age groups studied.

The major cellular population of the white pulp included lymphoblasts, lymphocytes of various sizes and reticulum cells arranged in the form of clumps separated by a meshwork composed of collagen, fibroblasts and reticulum cells in all the age groups studied. The follicular dendritic cells had a little heterochromatin. The mitochondria, rough and smooth endoplasmic reticulum were sparse in the cytoplasm.

The splenic red pulp was composed of anastomosing sinuses lined by endothelial cells. The pulp cords consisted of erythrocytes, reticular cells, lymphocytes of various sizes, macrophages, granulocytes, plasma cells and mast cells.

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References

Abe, M., Takehana, K., Iwasa, K. and Hiraga, T. *Scanning Electron Microscopic Studies on the Red Pulp of the Mink Spleen.* Nippon Juigaku Zasshi. 1989. 51; 775-81.

Banchereau, J. and Steinman, R.M. *Dendritic Cells and the Control of Immunity.* Nature. 1998. 392; 245-52.

Burke, J.S. and Simon, G.T. *Electron Microscopy of the Spleen. I. Anatomy and Microcirculation.* Am. J. Pathol. 1970. 58; 127-55.

Cross, P.C. and Mercer, K.L., 1993: *A Text Book on Cell and Tissue Ultrastructure.* New York: W.H. Freeman and Company.

Galindo, B. and Freeman, J.A. *Fine Structure of Splenic Pulp.* Anat. Rec. 1963. 147; 25-42.

Jeurissen, S.H.M. *Structure and Function of the Chicken Spleen.* Res. Immunol. 1991. 142; 352-55.

Moore, R.D., Mumaw, V.G. and Schoenbeg, M.D. *The Structure of the Spleen and Its Functional Implications.* Exp. Mol. Path. 1964. 3; 31-50.

Ogata, K., Sukumura, Y. and Kudo, N. *Developmental Studies on Embryonic and Post-Hatching Spleens in Chicken with Special Reference in Development of White Pulp.* Jap. J. Vet. Res. 1977. 25; 83-92.

Olah, I. and Glick, N. *Splenic White Pulp and Associated Vascular Channels in Chicken Spleen.* Am. J. Anat. 1982. 165; 445-80.

Weiss, L. *The White Pulp of the Spleen. The Relationships of Arterial Vessels, Reticulum and Free Cells in the Periarterial Lymphatic Sheath.* Bull Hopkins Hosp. 196. 115; 99-173.

Weiss, L., 1990: *Mechanism of Splenic Clearance of the Blood: A Structural Overview of the Mammalian Spleen. In the Spleen: Structure, Function and Clinical Significance.* Bowdler, A.J. (ed.) London: Chapman and Hall Medical. 103-26.

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Research Article | Open Access

Gross/Histopathological Impact of *Salmonella* Gallinarum Isolated from Layer Chickens in Jaipur and their Antibiogram Assay

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Abstract The study was conducted from April 2009 to March 2010 to assess the antimicrobial sensitivity pattern of eight *Salmonella* Gallinarum strains recovered from various sources in Jaipur of India by testing with eight different antimicrobials by disc diffusion method where the isolates showed least sensitive to tetracycline. Gross lesions incriminated to the pathogen, were comprised of caseous enteritis, hepatic necrosis, necrotizing myocarditis and oophoritis. Histopathological investigation revealed passive congestion of the visceral organs. Degenerative changes in hepatic lobules and consequential destruction of architectural arrangement of hepatocytes, necrotic foci in the enteric mucosa and in hepatic tissues were seen. Ovaries showed fibrinosuppurative to pyogranulomatous inflammation of ovarian follicles characterised by necrosis and fibrinosuppurative inflammation mixed with bacteria within the ovules to chronic pyogranulomatous inflammation. Heart characterised by locally extensive foci of myofibre necrosis with infiltration of heterophils mixed with few lymphocytes and plasma cells.
Keywords *Salmonella Gallinarum; Antibiogram; Histopathology; Layer Chickens*

1. Introduction

In poultry industry, salmonellosis creates intense economic losses along with reduction in productivity and high mortality (Khan et al., 1998). Out of different serovers, *Salmonella* Gallinarum produces Fowl Typhoid; a septicemic disease in poultry which may be characterized by anorexia, greenish-yellow diarrhoea with considerable mortality, also causes drop in egg production. *Salmonella* Gallinarum vertical transmission through eggs leads to higher mortality or producing weak chicks.

In recent times, antibiotic resistance in livestock becomes alarming in India, blaming the easy availability of antibiotics in markets and indiscriminate use of them. Research workers like Mir et al. (2010) and Taddele et al. (2012) reported isolation of *Salmonella* Gallinarum strains showing multiple drug resistance pattern in poultry in India.

In Jaipur, India, there is little systematic study on incidence and antibiotic resistance pattern, histopathological studies of Salmonella Gallinarum serovar. Therefore, the present study was conducted to isolate and identify the serotype *Salmonella* Gallinarum in the fresh carcasses of layer chickens in Jaipur area, their antimicrobial susceptibility pattern and documentation of associated gross and histopathological lesions.

2. Materials and Methods

2.1. Sample

In the department of pathology, Apollo College of Veterinary Medicine, seventy three necropsy tissue samples i.e. lungs, liver, intestine, heart, spleen and kidney from dead layer chickens with gross apparent lesions were subjected to microbiological as well as histopathological investigations for a duration of one year i.e. 2009-10.

2.2. Isolation of Salmonellae

For isolation of Salmonallae, one gram of minced tissue sample was aseptically transferred into sterile tube having 10 ml Tetrathionate Broth and/or Rappaport Vassiliadis Salmonella Enrichment Broth and inoculated at 42˚C for 24 hours aerobically. Then a loopful of broth culture was streaked onto Brilliant Green Agar plates (BGA), kept at 37˚C for 48 hours aerobically and Mac Conkeys Lactose Agar (MLA) plate were used to inoculate the suspected pinkish colonies on BGA. The plates were incubated at 37˚C for 24 hours aerobically for isolation of the salmonellae. Non lactose fermenter (pale) colonies on MLA, suspected for *Salmonella* were obtained in pure culture on nutrient agar slants by conventional method.

2.3. Gram's staining

The isolated bacteria were stained by Gram's Method to determine their staining characteristics and purity of the culture. Identification was done by IMViC reaction, TSI reaction, Nitrate reduction test, urease test, H_2S production test, and other fermentative or non-fermentative sugar tests (Edwards and Ewing, 1972; Cruickshank, et al., 1975). For Serotyping, the isolates were referred to National *Salmonella* and *Escherichia* centre, Central Research Institute, Kasauli (H.P.).

2.4. Antibiogram

In-vitro antibiotic sensitivity of the isolates were assessed using disc diffusion method (Bauer et al., 1966) while using disc coated with 12 antibacterial drugs (Hi-Media) viz. Ampicillin 10 mcg (A 10), Cephalexin 30 mcg (Cp 30), Ciprofloxacin 5 mcg (Cf 5), Enrofloxacin 5 mcg (Ex 5), Gentamicin 10 mcg (G 10), Kanamycin 30 mcg (K30), Ofloxacin 5 mcg (Of 5) and Tetracycline 30 mcg (T 30).

2.5. Histopathology

Tissue samples with grossly visible lesion were preserved in 10 per cent formalin. Histopathological investigation was done using standard protocol (Luna, 1968). The paraffin embedded tissues were cut into sections of 4 to 5 µ thickness. Sections were stained with Haematoxyline and Eosin (H&E) stain and microscopic lesions in different organ were documented.

3. Results and Discussion

Out of seventy three samples screened from layer birds, 8 (10.96 %) *Salmonella* could be recovered. All the isolated strains belong to Salmonella Gallinarum; antigenic structure 9, 12,--,--.

3.1. Antibiogram of the Isolates

In-vitro antibiotic sensitivity of the isolates revealed as (Table 1)

***Table 1**: Antibiogram of the Isolates*

Sl. No.	Antibiotic	Number Tested	Number Sensitive	(%)
1.	Ampicillin 10 mcg (A 10)	8	5	62.5
2.	Cephalexin 30 mcg (Cp 30)	8	6	75
3.	Ciprofloxacin 5 mcg (Cf 5)	8	8	100
4.	Enrofloxacin 5 mcg (Ex 5)	8	7	87.5
5.	Gentamicin 10 mcg (G 10)	8	7	87.5
6.	Kanamycin 30 mcg (K30)	8	6	75
7.	Ofloxacin 5 mcg (Of 5)	8	6	75
8.	Tetracycline 30 mcg (T 30)	8	1	12.5

The highest sensitivity was seen in Ciprofloxacin followed by Enrofloxacin, Cephalexin, gentamicin, kanamycin and ofloxacin. Tetracycline showed least sensitivity. Similar pattern was also reported by Taddele, et al. (2012). They found kanamycin, tetracycline as least sensitive with higher sensitivity towards gentamicin, amoxicillin/clavulanic acid, ciprofloxacin, ofloxacin and enrofloxacin. However, resistance to kanamycin was not seen in present study.

Knowing the resistance pattern of *Salmonella* Gallinarum strains towards different antimicrobial agents helps to offer a better and more effective treatment against fowl salmonellosis. *Salmonella* Gallinarum strains in the present study have shown higher resistance towards tetracycline. This can be due to reasons like resistance gained due to indiscriminate use of the antibiotic, improper antibiotic treatment time frame, use as growth promoter etc.

Clinical signs showed by death birds were anorectic, ruffled feathers, dullness, depression, dropiness, dehydrated, fall in egg production and vent feathers soil with diarrheic fecal materials.

Gross changes found in post mortem examination were recorded as follows-

Liver showed enlargement with severe congestion and focal haemorrhages in most of the cases, in few cases found focal grayish white necrotic foci in the parenchyma (Figure 4). Heart showed discrete nodular necrotic growth of variable size and shape of grayish white in colour (Figures 1 and 2). Intestine especially small intestine found severe congestion and hemorrhages and cecum of few birds showed caseated necrotic mass (Figure 1). Ovaries of all dead birds showed severe inflammatory changes in likes misshaped, hemorrhagic and discoloured cystic ova (Figure 3). The involved ova may contain oily and caseous material enclosed in a thickened capsule. These degenerative ovarian follicles closely attached to the ovary and few of them pedunculated and detached from the ovarian mass and embedded in the inner lining of the peritoneal cavity. The oviduct contains caseous exudates in the lumen with impacted uterus with degenerated eggs in few cases. Lung, kidneys and spleen also showed severe congestion and haemorrhages and enlargement of spleen in few were recorded. Fibrinous peritonitis and perihepatitis, with or without the involvement of the reproductive tract also recorded in some necropsied birds.

Histopathologically, the liver showed severe congestion in central vein and sinusoidal area with degeneration (Figure 6), focal necrosis with infiltration of mononuclear cells. The intestinal mucosa exhibited congestion and hemorrhages with infiltration of mononuclear cells in the submucosa and caseous necrosis in the centre. These types of histological lesions are supported for *Salmonella* infection by different investigators (Talha, et al., 2001; Habib-ur-Rahman, et al., 2003).

Ovaries showed fibrinosuppurative to pyogranulomatous inflammation of ovarian follicles characterised by necrosis and fibrinosuppurative inflammation mixed with bacteria within the ovules to chronic pyogranulomatous inflammation. In males, necrosis of the epithelial cells lining the seminiferous tubules may be seen, followed by fibrinosuppurative inflammation (Saif, et al., 2003).

Heart characterised by locally extensive foci of myofibre necrosis with infiltration of heterophils mixed with few lymphocytes and plasma cells (Figure 5). In later stages, these cells may be replaced by large numbers of fairly uniform mononuclear cells of the histiocytic type with irregular vesicular nuclei and faintly staining foamy eosinophilic cytoplasm. These cells may be arranged in solid sheets, forming nodules that often protrude from the epicardial surface. Such nodules, both grossly and microscopically, can be confused with certain tumours caused by Marek's disease virus or possibly retroviruses (Shivaprasad, 1997; Saif, et al., 2003)

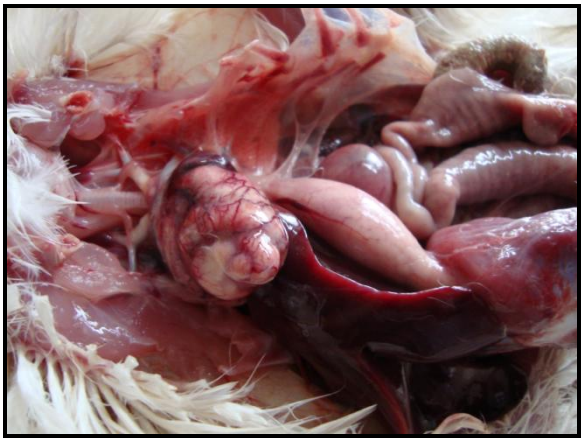

***Figure 1**: Gross Photograph of Layer Bird Showing Discrete Nodular Necrotic Growth on Heart and in Cecum Having Caseated Necrotic Mass*

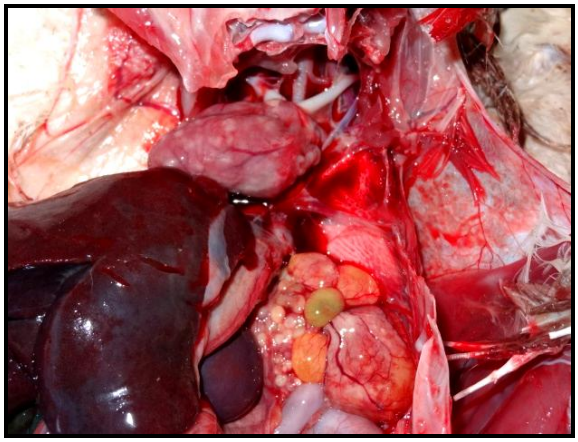

***Figure 2:** Gross Photograph of Layer Bird Showing Discrete Nodular Necrotic Growth on Heart, Severe Inflammatory Changes in Ovaries (Oophoritis), Severe Congestion and Haemorrhages in Liver*

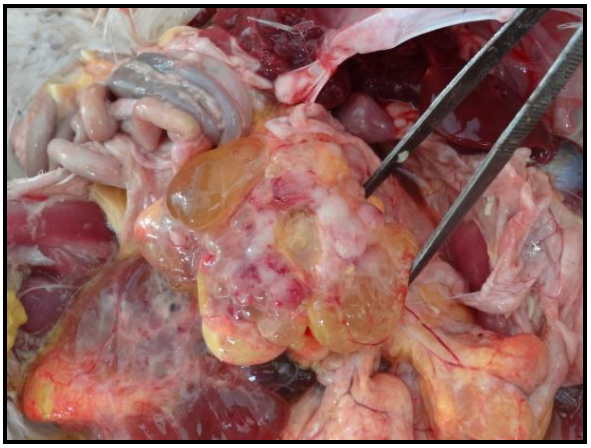

Figure 3: *Gross Photograph of Layer Bird Shows Severe Inflammatory Changes in Ovaries Likes Misshaped, Hemorrhagic and Cystic*

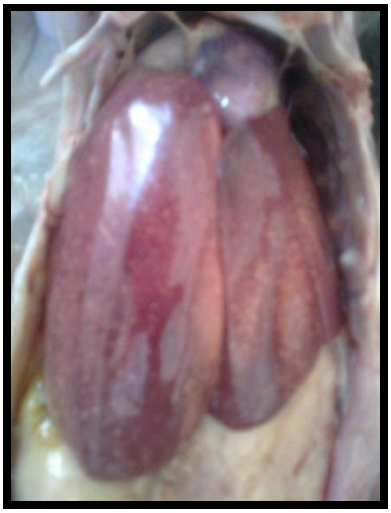

Figure 4: *Gross Photograph of Layer Bird Shows Grayish White Necrotic Foci in Hepatic Parenchyma*

3.2. Histopathological Investigation

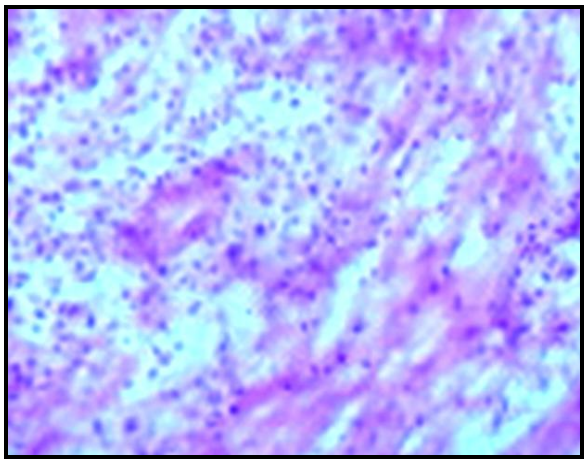

Figure 5: *Photo Micrograph of Heart Showing Fragmented and Necrosed Myocardium Which is Infiltrated by Heterophils and Mononuclear Cells. H&EX400*

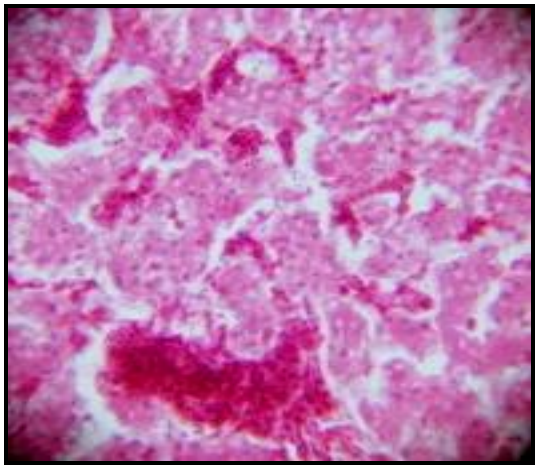

Figure 6: *Photo Micrograph of Liver Shows Severe Congestion in Central Vein and Sinusoidal Area with Degenerative Changes of Hepatocytes. H&EX400*

4. Conclusion

From the present study it was concluded that *Salmonella* spp. were isolated from 8 (10.96 %) samples comprising of different dead layer chicken samples. All the isolates belonged to serotype *Salmonella* Gallinarum. Antibiogram suggested that ciprofloxacin showed the maximum potential to be used as promising whereas least sensitivity towards tetracycline. It may pose potential danger to the poultry industry due to vertical transmission. Moreover, once it is introduced into the farm, become difficult to eliminate infection and becomes clinically evident with the frequent mortality and severe economic losses.

Acknowledgement

The authors are thankful to the Director, National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli (H.P.), India, for serotyping the *Salmonella* isolates. The authors are also thankful to the Dean, Apollo College of Veterinary Medicine, Jaipur for providing the facility for the present work.

References

Bauer, A.W., Kirby, W.M., Sherris, J.S. and Turck, M. *Antibiotic Susceptibility Testing by a Standardized Single Disk Method.* Am. J. Clin. Pathol. 1966 Apr. 45 (4) 493-496.

Cruickshank, R., Duguid, J.P., Marimion. B.P. and Swain, R.H., 1975: *Medical Microbiology*. 12th Ed., Vol. II, Churchill Livingstone, Edinburg, London and New York. 31-57; 96-218.

Edwards, P.R. and Ewing, W.H., 1972. *Identification of Enterobactriaceae*. 3rd Ed. Elsevier Burgess Publishing Company, Minneeapolis, Minnesota.

Habib-ur-Rahman, S., Sirzanin Hamayun, K., Saleem, K., Nazir, A. and Bhatti, W.M*. Incidence and Gross Pathology of Salmonellosis in Chicken in Hyderabad.* J. Asso. and Vet. Advances. 2003. 2; 581-584.

Khan, M.A.H.N.A., Bari, A.S.M., Islam, M.R., Das, P.M. and Ali, M.Y. *Pullorum Disease in Seminature Chicks and Its Experimental Pathology.* Bangladesh Veterinary Journal. 1998. 32; 124-128.

Luna, L.G., 1968: *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. 3rd Ed. New York: Mc Graw Hill.

Mir, I.A., Wani, S.A., Hussain, I., Qureshi, S.D., Bhat, M.A. and Nishikawa, Y. *Molecular Epidemiology and in Vitro Antimicrobial Susceptibility of Salmonella Isolated From Poultry in Kashmir.* Rev. Sci. tech. Off. Int. Epiz. 2010. 29 (3) 677-686.

Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R. and Swayne, D.E., 2003: Diseases of Poultry. 11th Ed. Iowa State University, Ames. 572-575.

Shivaprasad, H.L., 1997: Pullorum Disease and Fowl Typhoid. In: Diseases of Poultry. B.W. Calnek with H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds). 10th Ed. Iowa State University, Ames. 82-96.

Taddele, M.H., Rathore, R. and Dhama, K. *Antibiogram Assay of Salmonella Gallinarum and Other Salmonella enterica Serovars of Poultry Origin in India.* Asian Journal of Animal and Veterinary Advances. 2012. 7 (4) 309-317.

Talha, A.F.S.M., Hossain, M.M., Chowdhury, E.H., Bari, A.S.M., Islam, M.R. and Das, P.M. *Poultry Diseases Occurring in Mymensingh District of Bangladesh.* The Bangladesh Veterinarian. 2001. 18; 20-23.

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Research Article | Open Access

Effect of Different Freezing Rates and Thawing Methods on the Quality of Frozen Boar Semen

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Abstract A total of 30 ejaculates from three Hampshire crossbred boars were used to study the effect of freezing rates and thawing methods on quality of frozen boar semen. Semen was frozen using three freezing rates-30^{0}c/min, 50^{0}c/min and 70^{0}c/min in a programmable biological semen freezer. The semen characteristics *viz.,* sperm motility, live sperm, HOST reacted spermatozoa and intact acrosome were studied after thawing using three different thawing methods *viz.,* 37^{0}c for 30 sec, 50^{0}c for 12 sec and 70^{0}c for 10 sec. Out of three different freezing rates and thawing methods freezing rate 30^{0}c/min with thawing method 70^{0}c for 10 sec resulted better semen quality.
Keywords *Boar Semen; Freezing Rate; Thawing Method*

1. Introduction

Pig farming has been occupying a vital position in uplifting the rural economy by providing self-employment and supplementary income. The scarcity of superior boar and high cost for rearing them by the small and marginal farmers necessitated the use of artificial insemination (AI). At present liquid semen has been used in AI of pig. Since the first successful AI with frozen boar semen in 1971 (Crabo and Einarsson), several studies have been made (Bwanga, 1991; Wagner and Thibier, 2000; Baishya, 2013) on freezing and thawing procedure of boar spermatozoa, but the use of frozen thawed semen has remain very low. Preservation of semen for longer period in-vitro without lowering the inherent fertilizing ability of sperm is essential for success of AI and the current status of boar semen cryopreservation is still considered poor to fair. Frozen thawed boar sperm quality is influenced by many factors such as freezing and thawing protocols, composition of diluents used in the processes, susceptibility to cryoinjury of the spermatozoa etc (Johnson, et al., 2000). The present experiment was carried out to study the effect of different freezing rate and thawing methods on the quality of frozen boar semen.

2. Materials and Methods

A total of 30 ejaculates were collected by simple fist method, once in a week, from three Hampshire cross bred boars maintained at AICRP on pig, Assam Agricultural University, Khanapara, Guwahati, Assam in a thermo flask. Gel free semen sample was brought to the laboratory in the thermo flask at 35^0c and evaluated for its initial quality. The suitable semen sample was kept in BOD incubator at 24^0c in a conical flask for 3 hours. After 3 hours of holding time sample was centrifuged at 1500 rmp for 10 minutes. The supernatant fluid was discarded and the centrifugate was extended using Lactose egg yolk glycerol extender (Park and Pursel, 1985). The partially extended semen was cooled gradually at 5^0c at the rate of 1^0c per 3 minutes and finally extended at 5^0c with glycerolated extender (1: 1.5). The extended semen was then equilibrated for 1 hour. French medium straw (0.5ml) were used for filling the extended semen. Freezing was done with programmable freezing machine (IMV Technologies, XRP 60-S, Cryo Diffusion 49, Rue de Verdun, 27690 Lery, France) using three different freezing rates *viz.,* Freezing rate- I (30^0c/min), Freezing rate- II (50^0c/min) and Freezing rate-III (70^0c/min). After 16 hours of storage in liquid nitrogen, the frozen semen was thawed in warm water using three different thawing methods *viz.,* Thawing method- I (37^0c for 30 sec), Thawing method- II (50^0c for 12 sec) Thawing method-III (70^0c for 10 sec). The semen sample was evaluated for sperm motility, live sperm count, HOST reacted sperm and intact acrosome after thawing.

3. Results and Discussion

The result of different semen characteristics observed in the present study revealed that the better semen quality was observed in the freezing rate of 30^0c/min with the thawing method of 70^0c for 10 sec (Table 1). Statistical analysis revealed that the mean sperm motility, live sperm count and intact acrosome did not differ significantly between freezing rates, between thawing methods and due to freezing x thawing interaction while the mean HOST reacted spermatozoa differed

Table 1: *Percentage (Mean± SE)*Of Sperm Motility, Live Sperm, HOST Reacted Sperm and Intact Acrosome in Boar Semen after Freezing in Lactose Egg Yolk Glycerol Extender with Different Freezing Rates and Thawing Methods*

	Motility (%)			Live Sperm (%)			HOST Reacted Sperm (%)			Intact acrosome (%)		
	TM-I	TM-II	TH-III	TM-I	TM-II	TH-III	TM-I	TM-II	TH-III	TM-I	TM-II	TH-III
FR-I	45.30 ±0.88	44.40 ± 1.92	47.20 ±2.51	52.30 ±1.47	51.30 ±1.58	54.80 ±1.81	50.00 ±2.01	47.90 ±1.97	52.40 ±1.42	54.90 ±1.08	53.20 ±1.24	51.20 ±1.64
FR-II	42.30 ±2.12	44.00 ±1.99	43.40 ±2.09	53.60 ±1.50	53.30 ±0.72	52.00 ±1.11	49.40 ±1.75	46.90 ±1.97	51.10 ±0.91	52.10 ±1.57	54.00 ±1.16	51.80 ±1.91
FR-III	43.30 ±2.56	44.30 ±2.39	47.10 ±2.88	53.30 ±1.47	52.40 ±1.63	56.50 ±0.72	50.10 ±1.03	45.90 ±1.18	47.20 ±2.51	50.90 ±1.36	51.70 ±1.20	53.80 ±0.94

*Nos of observation=10, FR means Freezing Rate, TM means Thawing Method

significantly (P< 0.01) between thawing methods but did not differ significantly between freezing rates and due to freezing x thawing interaction. The significantly (P< 0.01) higher post thaw HOST reacted spermatozoa observed in freezing rate-I + thawing method –III in the present study was lower than the values reported by Eriksson and Martinez (2000) and Hernendez et al. (2007). The different semen qualities recorded in the present study did not differ significantly between freezing rates. It might be due to narrow range of freezing rate to demonstrate any significant effect on sperm survival which was in agreement with that of Eriksson and Martinez (2000). Result obtained in the present study led to the conclusion that out of the three freezing rates freezing rate of 30^0c/min was superior as apparently higher sperm quality was obtained. It was reported that the optimum freezing rate using 0.5 ml straws was 30^0c/min with 3% glycerol (Fiser and Fairfull, 1990) and for 0.25 ml straws was 50^0c/min with 1.5% glycerol (Woelders and Den Besten, 1993). Watson (1995) reported that boar spermatozoa tolerated a range of freezing rate and Martinez and Wallgren (2011) reported that the

optimum freezing rate for boar spermatozoa was in the range of 30 - 50^{0}c/min. The post thaw sperm motility, live sperm, HOST reacted spermatozoa increased along with the increase in thawing rate from 37^{0}c for 30 sec to 70^{0}c for 10 sec. This was in agreement with that of Fisher et al. (1993) and Hernandez et al. (2007). Hernandez et al. (2007) reported that fast thawing method of 70^{0}c for 8 sec (1800^{0}c/min) improved post thaw sperm quality than the thawing method 37^{0}c for 20 sec (1200^{0}c/min). However both temperature and timing for fast thawing method (70^{0}c for 10 sec) is very crucial to be applicable to commercial situation, since slightly longer period in the water bath at 70^{0}c would raise the temperature inside the straw to non-physiological limit causing irreversible damage to sperm.

4. Conclusion

It can be concluded from the present study that freezing rate 30^{0}c/min was found to be better than the freezing rates 50^{0}c/min and 70^{0}c/min. The thawing method 70^{0}c for 10 sec was found to be better than the thawing methods 50^{0}c for 12 sec and 37^{0}c for 30 sec.

References

Baishya, S.K., 2013: *Cryopreservation of Boar Semen with Special Emphasis on Cryo Damage and Its Mitigation.* Ph.D. thesis, Assam Agricultural University, Khanapara, Guwahati, Assam.

Bwanga, C.O. *Cryopreservation of Boar Semen I: A Literature Review.* Acta Vet Scan. 1991. 32; 431-453.

Crabo, B. and Einarsson, S. *Fertility of Deep Frozen Boar Spermatozoa.* Acta Vet Scand. 1971. 12; 125-127.

Eriksson, B.M. and Martinez-Rodriguez, H. *Effect of Freezing and Thawing Rates on the Post Thaw Viability of Boar Spermatozoa Frozen in Flat Packs and Maxi Straws Anim.* Reprod. Sci. 2000. 63; 205-220.

Fiser, P.S. and Fairfull, R.W. *Combined Effect of Glycerol Concentrations and Cooling Velocity on Motility and Acrosomal Integrity of Boar Spermatozoa Frozen in 0.5 Ml Straws.* Mole Reprod. Dev. 1990. 25; 123-129.

Fiser, P.S., Fairfull. R.W., Hansen, C., Panich, P.L., Shrestha, J.N. and Underhill, L. *The Effect of Warming Velocity on Motility and Acrosomal Integrity of Boar Sperm As Influenced By the Rate of Freezing and Glycerol Level.* Mol Reprod Dev. 1993. 34; 190-195.

Hernandez, M., Roca, J., Gil. M.A., Vazquez, J.M. and Martinez, M.A. *Adjustment on the Cryopreservation Conditions Reduce the Incidence of Boar Ejaculates With Poor Sperm Freezability.* Theriogenology. 2007. 67; 1436-1445.

Johnson, L.A., Weitze, K.F., Fiser, P. and Maxwell, W.M.C. *Storage of Boar Semen.* Anim Reprod. Sci. 2000. 70 (1) 30-32.

Martinez, H.R. and Wallgren, M. *Advances in Boar Semen Cryopreservation.* Veteri. Medi.Intern. 2011. 10; 4061.

Park, C.S. and Pursel, V.G. 1985: *Effect of Cooling Rate and Duration of Freezing Point Platue on Boar Sperm Frozen in Maxi Straws.* Proc. First Int. Conf. Deep freezing boar semen, Uppsala, Sweden, 25-27 August. 267.

Wagner, H.G. and Thibier, M. *World Statistics for Artificial Insemination in Small Ruminants and Swine.* Proceedings of the 14th International Congress on animal reproduction, Stockholm, Sweden, Abstr. 2000. 15; 3.

Watson, P.F. *Recent Development and Concepts in the Cryopreservation of Spermatozoa and the Assessment of Their Post Thawing Function.* Reprod. Fert. Dev. 1995. 7; 871-891.

Woelders, H. and Den Besten, M. *Cryopreservation of Boar Semen with Boar Variation of Post Thaw Sperm Survival.* Cryobiology. 1993. 30; 645.

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Research Article **Open Access**

Molecular Characterization of Sperm Antigens with *In Vivo* Developed Antisperm Antibodies in Variably Inseminated Cross Bred-Heifers/Cows

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Abstract Molecular characterization of sperm proteins and their immunoreactions to sera from subfertile/infertile animals may give deeper insight to the immunological reason of infertility. The purpose of this study was to characterize antigenic sperm proteins by reacting with blood serum and cervical mucus of variably inseminated heifers/cows using immunoblotting and immunofluorescence techniques. Immunoblot analysis of sperm proteins with blood serum/CM of variably inseminated cows/heifers revealed around 23 polypeptides in cattle sperm, but all proteins did not react with blood serum and cervical mucus of all tested animals. Number of sperm polypeptides that reacted with blood serum of non-inseminated, 1-3, 4-6 and >6 times inseminated heifers/cows were in the range of 11-14, 8-13, 3-14 and 5-13 respectively. Correspondingly cervical mucus of 1-3, 4-6 and >6 times inseminated heifers/cows also recognized about 2-7, 2-13 and 2-12 sperm polypeptides respectively. Immunofluorescence of sperm smears with blood serum and cervical mucus of variably inseminated heifer/cows indicated the presence of antisperm antibodies (ASA) mainly against acrosome surface, post –acrosome and principal piece surface proteins. There was not any difference in range of number of polypeptides detected with blood serum in regard to number of insemination. But range of number of polypeptides detected was higher with cervical mucus of >3 times inseminated animals, which indicated increase in ASA against antigenic sperm proteins in cervical mucus with increase in number of inseminations. It was concluded that ASA are produced in blood serum and cervical mucus of cows/heifers against sperm proteins, irrespective of number of inseminations. In future further characterization of sperm proteins and their homogeneity with bacterial proteins is needed to understand the mechanism of ASA mediated fertility impairment and to develop treatment protocol on the basis of purified antigens.

Keywords *Sperm; Antigens; ASA; Heifers; Cows*

1. Introduction

Infertility or sub fertility is a cause of concern in dairy industry. Infertility in the female animals may involve a number of factors, including problems of ovulation, obstruction of oviducts, presence of pathological lesions in the uterus and poor quality of cervical mucus [1]. Bovine infertility has also been associated with the presence of antisperm antibodies, which impairs the physiological process of reproduction. Antibodies against sperm prevent their motility through female reproductive tract and hamper the process of fertilization [2]. Antibodies to sperm or egg yolk have been suggested to be possible cause of sub fertility in cows [3, 4]. The association of sperm antibodies with infertility/sub fertility in zebu cattle [5, 6, 7], goat [8] and mare [9] has been studied using ELISA, immunoperoxidase assay or immunobead assay. During cryopreservation of semen, antigenic structure of sperm cell is changed due to addition of extender, freezing/thawing procedures and seminal plasma is also significantly reduced during semen preparation for AI. The varying degree to which the antibodies impair fertilization suggests that identification of relevant sperm surface antigens and their role in the fertilization process would lead to formulation of an effective therapeutic plan to treat infertility [10]. It was also demonstrated by Jawad [11] that elevated level of ASA can be treated with zinc sulfate. However, molecular characterization through intervention of electrophoretic separation of sperm polypeptides by SDS-PAGE and detection of their immunoreactions to sera from immunological infertile animals may give deeper insight to the immunological reason of infertility. Therefore, in the present study, sperm antigens were characterized by immunoblotting and indirect immunofluorescence with blood serum and cervical mucus of variably inseminated cows and correlated with the number of estrus / AI's.

2. Materials and Methods

2.1. Procurement of Semen

Frozen semen straws were procured from GADVASU, dairy farm.

2.2. Collection of Samples

Blood and cervical mucus (CM) were collected from 43 (3 non-inseminated heifers; 4 inseminated heifers and 36 inseminated cows) cross-bred cows (HF X Red dane X Sahiwal) from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University and private dairy farms around Ludhiana, Punjab, India. Blood was collected in sterilized vials from jugular vein without anticoagulant, centrifuged at 3000 rpm for 5 min to separate serum. At the time of estrus, CM was collected with the help of sterilized AI pipette, sonicated at 20 watts, 3 X 20 seconds. Serum and CM were inactivated at 56^{o} C for 30 min and stored in aliquots at -20^{o}C till further use. Number of estrus and AI's of the cows were also obtained from the farms. Out of 43 samples, collected, there were about 4, 8, 22 and 9 cows exhibiting about 0, 1-3, 4-6 and 6-14 times estrus viz a viz number of AI's, respectively.

2.3. Preparation of Sperm Extracts

Frozen semen straws were thawed, centrifuged and washed three times with PBS, pH 7.4. About 500 $x10^{6}$ spermatozoa were suspended in 1.0 ml of 2% SDS in 62.5 mM Tris-HCl (pH 6.8), containing 10 µl of cocktail protease inhibitors (SERVA), sonicated at 4^{o}C (20 W, thrice for 20 seconds each) and centrifuged at 16,000 g for 30 minutes to prepare sodium dodecyl sulphate sperm extract (SDS -SE).

2.5. SDS-PAGE [12] and Immunoblotting [13]

Blood serum and CM of variably inseminated heifers and cows were reacted with SDS-SE. Proteins separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose membrane using wet electrophoresis transfer apparatus at 100 V for 2.30 hrs. Transfer quality was checked by 0.2% ponceau dye and proteins were blocked in 2% BSA as blocking solution for overnight at 4^0C. After washing the membrane with PBS+0.05% Tween-20, it was incubated in blood serum (1:200) and CM (1:50) of variably inseminated heifers and cows for 2.5 hrs. Again washed thrice with PBS+0.05% Tween-20 and incubated with 1:10000 diluted HRP conjugated anti bovine IgG as secondary antibody for 45 min. Washed thrice with PBS + Tween-20 and incubated with substrate (0.05% Diaminobenzidine + 0.06% Hydrogen Peroxide) for 10 min. Gel images were captured on Syngene gel doc using GeneSnap image acquisition software and analyzed by using GeneTools gel analysis software (Syngene).

2.6. Immunolocalization of Antigenic Proteins using FITC Labeling [14]

Smears of frozen-thawed-washed cattle bull spermatozoa were prepared on glass slides, air-dried, and fixed in ethanol for 30 minutes. Slides were then covered with PBS containing 1% BSA for 45 minutes to block nonspecific antibody binding. They were then incubated at 37^0C in a humidified chamber for 2 hours with 1:200 blood serum, 1:50 CM of variably inseminated heifers and cows. Slides were then washed and incubated for 1 hour with 1: 100 diluted rabbit anti-bovine IgG for one hour. Slides were again washed and incubated with 1: 100 diluted goat anti rabbit-FITC-conjugated antibody (Sigma). After 3 washings, slides were mounted with PBS-glycerol (1:1 v/v) containing 1, 4 diazabicyclo (2.2.2) octane as anti-fade and observed under fluorescent microscope (olympus), blue filter and images were captured on digital camera.

3. Results and Discussion

3.1. Characterization of Sperm Surface Antigens with Blood Serum and Cervical Mucus of Variably Inseminated Cows/Heifers

The blood serum and CM of all cows irrespective of number of heats or artificial inseminations reacted with SDS-SE on immunoblots and indicated variation in immunodominant sperm proteins for development of antibodies in variably inseminated cows. Immunoblot analysis of SDS-SE with blood serum/CM of variably inseminated cows/heifers revealed around 23 polypeptides of 300, 270, 245, 230, 200, 170, 150, 130, 115, 100, 90, 80, 75, 70, 65, 48, 45, 42, 35, 24, 18, 16, 11 kDa in cattle sperm (Figure 1 and 2). However, all proteins did not react with blood serum and cervical mucus of all tested animals. Number of polypeptides of SDS-SE that reacted with blood serum of non-inseminated, 1-3, 4-6 and >6 times inseminated heifers/cows were in the range of 11-14, 8-13, 3-14 and 5-13 respectively. There was also difference in intensity of bands reacting with blood serum/ cervical mucus of tested animals. Only two polypeptides out of 16 sperm polypeptides reacted with blood serum of infertile cows [15]. Circulating anti-sperm antibodies in blood serum of infertile patients also reacted with 4 surface antigens of 35, 40, 47 and 65 kDa [10].

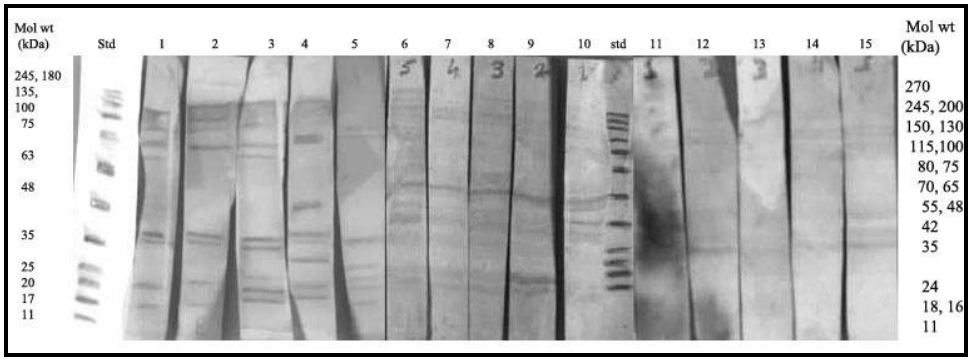

Figure 1: *Showing Reaction of SDS-SE Proteins with Blood Serum of Some of Variably Inseminated Cows/ Heifers on Immunoblot. SDS-SE were Separated on 10% Acrylamide Gel, Transferred to Nitrocellulose Membrane and Blot was developed with Blood Serum of Variably Inseminated Heifers/Cows*

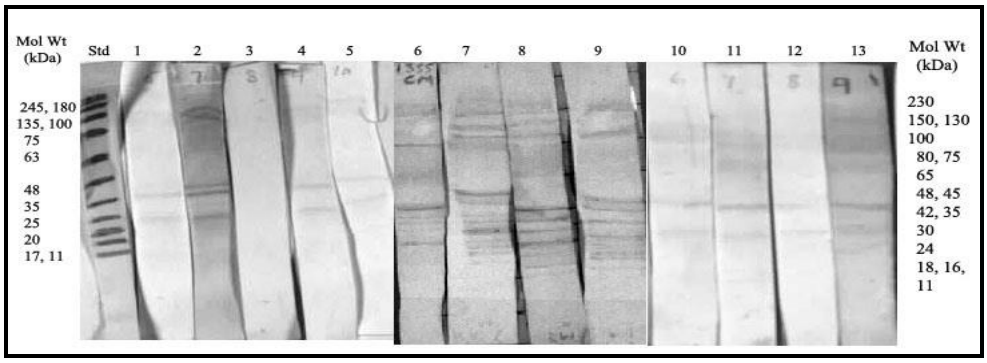

Figure 2: *Showing Reaction of SDS-SE Proteins with Cervical Mucus of Some of Variably Inseminated Cows/ Heifers on Immunoblot. SDS-SE were Separated on 10% Acrylamide Gel, Transferred to Nitrocellulose Membrane and Blot was developed with Cervical Mucus of Variably Inseminated Heifers/Cows*

Correspondingly cervical mucus of 1-3, 4-6 and >6 times inseminated heifers/ cows also recognized about 2-7, 2-13 and 2-12 polypeptides in SDS-SE respectively. Bohring et al. [16] identified a total of 18 antigens by 2-D western blotting using ASA from seminal plasma samples of infertile patients. Six of the recognized proteins were identified as heat shock proteins HSP70 and HSP70-2, the disulphide isomerase ER60, the inactive form of caspase-3 and two subunits of the proteasome. There were 11.6-13.9% and 8.3-19.4% cows irrespective of number of estrus/ AI, which had ASA against 5-9 and 2-7 sperm proteins in blood serum and cervical mucus respectively (Table 1). Percentage of cows having ASA against higher number of sperm proteins i.e. 10-14 and 8-14 was only 0-9.3% and 2.7-5.5% in blood serum and CM respectively. Cervical mucus of only 2.7% tested cows could not recognize any polypeptide in SDS-SE.

Table 1: *Number of Sperm Polypeptides Detected with Blood Serum/ Cervical Mucus and Percentage of Cows*

Number of Proteins		0	2	3	4	5	6	7	8	9	10	11	12	13	14
%	**Serum**	-----	-----	4.6	---	**11.6**	**11.6**	**13.9**	**13.9**	**13.9**	4.6	6.9	4.6	9.3	4.6
cows	**CM**	2.7	**11.1**	**16.6**	**8.3**	**11.1**	**19.4**	**16.6**	2.7	2.7	**----**	**----**	2.7	5.5	**---**

3.5. Immunolocalization of Sperm Antigens with Blood Serum and Cervical Mucus of Variably Inseminated Cows/Heifers

Immunofluorescence of sperm smears with blood serum/cervical mucus of variably inseminated animals gave variable signals. A very strong signal was observed on acrosome surface, post-acrosome region and principal piece of spermatozoa (Figure 3). It indicated the presence of ASA

against mainly acrosome surface, post –acrosome and principal piece surface proteins in the blood serum/ cervical mucus of variably inseminated animals. Milovanovic et al. [17] also observed fluorescence on the head, tail or neck of the sperm cell with blood serum and cervical mucus of artificially inseminated Holstein cows. They confirmed the hypothesis that immune mechanisms may be involved in reproductive disturbances due to high levels of ASA of Ig A class and found high levels of ASA in animals with longer open day's period. The mean age at first calving and the mean intercalving interval were significantly higher in the group positive for sperm antibodies compared to the negative animals [5].

Therefore, immunofluorescence as well as immunoblotting revealed the presence of antisperm antibodies in the blood serum and cervical mucus of all tested animals irrespective of number of inseminations. However, there was not any difference in range of number of polypeptides detected with blood serum in regard to number of insemination. But range of number of polypeptides detected was higher with cervical mucus of >3 times inseminated animals. It indicated increase in ASA in cervical mucus with increase in number of inseminations. The cows having more unsuccessful inseminations also showed higher ASA with high titre in serum and mucus [7]. Farahani et al. [18] found agglutinating and immunofluorescent antibodies in serum from repeat breeder, fertile cows and virgin heifers with no sperm-immobilizing antibodies. The antibodies were assumed to be produced naturally with no need of female exposure to sperm antigens as all virgin heifers also demonstrated agglutinating and immunofluoresence antibodies in their serum. Similarly, in our study 11-14 polypeptides were detected even with blood serum of virgin heifers (<6 months), which were reduced to 8-13, 3-14 and 5-13 in 1-3 and >3 times inseminated animals. Reaction of 11-14 sperm polypeptides with blood serum of virgin heifers indicated the presence of cross reacting antibodies in their blood serum. Paolichhi et al. [19] also postulated that cross reactive antibodies may develop in animals and impair fertility. Many species of bacteria and anaerobic microorganisms inhabit the vagina, uterus, cervix of cows and many bacteria were isolated from the uteri of cows with a history of repeat breeding, retained placenta and metritis [20]. Recently Thapar et al. [21] were of the opinions that cross reactivity between certain epitopes on the bacterial surface & spermatozoa particularly involving carbohydrate determining domains might be potential triggering mechanism for induction of ASA in male and female. An amazing analogy between the chlamydial heat shock proteins and human proteins; the relationships between *Ureaplasmaurealyticum* infection, antisperm antibodies, and infertility, homology between Ure-G and Nuclear sperm autoantigenic protein was noticed in human in different studies [22]. Therefore, reaction of sperm proteins with blood serum/cervical mucus of virgin heifers and cows may also be due to the presence of cross reacting antibodies against microorganisms.

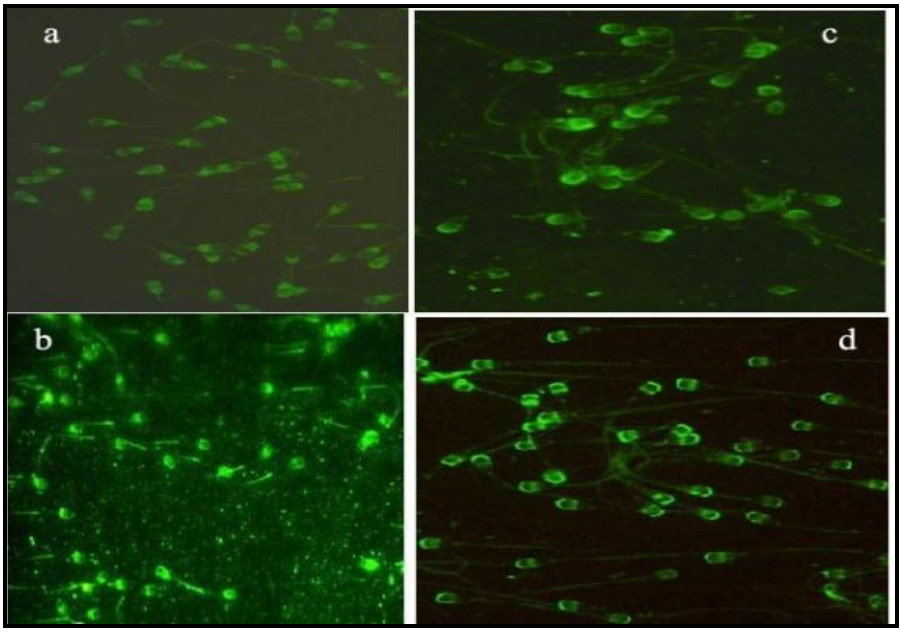

Figure 3: *Showing Localization of Sperm Antigens upon Reaction of Sperm Smears with Blood Serum (A, B) and Cervical Mucus (C, D) of Variably Inseminated Cows. Slides Fixed in Ethanol were Reacted with Blood Serum / Cervical Mucus and Fluorescence was Developed with Goat Anti Rabbit-FITC-Conjugated Antibody and Observed at 400 X*

4. Conclusions

It was concluded that ASA are produced in blood serum and cervical mucus of cows/heifers against sperm proteins, ranging from 11-300 kDa, irrespective of number of inseminations. In future further characterization of sperm proteins and their homogeneity with bacterial proteins is needed to understand the mechanism of ASA mediated fertility impairment and to develop treatment protocol on the basis of purified antigens.

Acknowledgement

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References

[1] Shulman, S. *Therapy of Immunological Infertility.* Journal of Immunology and Immunopharmacology. 1986. 6; 95.

[2] Tripti, N., Palra, P.K. and Sahu, J.P. *Evaluation of Serum Antisperm Antibodies in Infertility*. The Journal of Obstetrics and Gynaecology of India. 2011. 61; 307-316.

[3] Hunter, A.G., 1972: *Immunological Aspects of Reproduction Associated with Repeat Breedings.* In: Proceedings of 4th Technical Conference on Animal Reproduction and Artificial Insemination, National Association of Animal Breeders USA. 2-7.

[4] Coulter, G.H., Foote, R.H., Schiavo, J.J. and Braun, R.K. *Antibodies to Egg Yolk in Blood Serum of Rabbits and Cattle and Cervical Mucus of Cattle Inseminated Artificially.* Theriogenology. 1976. 60; 585-589.

[5] Fayemi, O. *Sperm Antibodies and Reproductive Efficiency in the Zebu Cattle in South-Western Nigeria.* Pakistan Veterinary Journal. 2005. 25; 111-114.

[6] Lazarevic, M., Milanovic, S., Kirovski, D. and Milovanovic, A. *Antisperm Antibodies of the IG a Class in the Cervical Mucus and Sera of Artificially Inseminated Cows.* Acta Vetenaria (Beograd). 2003. 53; 311-319.

[7] Sarna, D.K., Baishya, N., Sharma, D.K., Deka, B.C. and Bhuyan, D. *Antisperm Antibodies in Serum and Cervical Mucus of Normal and Repeat Breeding Cows.* Indian Journal of Animal Reproduction. 2009. 30; 54-56.

[8] Waziri, M.A. and Fayemi, O.E. *Seroprevalence of Sperm Antibodies in Goats*. Veterinarski. Arhiv. 2000. 70; 95-102.

[9] Risvanli, A., Cetin, H., Apaydin, M., Korkmaz, O., Atli, O. and Timurkan, H. *Prevalence of Anti-Sperm Antibodies in Mares in the South-Eastern Anatolian of Turkey.* Bulletin of Veterinary Institute Pulawy. 2005. 4; 45-48.

[10] Feng, H.L., Han, Y.B, Sparks, A.E.T. and Sandlow, J.I. *Characterization of Human Sperm Antigens Reacting with Antisperm Antibodies from an Infertile Female Patient's Serum.* Journal of Andrology. 2008. 29; 440-448.

[11] Jawad, H.M. *Zinc Sulphate Treatment of Secondary Male Infertility Associated with Positive Serum and Seminal Plasma Antisperm Antibodies Test.* Middle East Fertility Society. 2013. 18; 24-30.

[12] Laemmli, U.K. *Cleavage of Structural Proteins during Assembly of the Head of Bacteriophage T4.* Nature. 1970. 227; 680-685.

[13] Towbin, H., Staehelint, T. and Gordon, J. *Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications.* Proceedings of National Academy of Sciences. USA. 1979. 76; 4350-4354.

[14] Verdier, Y., Rouet, N., Artois, M. and Bou, F. *Partial Characterization of Antigenic Sperm Proteins in Foxes (Vulpes Vulpes).* Journal of Andrology. 2002. 23; 529-536.

[15] Tripathi, D., Sharma, N.C., Singh S.K. and Gupta L.K. *Identification of Bovine Sperm Specific Polypeptides Reactive with Antisperm Antibodies.* Indian Journal of Experimental Biology. 1999. 37; 655-661.

[16] Bohring, C.E., Krause B. Habermann and Krause, W. *Isolation and Identification of Sperm Membrane Antigens Recognized by Antisperm Antibodies and their Possible Role in Immunological Infertility Disease.* Molecular Human Reproduction. 2001. 7; 113-118.

[17] Milovanovic, A., Lazarevic, M., Milanovic, S., Kirovski, D. and Jovicin, M. *Open Days Period and Antispermatozoal Antibodies in Artifically Inseminated Cows.* Acta Vetenaria (Beograd). 2005. 55; 449-460.

[18] Farahani, J.K., Tompkins W. and Wagner W.C. *Reproductive Status of Cows and Incidence of Antisperm Antibodies.* Theriogenology. 1981. 15; 605-612.

[19] Paolichhi, F.A., Casaro P.A., Gimeno G.J., Kartebani L.G. and Mozzolli A.B. *Antisperm Response in Rams Experimentally Infected with Brucella Ovis.* Small Ruminant Research. 2000. 36; 7-15.

[20] AbdEl Kader, H.A. and Shahata S.H. *Bacteriological Evaluation of Vaginal Discharges in Cows with Endometritis and Clinically Healthy Heifers in Assiut Governorate.* Assiut University Bulletin for Environmental Researchers. 2001. 4; 45-53.

[21] Thaper, D., Vander, H. and Prabha, V. *Association of Antisperm Antibodies with Bacterial Infection: An Insight to Infertility. Androl.* Gynecology: Current Research. 2014. 2; 1-6.

[22] Haidl, G. *Characterization of Fertility Related Antisperm Antibodies—A Step towards Causal Treatment of Immunological Infertility and Immuno-Contraception.* Asian Journal of Andrology. 2010. 12; 793-794.

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Case Study | Open Access

A Report of Gastric Adenocarcinoma in a Dog

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Abstract An 11 year old male non-descript dog was presented with the history of frequent vomition and progressive weight loss for the past one month. The dog was dull, dehydrated, anaemic and emasciated. Positive contrast survey radiographs, revealed delayed gastrointestinal transit time and absence of foreign body. Exploratory laparatomy was done which revealed thickening of entire layer of the gastric wall with pylorus and the histopathology revealed gastric adenocarcinoma. Since the tumor was spread to the full thickness of the stomach wall including pylorus, the prognosis was informed to the owner and euthanasia was advised.
Keywords *Case Report; Canine; Gastric Adenocarcinoma; Histopathology*

1. Introduction

Adenocarcinoma is the most common gastric neoplasm, comprising 1 to 2% of all the malignant neoplasms [1] and 47 to 72 % of all gastric malignancies [10] in canine. Canine gastric cancer was more prevalent in dogs than in domestic animals [4]. The majority of gastric malignancies in dogs were carcinomas accounting for 50-90%, followed by leiomyosarcomas and malignant lymphoma [2, 3]. This is a report of a case of gastric adenocarcinoma in a dog.

2. Case History and Observation

A 11 year old non-descript dog was presented to SAC OP surgery unit of Madras Veterinary College Teaching Hospital with the history of chronic vomition for the past one month after taking food and was treated symptomatically for gastritis with no improvement. The dog was progressively losing its body weight. On clinical examination the dog was dull, depressed and dehydrated. Heart rate and respiratory rate were within the normal limit. Haematology and serum biochemical profiles revealed reduction in total red blood cell count and haemoglobin with neutrophilia and leucocytosis.

Plain radiography of the thorax lateral view revealed early metastatic lesion (Figure 1) and abdomen lateral view revealed no abnormalities. Hence positive contrast radiography using barium meal were taken at 5, 10, 20 minutes and 24 hrs which revealed delayed gastrointestinal transit time and absence of radio opaque foreign body. Ultrasonography revealed a prostatic cyst of about 3.04 mm

diameter and thickening of the stomach wall and pylorus was noticed. Hence exploratory laparatomy was performed to find out the cause of chronic vomition.

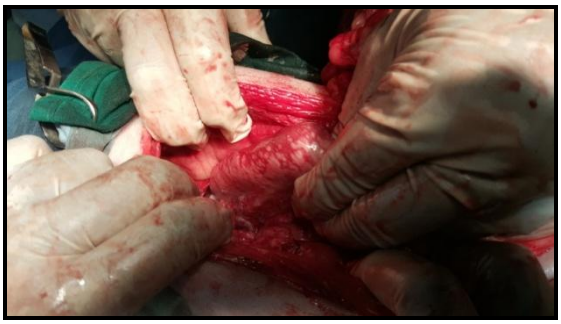

Figure 1: *Gastric Neoplasm*

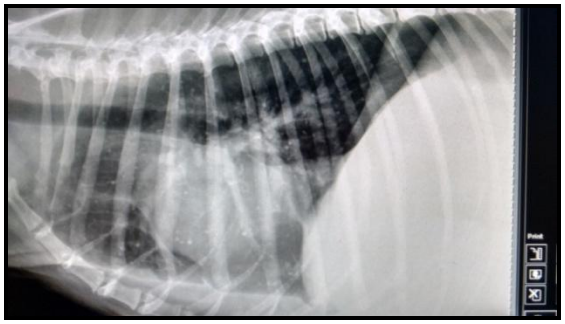

Figure 2: *Plain Radiography-Lung Metastasis*

3. Treatment and Discussion

The dog was premedicated with Diazepam @ 0.2 mg / kg intravenously. General anaesthesia was induced using propofol @ 4 mg / kg intravenously and maintained with isoflurane (2.5%) in oxygen using Boyles anaesthetic apparatus with assisted ventilation.

Cefotaxime @ 10 mg / kg and tramadol @ 2 mg / kg was administered intravenously before surgery.

The dog was placed on dorsal recumbency and the surgical site was prepared following aseptic procedures. A midventral skin incision was made from xiphoid to prepubic region and the subcutaneous tissue and fascia dissected. The linea alba was incised and the abdominal viscera exposed. On examination, the entire lesser curvature of stomach wall and pylorus was highly thickened and discoloured serosal patches were seen suspecting for gastric neoplasm (Figure 2). The lesion was spread throughout the serosal and mucosal surface of the stomach wall and pylorus. Fine needle aspiration cytology of the mass was taken and another tissue sample was collected in 10 % formalin for histopathological examination. The prognosis was informed to the owner regarding the condition and euthanasia was advised.

Adenocarcinoma is the most common type of gastric carcinoma [8]. This type of carcinoma forms tubular structures and exhibit various patterns at different levels of invasion of the stomach wall. Among dogs, gastric carcinoma has been reported to affect males more frequently than females [11]. Gastric carcinoma is a disease of older dogs, but has been reported in dogs in the age range of 3-20 yrs. In the above discussed case the age of the dog was 11 yrs. Most common location of gastric carcinoma was the lesser curvature and pyloric region of the stomach [12]. The most common clinical signs associated with gastric carcinoma were vomiting, anorexia, progressive weight loss. haematemesis, melaena, anaemia, lethargy, ptyalism, polydipsia, abdominal distension, and abdominal discomfort [2, 3, 5]. The prognosis in case of gastric carcinoma was poor. 70-90 % of gastric carcinomas have metastasized by the time of diagnosis or euthanasia. The preliminary diagnosis of gastric carcinoma was usually obtained by ultrasound examination, in which thickening of gastric wall and loss of gastric wall layering was observed [6, 9]. The most common sites of metastases are the regional lymphnode, omentum, duodenum, liver, spleen, pancreas and lungs [13]. According to World Health Organisation tumor staging system for gastric tumors [5, 7] the primary tumor in the above case invaded the serosa (T-3), there was evidence of lymphnode involvement (N-2) and evidence of distant metastasis (M-2).

Microscopically poorly demarcated unencapsulated irregular pleomorphic glands involving mostly the submucosa and muscularis mucosa with excessive desmoplasia with nests of neoplastic acini and

cells were seen (Figure 5). Neoplastic cells were cuboidal with moderate eosinophilic cytoplasm. Nuclei round to oval with stippled chromatin, dysplastic, mild anisokaryosis and mitotic figures, 2-3 / high power field (Figure 3). Glandular lumen contained eosinophilic secretary product with sloughed neoplastic cells. Lymphatic vessels contained neoplastic emboli (Figure 4).

4. Summary

Due to the advanced stage of disease at the time of diagnosis and the high frequency of metastasis, early detection is essential, if the treatment of canine gastric neoplasm was to be attempted. As the presenting signs were similar to those observed in cases of chronic gastritis, some dogs were symptomatically treated for gastritis for prolonged periods and the suspicion of gastric neoplasm may first arise when this form of treatment failed. It is therefore important to obtain a correct diagnosis as early as possible. This paper describes about the advanced stage of gastric adenocarcinoma in a dog.

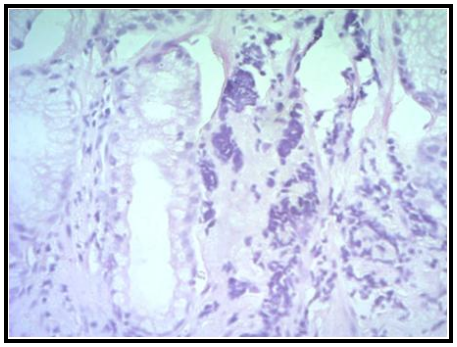

Figure 3: *Dysplastic Nuclei in the Gland*.

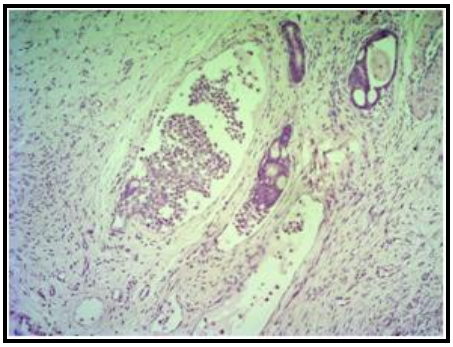

Figure 4: *Tumor Emboli in Veins*

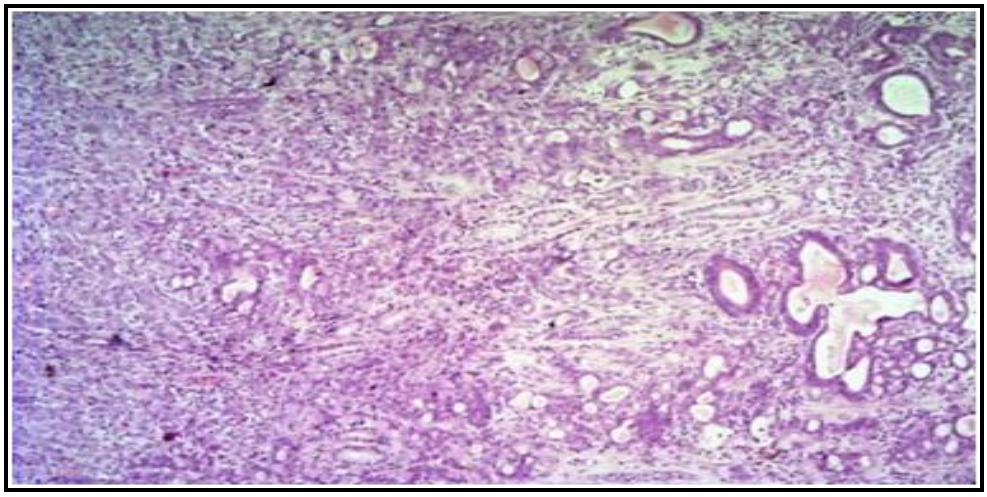

Figure 5: *Pleomorphic Glands with Nests of Neoplastic Acini*

References

[1] Brodey, R.S. and Cohen, D., 1964: *An Epizootiologic and Clinicopathologic Study of 95 Cases of Gastrointestinal Neoplasms in the Dog.* Scientific Proc Am Vet Med Assoc. 10Ist Ann Meeting. 167-169.

[2] Couto, C.G., Rutgers, H.C., Sherding, R.G. and Rojko, J. *Gastrointestinal Lymphoma in 20 Dogs.* J Vet Intern Med. 1989. 3; 73-78.

[3] Fonda, D., Gualtieri, M. and Scanziani, E. *Gastric Carcinoma in the Dog – A Clinicopathological Study of 11 Cases.* J Small Anim Pract. 1989. 30; 353-360.

[4] Head, K.W. *Tumours of Lower Alimentary Tract.* B World Health Organ. 1976. 53; 167-186.

[5] Jonathan, Bray and Reto, Neiger, 2011: Tumors of Stomach. In: BSAVA Manual of Canine and Feline Oncology. 3rd Edn. Jane M., Dobson, B., Duncan X. (Eds.) Lanceller, BSAVA, England. 209-211.

[6] Kelley, J.R. and Duggan, J.M. *Gastric Cancer Epidemiology and Risk Factors.* J Clin Epidemiol. 2003. 56; 1-9.

[7] Lingeman, C.H., Garner, F.M. and Taylor, DON. *Spontaneous Gastric Adenocarcinomas of Dogs - Review.* J Natl Cancer Inst. 1971. 47; 137.

[8] Patnaik, A.K., Hurvitz, A.I. and Johnson, GF. *Canine Gastrointestinal Neoplasms.* Vet Pathol. 1977. 14; 547-555.

[9] Penninck, D.G., Moore, A.S. and Gliatto, J. *Ultrasonography of Canine Gastric Epithelial Neoplasia.* Vet Radiol Ultrasoun. 1998. 39; 342-348.

[10] Sautter, J.H. and Hanlon, G.F. *Gastric Neoplasms in the Dog: A Report of 20 Cases*. J Am Vet Med Assoc. 1975. 166 (7) 691-696.

[11] Sullivan, M., Lee, R., Fisher, E.W, Nash, A.S. and Mccandlish, I.A.P. *A Study of 31 Cases of Gastric Carcinoma in Dogs.* Vet Rec. 1987. 120; 79-83.

[12] Swann, H.M. and Holt, D.E. *Canine Gastric Adenocarcinoma and Leiomyosarcoma: A Retrospective Study of 21 Cases (1986–1999) and Literature Review.* J Am Anim Hosp Assoc. 2002. 38; 157-164.

[13] Scanziani, E., Giusti, A.M., Gualtieri, M. and Fonda, D. *Gastric Carcinoma in the Belgian Shepherd Dog.* J Small Anim Pract. 1991. 32; 465-469.

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Research Article | Open Access

Guinea Fowl Mortality Associated with *Ascaridia numidae* Infection

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Abstract In an organized poultry farm 8-12 weeks old grower flocks of pearl variety guinea fowls have shown symptoms like anorexia, diarrhea, lethargy and emaciation. There was also 3.5 % morality in the flock. Postmortem revealed the highly inflamed mucosa severely studded with ascarid larvae at the jejunum and ileum region. Few adult parasites were present in the lumen and were identified as *Ascaridia numidae* based on their microscopic morphology. Pooled droppings from pens were examined for EPG. Histopathology revealed numerous larvae in the lumen, mucosa and submousa of the intestine. Treatment with piperazine adipate significantly reduced the mortality to 0.8 %. EPG was significantly reduced after treatment. All these findings indicated that the cause of morbidity and mortality in pearl variety guinea fowls was due to *A. numidae* infection.
Keywords *Guinea Fowl; Ascaridia Numidae; Morbidity; Mortality; EPG; Histopathology*

1. Introduction

The Guinea fowl (*Numida meleagris*) is one of the important and interesting gallinaceous birds being farmed for centuries in the Indian subcontinent. Because of its low input requirements and better forage utilization capacity, guinea fowls hold a unique status for alternate poultry production for the rural unemployed youths and women. It is reared for food, game, fancy and also as a pet. The guinea fowls are highly susceptible for gastro intestinal helminth parasites because of the rearing methods and feeding habits. Helminthiasis is considered as one of the most significant constraints on poultry production in tropical climatic conditions of India which are favourable for faster propagation and development of the larval stages of helminth parasites (Matta and Ahluwalia, 1981; Kulkarni et al., 2001). Ascarid worms are one of the more common parasites infecting poultry such as guinea fowls, chickens and turkeys. The eggs of these worms were excreted in the droppings and the eggs can survive in the environment for a couple of years. The susceptible birds get infection by ingestion of these eggs. These worms have significant economic impact on the poultry farming due to the reduced feed conversion efficiency, decreased growth rate and mortality in the worm infected birds (Katoch et al., 2012).

Different helminth parasites including *Ascaridia numidae* infecting guinea fowls have been reported from South Africa (Junker et al., 2008) and Ghana (Hodasi et al., 1976). In Turkey *A. numidae* infection has been reported from rock partridges (Avcioglu et al., 2008). It is found in the lumen of small intestine or caecum (Saif et al., 2003) of guinea fowl. *Ascaridia numidae* are much smaller than *A. galli* which is a very common parasite of poultry. *Ascaridia numidae* infection in guinea fowl have been reported in northern parts of India (Gupta and Acharya, 1971; Matta and Ahluwalia, 1979) but it has not been reported from southern peninsula of India. This report describes mortality in 8-12 weeks old pearl variety guinea fowl flock in Institute of Poultry Production and Management, TANUVAS due to *A. numidae* infection.

2. Materials and Methods

2.1. History

Pearl variety guinea fowls of 8-12 weeks grower age group had shown symptoms like anorexia, diarrhea, lethargy and emaciation. During this period, there were 74 mortalities per week in this flock which was 3.5% of the total stock. Morbidity was around 50% of the flock. These guinea fowls were fed with commercial grower feed. They were grown in eight pens with 60 -70 birds /pen.

2.2. Faecal Examination

Pooled droppings from eight individual pens were collected from the farm to rule out gastro intestinal parasitism. These samples were examined for Egg per Gram of feces using Modified Mc Master technique for greater sensitivity. Briefly four gram of droppings was suspended with 26 ml of saturated salt solution and mixed thoroughly. This was loaded in to a three chambered Mc Master slide and kept undisturbed for 2 minutes for the eggs to float to the top layer. Total number of eggs present under grid lines were counted and multiplied by 25 to get the EPG.

2.3. Necropsy

Necropsy was conducted on three guinea fowl carcasses received from the farm as per standard procedure.

2.4. Parasites Identification

Parasite samples preserved in 95% ethanol were treated with Lactophenol and examined under trinocular microscope for morphological characteristics.

2.5. Histopathology

Tissues fixed in 10% formalin were embedded in paraffin. Using microtome, thin sections were made after standard processing. These sections were stained with haematoxylin and eosin and examined under trinocular microscope with image capture software.

2.6. Treatment

Birds were treated with Albendazole @ 10mg/kg body weight in water (40 litres / 1000 birds). But mortality was not controlled after treatment with Albendazole then birds were treated with Piperazine adipate @ 300mg/kg body weight. Second dose was administered 14 days after the first treatment.

3. Results

3.1. Fecal Examination

EPG ranged from 1100-1500. Eggs of *A. numidae* are oval in shape with smooth shells and unsegmented. They measured 61 µm in length and 32 µm in width.

3.2. Necropsy

Carcasses were emaciated and poor in condition. There was paleness of visible mucous membrane. Liver showed multifocal pale areas. Intestinal contents were scanty as there was anorexia. Mucosa of intestine was heavily studded with ascarid larvae (Figure 1) both in the jejunum and ileum region in all the three birds. There were few adult parasites in the ileum region. No significant lesions were noticed in other organs.

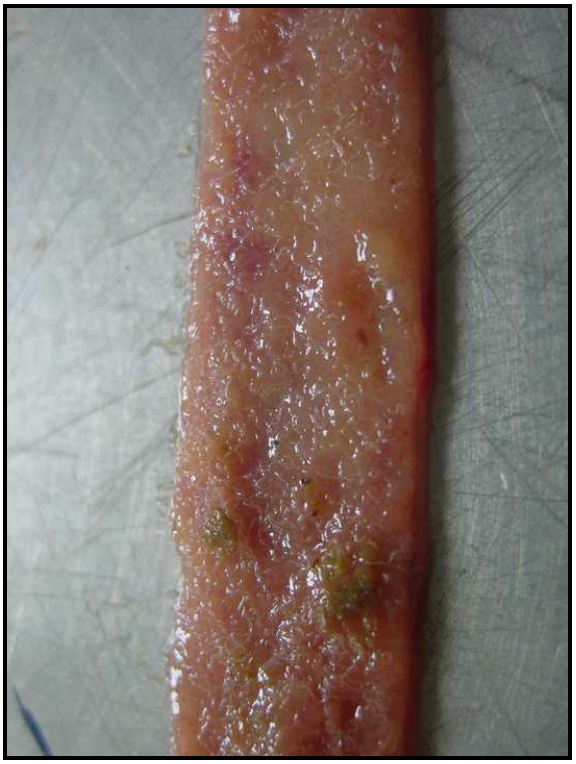

Figure 1: *Intestinal Mucosa Heavily Studded with Ascarida numidae*

3.3. Parasite Identification

Biometry of male and female parasites was 21 mm length and 0.9 mm width. And 29 mm in length and 1.3 mm in width respectively. Oesophagus length of male nematode was 1.4 mm and female was 1.7 mm. In male nematode there was a single papilla on the pre anal sucker (Figure 2). It is an important morphological feature of *A. numidae* which differentiates it from *A. galli* beside the difference in size.

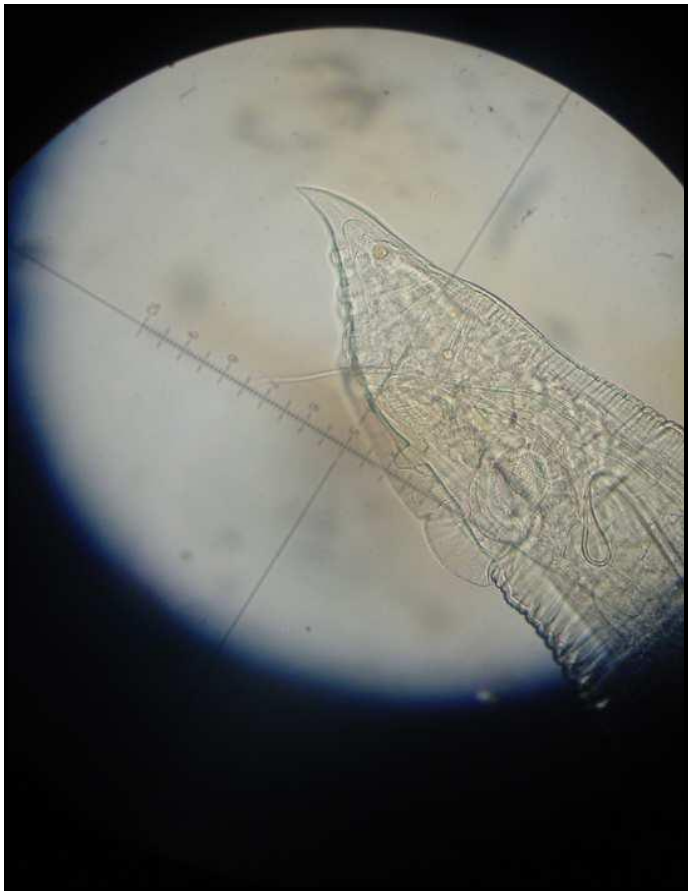

Figure 2: *Male Ascaridia numidae Helminth with Single Papillae on the Pre Anal Sucker*

3.4. Histopathology

There was catarrhal inflammation and diffuse lymphocytic infiltration in the mucosa and submucosa. Numerous second stage larvae were present in the mucosa and submucosa (Figure 3 and 4). There was also hyperplasia of goblet cells in the mucosa and submucosa. Clusters of bacilli were observed in the cellular debris of the lumen. In liver, there was periportal hepatitis and diffuse vacuolar degenerative charges in the hepatocytes.

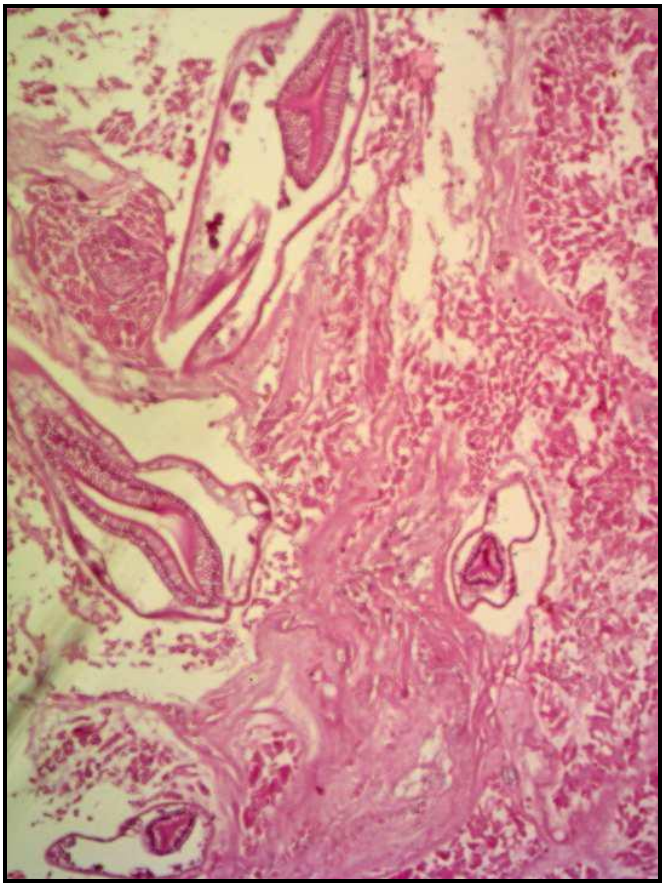

Figure 3: *Numerous Second Stage Larvae were Present in the Mucosa and Submucosa*

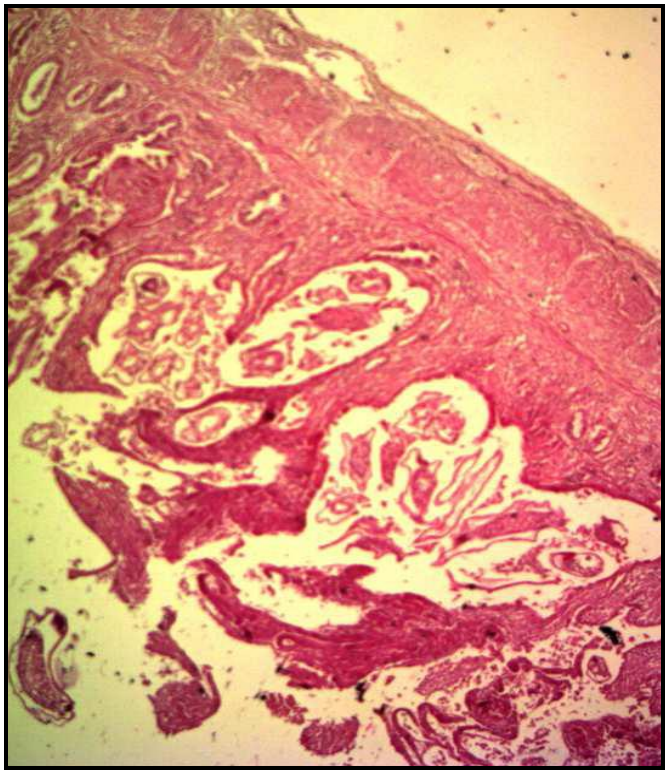

Figure 4: *Numerous Second Stage Larvae with Hyperplasia of Goblet Cells in the Mucosa and Submucosa*

3.5. Treatment

Initial treatment with Albendazole @ 10 mg/kg body weight in water (40 litres / 1000 birds) did not control mortality and then the birds were treated with Piperazine adipate @ 300mg/kg body weight with the second dose at 14 days after the first treatment. It was advised to change the litter as it was the source of infection. 14 days after the first treatment pooled droppings were examined for EPG. It was significantly reduced to 0-100. Mortality was also gradually reduced to 0.8 % after treatment.

4. Discussion

Infection with *A. numidae* was the cause of mortality and morbidity in this pearl variety guinea fowls. Absence of diagnostic lesions in organs other than intestines in necropsy, microscopic morphology of adult parasites, histopathology and response to piperazine treatment suggest this association. The identification of *A. numidae* was based only on the microscopic morphology of the adult parasite recovered during postmortem.

A. numidae infection is relatively host specific (Bush, 1990) but the entry of infection into this organized farm is unknown. Multiple stages of nematode parasite in the same host indicate continuous reinfection from the source of infection. Weight loss and emaciation in *A. numidae* infected birds which has been reported might be due to diarrhea and alteration in the feed conversion ratio.

Tissue stages in the development of *A. numidae* from the guinea fowl has been reported from Georgia as early as 1973 (Mabon and Reid, 1973). Larval stages of *A. numidae* were found in the mucosa and submucosa has also been reported by the above authors. The hyperplasia of goblet cells in the mucosa and submucosa has been reported in *A. galli* infection in chickens (Soulsby, 1986). But it has not been previously reported in guinea fowls with *A. numidae* infection. Lesions in the liver and presence of cluster of bacilli in the intestine observed on histopathology might be secondary to the damage caused by penetrating larvae. No significant bacterial growth from the routine heart blood swab culture confirms the above finding. No other lesions in the liver, lung and other internal organs of the host coincide with the report of Mabon and Reid (1973).

There is a report on response to piperazine treatment for *Ascaridia numidae* infection (Robbins et al., 2011). Treatment with Levamisole @ 30 mg/kg body weight (Avcioglu et al., 2008), Fenbendazole have been suggested (Robbins et al., 2011). But in our study birds did not respond to Albendazole treatment.

Guinea fowl rearing is an important alternate poultry farming practiced in Indian subcontinent to utilize the poorly cultivable lands and this practice generate employment opportunity and revenue to the rural youth besides catering to the protein needs of the rural farmers. This report on *A. numidae* infection is first of its kind in Indian guinea fowls. This paper emphasizes the economic importance of *A. numidae* infection in guinea fowls as it causes heavy morbidity and mortality.

Prophylactic measures such as periodical removal of litters, stacking of litters for several days to allow heating before placing them in pens, good ventilation, feed trough and drinking water hygiene along with treatment may be useful in effective control of *Ascaridia numidae* infection in guinea fowls.

5. Conclusion

This study reported a significant mortality rate of 3.5% in grower age group of guinea fowl due to *Ascaridia numidae* infection. The diagnosis was established through identification of eggs in feces, EPG of feces and numerous adult worms in the intestine. Histopathology revealed a severe damage

to mucosa and submucosa of intestine by second stage larvae. Treatment with piperazine and prophylactic measures significantly decreased the mortality rate.

References

Avcioglu, H., Burgu, A. and Bolukbas, C. *Ascardiae Numidae (Leiper, 1908: Travassor, 1913) in Rock Partridge (Alectoris Chukar) in Turkey.* Parasitol. Res. 2008. 102; 527-530.

Bush, A.O., 1990: *Helminth Communities in Avian Hosts: Determinants of Pattern in Parasite Communities: Patterns and Processes.* Edited by G.W. Esch, A.O. Bush & J.M. Aho London & New York. Chapman & Hall.

Gupta, N.K. and Acaharya, A.K. *Some Ascarids of Poultry from North India.* Research Bulletin of the Punjab University. 1971. 21; 379-391.

Hodasi, J.K.M. *The Helminth Parasites of the Helmet Guinea Fowl (Numida Meleagris Galeata Pallas) in Ghana.* Bulletin of Animal Health and Production in Africa. 1976. 24; 81-87.

Junker, K. and Boomker, A. *Check List of the Helminthes of Guinea Fowls (Numidae) and a Host of List of These Parasite.* Onderstepourt J. Vet. Res. 2007. 74; 315-337.

Katoch, R., Yadav, A., Godara, R., Khajuria, J.K., Borkataki, S. and Sodhi, S.S. *Prevalence and Impact of Gastrointestinal Helminths on Body Weight Gain in Backyard Chickens in Subtropical and Humid Zone of Jammu, India.* J. Parasit Dis. 2012. 36; 49-52.

Kulkarni, G.M., Narladkar, B.W. and Deshpande, P.D. *Helminthic Infections in Desi Fowl (Gallus Gallus Domesticus) in Marathwada Region.* J. Vet. Parasitol. 2001. 15; 137-139.

Mabon, J.L. and Reid, W.M. *Tissue Stages in the Development of Ascaridia Numidae from the Fowl (Numida Meleagris).* J. Parasitol. 1973. 59; 1012-1015.

Matta, S.C. and Ahluwalia, S.S. *Note on the Pathogenesis of Ascaridia Numidae Larva in the Gut of Guinea Fowls.* Indian J. Anim. Sci. 1979. 49; 72-74.

Matta, S.C. and Ahluwalia, S.S. *Note on the Survey of Gastrointestinal Helminths of Domestic Fowls in UP.* Indian J. Anim. Sci. 1981. 51; 1013-1015.

Robbins, K.M., Ye, W. and Fletcher, O.J. *Identification of Ascaridia Numidae in Guinea Fowl (Numida Meleagris) and Association with Elevated Mortality.* Avian Diseases. 2011. 55; 151-154.

Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R. and Swayne, D.E., 2003: *Eds. Disease of Poultry.* Ames, USA. Iowa State University Press.

Soulsby, E.J.L., 1986: *Helminths, Arthropods and Protozoa of domesticated Animals.* Seventh Edition.

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Research Article **Open Access**

Biosynthesis and Structural Characteristics of Selenium Nanoparticles using *Lactobacillus Acidophilus* Bacteria by Wet Sterilization Process

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Abstract A facile ecofriendly biological approach for the synthesis of selenium nanoparticles using the probiotic *Lactobacillus acidophilus* bacteria and its characterization is reported in this paper. The synthesized selenium nanoparticles were characterized using UV spectrometer, X-ray diffraction and transmission electron microscopy. The results of X-ray diffraction and transmission electron microscopic studies showed that the nanoparticles were in the uniform size range of 15-50 nm. The present study showed that no chemical changes occurred in selenium nanoparticles during the wet sterilization process and therefore, the wet sterilization method can effectively use to recover the elemental selenium from bacterial cells.

Keywords *Selenium Nanoparticles; Lactobacillus Acidophilus; X-Ray Diffraction; Transmission Electron Microscopy; Wet Sterilization*

1. Introduction

Bio nanotechnology is an emerging important technical tool for the development of reliable eco-friendly methodology for the synthesis of materials in nanoscale using biological sources. Nano sized particles possess unique properties due to their larger surface to volume ratio and higher surface energy. Metal nanoparticles have diverse broad ranging tremendous applications in the field of chemistry, electronics, diagnostics, biomedical and material science. Production of nanomaterials can be achieved through conventional physical and chemical methods. Physical methods employ cumbersome high cost equipment and techniques involving elevated temperatures and high pressures. While chemical approaches are the most popular methods for the production of nanoparticles some chemical synthesis protocols involve the use of toxic chemicals which are hazardous to both the environment and the biological system in which they are used.

Biological methods of nanoparticles synthesis using microorganisms, enzymes, and plant or plant extracts deserve merit and have been suggested as possible clean, nontoxic and eco-friendly alternatives to chemical and physical methods especially if they are intended for invasive applications in biological systems and medicine.

Among the biological methods of synthesis, the methods based on microorganisms have been widely reported (Dhillon et al., 2012; Kaler et al., 2011; Li et al., 2011; Sanghi and Verma, 2010). Microbial synthesis is readily scalable, environmentally benign and compatible with the use of the product for medical applications. Biosynthetic routes provide nanoparticles of a better defined size and morphology than some of the physicochemical methods of production (Raveendran et al., 2003). The application of bacteria strains for bio manufacturing of nanoparticles have advantage over other biological sources such as easy handling and short cultivation period. Zhang et al. (2011) reported a very simple, clean and ecofriendly biological method to synthesize monoclinic selenium nanoparticles with well-defined dimensions (200 nm) and disparity using *Pseudomonas alcaliphila* under 28°C with ambient pressure.

Dwivedi et al. (2013) synthesized selenium nanoparticles involving a biological reduction process by the selenium oxide tolerant bacteria *Pseudomonas aeruginosa* strain JS-11 grown in Luria-Bertani broth finally yielding predominantly monodispersed and spherical selenium nanoparticles of an average size of 21 nm.

In this paper, we report a facile, economical and green protocol to synthesize Se nanoparticles (SeNPs) using *Lactobacillus acidophilus* bacteria using wet sterilization process, which, holds promising alternative for the large-scale commercial synthesis of selenium nanoparticles.

Nanoselenium has attracted widespread attention for use in livestock supplementation. Due to its high bioavailability and low toxicity because nanometer particulates exhibit novel characteristics, such as great specific surface area, high surface activity, a lot of surface active centers, high catalytic efficiency and strong adsorbing ability and low toxicity of routine Se (Wang et al., 2007; Zhang et al., 2008). Since surface area-to-volume ratio increases with decreasing particle size, selenium nanoparticles have high biological activity (Zhang et al., 2005), including anti-hydroxyl radical property (Gao et al., 2002) and a protective action against the oxidation of DNA (Huang et al., 2003).

2. Experimental Methods

2.1. Preparation of Selenium Nanoparticles

A primary stock culture of *Lactobacillus acidophilus* (NCDC 15) bacteria purchased from National Dairy Research Institute, Karnal was revived and fresh subcultures were prepared (Eszenyi et al., 2011). The subcultures of *L. acidophilus* were used to prepare nanoselenium. Lactobacillus MRS broth (55.15 g) was dissolved in 1000 ml of double distilled water and boiled for 30 min at 120°C. After cooling down to 25°C, 20 mg of sodium selenite (Sigma, USA) dissolved in 20 ml of distilled water was added to the 980 ml of broth. Ten millilitre of fresh L. acidophilus bacterial culture was added to 1000 ml of MRS broth containing sodium selenite solution. The fermentation bottle was placed in shaking incubator for 48 h at 37°C. At the end of fermentation process, the culture medium turned red, indicating the production of nanoselenium. The medium was centrifuged at 6,000 rpm for 15 min and then the supernatant was discarded. The bacterial culture which formed a pellet at the bottom was taken in 50 ml of distilled water. The culture medium was autoclaved at 121°C for 20 min to disrupt the bacterial cell wall and release the red nanoselenium particles. The medium was centrifuged at 14,000 rpm for 15 min and washed thrice with distilled water. Then the sample was ultrasonicated for 15 min. Finally the nanoselenium containing solution was passed through vacuum filter, dried at 70°C and stored in sealed tubes for further characterization.

2.2. Sample Characterization

2.2.1. X-Ray Diffraction Analysis

Compositional analysis of the samples were studied based on the energy dispersive analysis of X-Rays using PANalytical X-Ray diffractometer (JEOL Model JED-2300).

2.2.2. Transmission Electron Microscopic Analysis

Samples for transmission electron microscopy (TEM) analysis were prepared by drop-coating selenium nanoparticles solution on to carbon-coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 min. The extra solution was removed using a blotting paper and the grid was dried prior to measurement. Transmission electron micrographs were obtained on JEM- 2100F (JEOL Inc., Japan) instrument with an accelerating voltage of 80 kV.

2.2.3. UV Spectroscopic Analysis

Absorption spectra of the synthesized nanoparticles were studied using a UV–VIS spectrophotometer (Systronics, Model 2202, India) at a wavelength range of 200-800 nm.

3. Results and Discussion

The X-ray diffraction pattern of nanoselenium is shown in Figure 1. The diffraction peaks at 2θ (degrees) of 23.28°, 25.01°, 27°, 29° and 32.22° were indexed as the (211, 202, 221, 230 and 311) planes of Se respectively. All the diffraction peaks in the 2θ range measured corresponded to the trigonal structure of Se with lattice constants a = 4.352Å and c = 4.929Å and were in good agreement with those on the standard data card. The sharpness of the diffraction peaks revealed that the product was well crystallized. The crystallite size of selenium was calculated using Scherrer's equation.

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where D is the grain size, K is the constant taken to be 0.94, λ is the wavelength of the X-ray radiation, β is the line broadening at half the maximum intensity, θ (Bragg angle) is the angle of diffraction. The calculated crystallite size of the nanoselenium was found to be in the range of 23 - 31nm.

The morphology of the prepared nanoparticles was investigated by TEM analysis which clearly showed that the particle sizes of spherical selenium prepared were in the range of 15-50 nm (Figure 2). The nanoparticles obtained in the present study were of relatively smaller size than that reported by Eszenyi et al. (2011) who synthesized nanoselenium using *Lactobacillus spp* and obtained nanoparticles with the sizes of 100-200 nm. The reduction in the size of the nanoselenium obtained could be due to the variation in the strain of bacteria used, which differed in their protein characteristics. The bacterial proteins play a major role in controlling the size and shape of nanoparticles (Dobias et al., 2011).

UV Spectroscopic Analysis

The nanoselenium particles prepared showed peak absorption values between 280-353 nm (UV range). This clearly indicated that all the nanoselenium particles had size below 100 nm, as recorded by Lin and Chris Wang (2005) who stated that the particle size could be correlated with the nature of

the UV-visible spectra and if the particle size below was 100 nm or less, it showed a clear absorption maximum in the UV range.

The present results concurred with the findings of Fesharaki et al. (2010) who reported the absorption band between 200-300 nm for the nanoselenium synthesized using *Klebsiella pneumonia*. Similar observations were reported by Zhang et al. (2011) and Harikrishnan et al. (2012) who synthesized nanoselenium using *Pseudomonas alcaliphila* and *Saccharomyces cerevisiae* respectively which exhibited absorption band between 200-300 nm.

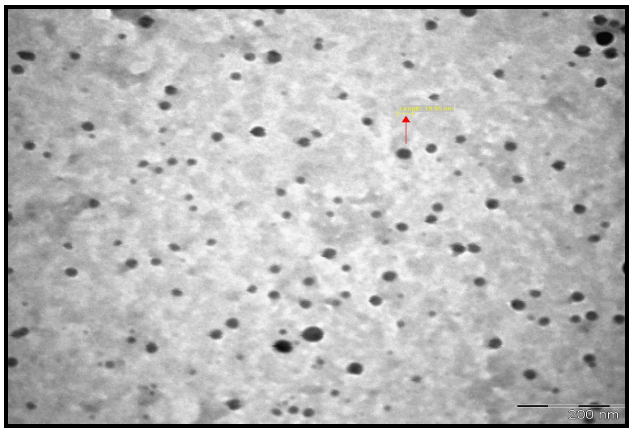

Figure 1: *Transmission Electron Microscope Image of Nanoselenium*

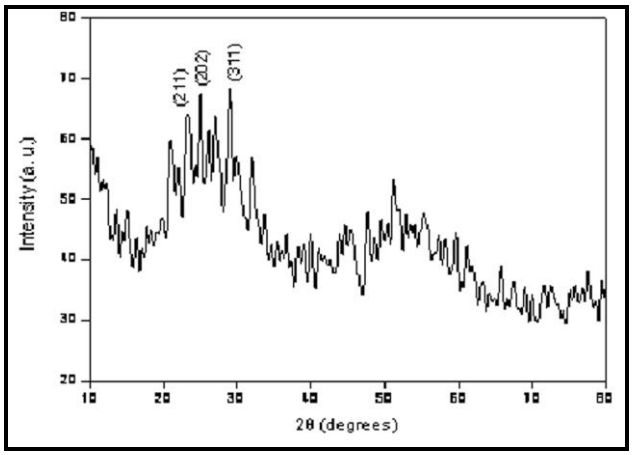

Figure 2: *X-Ray Diffraction Pattern on Nanoselenium*

4. Conclusion

Use of microorganisms for the production of nanomaterials is rapidly gaining significance owing to its growing successes, cost effective procedure and simplicity. There are several potential advantages around the microbe's ability to grow in aerobic conditions which include rapid ability to generate more number of bacterial cells within a short time period and less stringent culture conditions.

This green route of biosynthesis of selenium nanospheres is a simple, economically viable and an ecofriendly process resulting in nearly monodispersed highly stable selenium nanoparticles. In this process, the proteins secreted by the bacteria play an important role in the stabilizing and determining the size of nanoparticles.

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References

Dhillon, G.S., Brar, S.K., Kaur, S. and Verma, M. *Green Approach for Nanoparticle Biosynthesis by Fungi: Current Trends and Applications.* Critical Reviews in Biotechnology. 2012. 32; 49-73.

Dobias, J., Suvorova, E.I. and Bernier-Latman, R., 2011: *Role of Proteins in Controlling Selenium Nanoparticle Size.* Nanotechnology. 22.

Dwivedi, S., AlKhedhairy, A.A., Ahamed, M. and Musarrat, J., 2013: *Biomimetic Synthesis of Selenium Nanospheres by Bacterial Strain JS-11 and Its Role as a Biosensor for Nanotoxicity Assessment: A Novel Se-Bioassay.* PLOS ONE, 8.

Eszenyi, P., Sztrik, A., Babka, B. and Prokisch, J. *Elemental, Nano-Sized (100-500 Nm) Selenium Production by Probiotic Lactic Acid Bacteria.* International Journal of Bioscience, Biochemistry and Bioinformation. 2011. 2; 148-152.

Fesharaki, P.J., Nazari, P., Shakibaie, M., Rezaie, S., Banoee, M., Abdollahi, M. and Shahverdi, A.R. *Biosynthesis of Selenium Nanoparticles Using Klebsiella Pneumoniae and Their Recovery by a Simple Sterilization Process.* Brazilian Journal of microbiology. 2010. 41; 461-466.

Gao, X.Y., Zhang, J. and Zhang, L. *Hollow Sphere Selenium Nanoparticles: Their in Vitro Anti Hydroxyl Radical Effect.* Advanced Material. 2002. 14; 290-293.

Harikrishnan, H., Abdullah, N.A., Ponmurugan, K. and Shyam Kumar, R. *Microbial Synthesis of Selenium Nanocomposite using Saccharomyces Cerevisiae and its Antimicrobial Activity against Pathogens Causing Nosocomial Infection.* Chalcogenide letters. 2012. 9; 509-515.

Huang, B., Zhang, J., Hou, J. and Chen, C. *Free Radical Scavenging Efficiency of Nano-Se in Vitro.* Free Radical Biol. Med. 2003. 35 (7) 805-813.

Kaler, A., Nankar, R., Bhattacharyya, M.S. and Banerjee, U.C. *Extracellular Biosynthesis of Silver Nanoparticles using Aqueous Extract of Candida Viswanathii.* Journal of Bionanoscience. 2011. 5; 53-8.

Li, X., Xu, H., Chen, Z.S. and Chen, G. *Biosynthesis of Nanoparticles by Microorganisms and their Applications.* Journal of Nanomaterial. 2011. 6 (1) 121-124.

Lin, Z.H. and Chris Wang, C.R. *Evidence on the Size-Dependent Absorption Spectral Evolution of Selenium Nanoparticles.* Material Chemistry Physics. 2005. 92; 591-594.

Raveendran, P., Fu, J. and Wallen, S.L. *Completely "Green" Synthesis and Stabilization of Metal Nanoparticles.* Journal of American chemical society. 2003. 125; 13940-1.

Sanghi, R. and Verma, P. *Microbes as Green and Eco-Friendly Nanofactories.* Green Chemistry Environment Sustainable. 2010. 15; 315-39.

Wang, H., Zhang, J. and Yu, H. *Elemental Selenium at Nano Size Possesses Lower Toxicity Without Compromising the Fundamental Effect on Selenoenzymes: Comparison with Selenomethionine in Mice.* Free Radical Biology Medicine. 2007. 42; 524-533.

Zhang, J.S., Wang, H.L., Yan, X.X. and Zhang, L.D. *Comparison of Short Term Toxicity between Nano-Se and Selenite in Mice.* Life Science. 2005. 76; 1099-1109.

Zhang, J., Wang, X. and Xu, T. *Elemental Selenium at Nano Size (Nano-Se) as a Potential Chemopreventive Agent with Reduced Risk of Selenium Toxicity: Comparison with Se-Methylselenocysteine in Mice.* Toxicology Science. 2008. 101; 22-31.

Zhang, W., Chen, Z., Liu, H., Zhang, L., Gao, P. and Li, D. *Biosynthesis and Structural Characteristics of Selenium Nanoparticles by Pseudomonas Alcaliphila.* Colloids and Surface. B: Biointerfaces. 2011. 88; 196-201.

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Research Article **Open Access**

A Study on Prevalence of Bovine Paratuberculosis in Semen Station in India

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Abstract A study was conducted on bovine bulls in two bio-secure farms in India using Delayed Type of Hypersensitivity (DTH) to determine the prevalence of bovine paratuberculosis or Johne's disease (JD). The DTH was carried out on the neck of the animals, conducted as per OIE Terrestrial manual 2008. The results of DTH carried out for JD in two farms in western and northern India for a period of five years is being described. Prevalence of JD was found in Jun-10 (0.62%), Oct-10 (0.52%), Dec-10 (0.51%) and May-15 (1.09%) in farm at northern India whereas in western India farm, in Aug-10, May-11 and Mar-12, JD incidence rate was 0.34%, 1.86% and 0.54% respectively. It can be concluded from the present study that JD was present in both the herds with low incidence rate of 0-1.86 percent during the five years.
Keywords *JD; DTH; Semen Station; Bull*

1. Introduction

Bovine paratuberculosis or Johne's disease (JD) is a chronic debilitating disease caused by Mycobacterium avium *subsp.* paratuberculosis and causes heavy economic losses. High prevalence of JD (Raveendran et al., 2011) with low systematic monitoring of the same makes it more significant in India. The common source of infection may be faeco-oral route, contaminated feed, water, milk or colostrums for the calves (Sweeney, 1996). Spreading of the organism through vertical infection to the fetus (Larson *and* Kopecky, 1970) and infection in semen (Munster et al., 2012) has also been reported. Trangadia et al. (2012) found apparent prevalence of JD in Gujarat and Andhra Pradesh 13.39% (true prevalence, 15.68%) and 16.26% (true prevalence, 19.31%) respectively in serological diagnosis. Therefore, it is important that bulls in semen station should ideally be free from JD. Kalis et al. (2003) described the Delayed Type of Hypersensitivity (DTH) for JD as a specific and low-cost test for the early diagnosis of the same in dairy herds. Hence in the present study, DTH was used to investigate the comparative prevalence of JD in bovine bulls in two farms. Both the farms are involved in frozen semen production activities, one located in western India whereas other in northern India. The JD status of the herd was monitored in farms for 5 years i.e. 2010 - 2015. The status of the herds was monitored along with the practices of periodic testing and culling of reactors.

2. Materials and Methods

One hundred and sixty two bulls in semen station located in northern India were tested in Jun 2010. The farm introduced and also culled bulls from the main herd periodically, hence the population tested in this farm varied. In Jun-10, Oct-10, Dec-10, Jun-11, Feb-12, April-12, Aug-12, Feb-13, Aug-13, Feb-14, Dec-14 and May-15, the numbers of animals tested were 162, 194, 197, 204, 214, 218, 245, 257, 260, 286, 316 and 274 respectively.

The farm located in western India (semen station) had 298 bulls in August 2010. The farm introduced animals periodically into the main herd under various breeding programmes with regular culling practices, resulting in changes in the population of bulls. In Aug-10, Nov-10, May-11, Aug-11, March-12, May-12, Nov-12, May-13, Dec-13, Jul-14 and Jan-15, the numbers of animals tested were 298, 277, 323, 360, 372, 364, 378, 389, 421, 432 and 442 respectively.

Proper practices of isolation, sanitation, traffic control, personnel management and herd health management were adopted in the farms.

Screening of exotic Pure Bred (HF, Jersey), indigenous Pure Bred, crossbreds and Buffaloes against JD was carried out half yearly by DTH between 2010 and 2015. Bovine Johnin PPD was obtained from the Indian Veterinary Research Institute (IVRI), Izatnagar, UP. The test was conducted as per OIE Terrestrial manual 2008. In case of any positive reactors, testing of the whole herd was repeated after two months with the objective of keeping the incidence rate of JD less than 1%. All the positive reactors were culled immediately.

3. Results

During the period of 2010-15, a total of 15 animals were found positive for JD consisting of 7 pure exotic, 3 cross breeds and 5 Murrah buffaloes (Table 1 and 2).

In the farm located in northern India, 12 rounds of testing were conducted from 2010 to 2015. Overall prevalence of JD found was: Jun-10 (0.62%), Oct-10 (0.52%), Dec-10 (0.51%) and May-15 (1.09%) (Table 1). Breed wise incidence was found highest in pure exotic (4.23%) followed by crossbred (2.17%) and buffaloes (1.85%) (Table 1).

In the Farm located in western India, 11 rounds of testing were conducted from 2010 to 2015. Over all prevalence of JD was found relatively higher during testing in May-11 (1.86%) followed by Mar-12 (0.54%) and Aug-10 (0.34%) (Table 2). Breed wise, the incidence was found relatively higher in pure exotic (5.08%) followed by crossbreds (1.61%) and buffaloes (0.97%) (Table 2).

4. Discussion

An overall comparison of the positive animals from our data on DTH shows relatively higher incidence (Table 1 and 2) of JD in the pure exotic breeds followed by crossbreds and buffaloes in both the semen stations. Rawat et al. (2014) reported outbreak of paratuberculosis in a HF dairy farm in Rajasthan. Singh et al. (2004) found the prevalence of bovine paratuberculosis at 2.71% in organized dairy farms in Ludhiana using DTH test. Sharma et al. (2007) found overall prevalence of paratuberculosis 1.72% with 2.31 and 0.33% in cows and buffaloes, respectively in Punjab. In the present study, JD was present in both the herds with incidence rate ranging between 0-1.86 percent which is in accordance with the observations of the previous authors.

Table 1: *Breed Wise Distribution of JD in Northern India Farm*

Sl No	Month and year of testing	Breed wise												Total animal tested	Total positive	% positive
		Pure exotic			Indigenous			Cross Bred			Buffalo					
		Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive			
1	Jun-10	55	0	0.00	21	0	0.00	46	1	**2.17**	40	0	0.00	162	1	**0.62**
2	Oct-10	66	0	0.00	28	0	0.00	43	0	0.00	57	1	**1.75**	194	1	**0.52**
3	Dec-10	65	0	0.00	29	0	0.00	49	0	0.00	54	1	**1.85**	197	1	**0.51**
4	Jun-11	73	0	0.00	27	0	0.00	50	0	0.00	54	0	0.00	204	0	0.00
5	Feb-12	69	0	0.00	29	0	0.00	55	0	0.00	61	0	0.00	214	0	0.00
6	Apr-12	71	0	0.00	29	0	0.00	53	0	0.00	65	0	0.00	218	0	0.00
7	Aug-12	73	0	0.00	34	0	0.00	68	0	0.00	70	0	0.00	245	0	0.00
8	Feb-13	88	0	0.00	31	0	0.00	66	0	0.00	72	0	0.00	257	0	0.00
9	Aug-13	86	0	0.00	31	0	0.00	66	0	0.00	77	0	0.00	260	0	0.00
10	Feb-14	94	0	0.00	31	0	0.00	71	0	0.00	90	0	0.00	286	0	0.00
11	Dec-14	101	0	0.00	37	0	0.00	72	0	0.00	106	0	0.00	316	0	0.00
12	May-15	71	3	**4.23**	34	0	0.00	64	0	0.00	105	0	0.00	274	3	**1.09**

Table 2: *Breed Wise Distribution of JD in western India Farm*

Sl No	Month and year of testing	Breed wise												Total animal tested	Total positive	% positive
		Pure exotic			Indigenous			Cross Bred			Buffalo					
		Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive	Nos. tested	Nos. positive	% positive			
1	Aug-10	58	0	0.00	34	0	0.00	103	0	0.00	103	1	**0.97**	298	1	**0.34**
2	Nov-10	54	0	0.00	35	0	0.00	92	0	0.00	96	0	0.00	277	0	0.00
3	May-11	59	3	**5.08**	36	0	0.00	124	2	**1.61**	104	1	**0.96**	323	6	**1.86**
4	Aug-11	65	0	0.00	43	0	0.00	141	0	0.00	111	0	0.00	360	0	0.00
5	Mar-12	79	1	**1.27**	45	0	0.00	131	0	0.00	117	1	**0.85**	372	2	**0.54**
6	May-12	76	0	0.00	42	0	0.00	129	0	0.00	117	0	0.00	364	0	0.00
7	Nov-12	78	0	0.00	45	0	0.00	137	0	0.00	118	0	0.00	378	0	0.00
8	May-13	79	0	0.00	44	0	0.00	138	0	0.00	128	0	0.00	389	0	0.00
9	Dec-13	81	0	0.00	45	0	0.00	148	0	0.00	147	0	0.00	421	0	0.00
10	Jul-14	80	0	0.00	50	0	0.00	142	0	0.00	160	0	0.00	432	0	0.00
11	Jan-15	81	0	0.00	50	0	0.00	162	0	0.00	149	0	0.00	442	0	0.00

The influence on specificity by apparent presence of environmental mycobacterium may also be a factor resulting in false-positive skin test. The quality of sawdust used as animal bedding may perhaps have some influence on the proportion of false-positive skin tests. Fodstad et al. reported sawdust as a source of *M. avium*, causing false-positive tests in cattle and pigs. According to Vinodh et al., the diagnostic sensitivity and specificity of Johnin DTH over gamma interferon assay was 38.09% and 93.75% respectively. Also, the performance of DTH may also be significantly affected by different batches of antigen due to occurrence of minor antigenic differences (Kalis et al., 2003). So with a lesser sensitivity, eradication of JD from a herd may not be possible solely by DTH and culling of positives. However, the practice of performing DTH bi-annually in both the farms with regular culling practices helped lower the incidence of JD in the semen stations.

5. Conclusion

It can be concluded from the present study that JD was present in both the herds with incidence rate ranging between 0-1.86 percent during the five years under study. Strict bio-security, regular disease monitoring and herd management protocol at farm helps in lowering the incidence of bovine JD.

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Competing Interest

The authors declare that they have no competing interests.

References

Fodstad, F.H. *Tuberculin Reactions in Bulls and Boars Sensitized with Atypical Mycobacteria From Sawdust.* Acta Vet. Scand. 1977. 18; 374-383.

Kalis, C.H.J., Collins, M.T., Hesselink, J.W. and Barkema. H.W. *Specificity of Two Tests for the Early Diagnosis of Bovine Paratuberculosis Based On Cell-Mediated Immunity: The Johnin Skin Test and the Gamma Interferon Assay.* Veterinary Microbiology. 2003. 97; 73-86.

Larson, A.B. and Kopecky, K.E. *Mycobacterium Paratuberculosis in Reproductive Organs and Semen of Bulls.* Am. J. Vet. Res. 1970. 31; 255-258.

Munster, P., Volkel, I., Wemheuer, W., Schwarz, D., Doring, S. and Czerny, C.P., 2012: A Longitudinal Study to Characterize the Distribution Patterns of *Mycobacterium avium* ssp. *paratuberculosis* in Semen, Blood and Faeces of a Naturally Infected Bull by IS 900 Semi-Nested and Quantitative Real-Time PCR. Transboundary and Emerging Diseases. 2013. 60 (2) 175-87.

OIE, 2008: Paratuberculosis (Johne's disease). OIE *Terrestrial Manual.* Chapter 2.1.11; 276-291.

Rawat, K.D., Chaudhary, S., Kumar, N., Gupta, S., Chaubey, K.K., Singh, S.V., Dhama, K., and Deb, R. *Economic Losses in a Commercial Dairy Farm Due to the Outbreak of Johne's Disease in India*. Res. J. Vet. Pract. 2014. 2 (5) 73-77.

Remya Raveendran, Priya, P.M., John, K, Nair, G.K. and Vijayakumar, K. *Detection of Mycobacterium Avium Subsp. Paratuberculosis in Asymptomatic Bovines by IS900 Polymerase Chain Reaction.* Veterinary-World. 2011. 4 (6) 248-249.

Singh, B.B, Gumber, S., Randhawa, S.S, Aradhana, Dhand, N.K. *Prevalence of Bovine Tuberculosis and Paratuberculosis in Punjab.* Indian-Veterinary-Journal. 2004. 81 (11) 1195-1196.

Sweeney, R.W. *Transmission of Paratuberculosis.* Vet. Clin. N. Am. Food Anim. Pract. 1996. 12; 305-313.

Trangadia, B.J., Rana, S.K., Nagmani, K., Srinivasan, V.A. *Serological Investigation of Bovine Brucellosis, Johne's Disease and Infectious Bovine Rhinotracheitis in Two States of India.* Journal of Advanced Veterinary Research. 2012. 2 (1) 38-41.

Vinodh, Kumar O.P., Gunaseelan, L., Ronald, B.S.M., Rishikesavan, R., Senthil, N.R. and Gopi, H. *Comparative Evaluation of John in Intradermal Test and Gamma Interferon Assay in Bovine Paratuberculosis.* Indian J. Vet. Med. 2011. 31 (1) 30-31.

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In Vitro Studies on the Effect of Ethanol Extract of *Syzygium Aromaticum* on the Carbohydrate Metabolism of *Cotylophoron Cotylophorum*

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Abstract Paramphistomosis is one of the major pathogenic diseases in domestic animals and responsible for heavy economic loss in terms of reduced milk, meat and wool production. *Cotylophoron cotylophorum* is more prevalent in Tamilnadu. In the present investigation, the effect of *Syzygium aromaticum* ethanol extract on the enzymes of carbohydrate metabolism viz. pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase, malate dehydrogenase, fumarate reductase and succinate dehydrogenase of *Cotylophoron cotylophorum* was studied *in vitro*. The parasites were incubated in five different sub-lethal concentrations of ethanol extract of *Syzygium aromaticum* viz. 0.005, 0.01, 0.05, 0.1 and 0.5mg/ml for 2, 4 and 8 h. The activity of the enzymes was assayed using standard procedures. The enzyme activity was expressed in terms of protein. The data obtained were analyzed statistically. Ethanol extract of *Syzygium aromaticum* significantly inhibited the enzymes of carbohydrate metabolism and the percentage of inhibition was dose and time dependent. Inhibition of these enzymes leads to decreased ATP production which may be fatal to the parasites. The present study validates the anthelmintic property of ethanol extract of *Syzygium aromaticum* against *C. cotylophorum.*

Keywords *Syzygium Aromaticum; Cotylophoron Cotylophorum; Pyruvate Kinase; Phosphoenolpyruvate Carboxykinase; Lactate Dehydrogenase; Malate Dehydrogenase; Fumarate Reductase; Succinate Dehydrogenase*

1. Introduction

The most important and reliable source of animal proteins in India is meat from goat and sheep. Sheep production has remained an integral part of cultural life and farming system of the rural population. Helminthiasis affects the production potential through mortality, weight loss, reduced milk yield and wool production [1]. Paramphistomosis is a major disease caused by amphistomes. The paramphistome *Cotylophoron cotylophorum* lives in the rumen and reticulum of sheep, goats, cattle and other domestic ruminants [2, 3].

Vast array of synthetic anthelmintics are used to combat paramphistomosis. However, problems have emerged with the use of synthetic anthelmintics, notably the development of resistance in helminths to various anthelmintic compounds and classes, as well as chemical residues and toxicity problems [4, 5]. In addition, recognition of the antigenic complexity of parasites has slowed vaccine development. These disadvantages have stimulated a search for alteration control methods such as the use of traditional medicinal plants. Plants are known to provide a rich source of potent botanical anthelmintics. The use of medicinal plants for the prevention and treatment of gastro intestinal parasitism has its origin in ethno veterinary medicine [6, 7].

Syzygium aromaticum commonly called clove belongs to the family myrtaceae. Clove bud oil has biological activities, such as antibacterial, antifungal, antiviral, antimicrobial, anticancer, antiseptic, anesthetic, insecticidal, analgesic, antispasmodic, anticarminative and antioxidant properties [8]. Clove oil is also active against plant-parasitic nematodes [9]. The major constituents in bud oil are eugenol and *β*-carophyllene, vanillin, crategolic acid, gallotannic acid, methyl salicylate (pain-killer) eugenin, kaempferol, rhamnetin, eugenitin, oleanolic acid, stigmasterol, campesterol and several sesquiterpenes [10, 11] Kumar and Singh, Manoj Dhanraj and Veerakumari reported the anthelmintic activity of *Syzygium aromaticum* against *Fasciola gigantica* and *Cotylophoron cotylophorum* [12, 13].

Carbohydrate is an essential energy source in all adult parasitic helminths and its metabolism is often predominantly anaerobic, even in the presence of oxygen. They depend on carbohydrate either in the form of glycogen or glucose. The inhibition of energy metabolism is the most important mode of anthelmintic action of various groups of drugs since the parasitic trematodes depend on carbohydrates for their energy metabolism and glucose is the only direct source of energy [14, 15]. Glucose is absorbed from the host via the glucose transporters located in the tegument and intestinal epithelium of trematodes [16, 17]. The main storage of carbohydrate in parasitic helminths is glycogen [18].

Carbohydrate metabolism of the helminth parasite resembles Embden-Meyerhof glycolytic pathway of their host animals, until the formation of phosphoenol pyruvate (PEP). PEP obtained from glycolysis can either be carboxylated to oxaloacetate (OAA) by phosphoenolpyruvate carboxykinase (PEPCK), or dephosphorylated to pyruvate by pyruvate kinase (PK). Pyruvate so formed is further reduced to lactate by lactate dehydrogenase (LDH) and OAA is reduced to malate by malate dehydrogenase (MDH). Malate permeates into the mitochondrion where it undergoes dismutation in which one-half of malate is oxidized to pyruvate by malic enzyme (ME) and the other half is dehydrated to fumarate by fumarase (FM), which is further reduced to succinate by fumarate reductase (FR). Succinate oxidized to fumarate by succinate dehydrogenase (SDH). Decarboxylation of pyruvate and succinate results in the final end products of acetate and propionate respectively [18, 19, 20]. Keeping this in view, an attempt has been made to the assess the anthelmintic efficacy of ethanol extract of *Syzygium aromaticum* against *Cotylophoron cotylophorum* based on its effect on the enzyme involved in carbohydrate metabolism.

2. Materials and Methods

2.1. *In Vitro* Maintenance of *Cotylophoron Cotylophorum*

Cotylophoron cotylophorum were collected from the rumen of infected sheep, slaughtered at Perambur abbatoir, Chennai. Adult live worms were collected, washed thoroughly in physiological saline and maintained in Hedon-Fleig solution, which is the best medium for *in vitro* maintenance [21]. It is prepared by dissolving 7gm of sodium chloride, 0.3gm of potassium chloride, 0.1gm of calcium chloride, 1.5gm of sodium bicarbonate, 0.5gm of disodium hydrogen phosphate, 0.3gm of magnesium sulphate and 1gm of glucose in 1000ml of distilled water.

2.2. Preparation of Plant Extracts

The buds of *Syzygium aromaticum* were collected from a local shop at Chennai, and were authenticated in the Department of Botany, Pachaiyappa's college; Chennai and vouchered specimens are deposited in the herbarium of Pachaiyappa's College, Chennai-30. The extraction of plant materials was done following the method of Harborne [22].

2.3. Sample Preparation

Adult *C. cotylophorum* were incubated in various concentration of *Syzygium aromaticum* ethanol extract (*Sa*EE) (0.005, 0.01, 0.05, 0.1 and 0.5mg/ml) for 2, 4 and 8h. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extract. After incubation, the parasites were rinsed in distilled water. The parasites were weighed wet and a 10% (W/V) homogenate was prepared by homogenising the flukes in ice-cold 0.25 M sucrose solution containing 0.15 M Tris-HCl (pH-7.5). This homogenate was centrifuged at 1000 rpm for 10 min. The supernatant was used as the enzyme source. The cytosolic and mitochondrial fractions of *C. cotylophorum* were prepared following the method of Fry et al. [23].

2.4. Enzyme Assay

2.4.1. Pyruvate Kinase (PK)

Pyruvate kinase (PK, EC 2.7.1.4) activity in the cytosolic fraction was assayed following the method of McManus and Smyth [24]. The reaction mixture contained 1 ml of 300 mM Tris-HCl buffer (pH 7.8) [25], 0.5 ml of 42 mM magnesium sulphate (MgSO4), 0.5 ml of 450 mM potassium chloride (KCl), 0.3 ml of 50 mM adenosine diphosphate (ADP), 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM nicotinamide adenine dinucleotide reduced (NADH), 0.025 ml of 48 mM fructose biphosphate (FBP), 0.025 ml of 15 units of LDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised / min / mg protein.

2.4.2. Phosphoenolpyruvate Carboxykinase (PEPCK)

The activity of phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) was assayed according to the method of McManus and Smyth [24]. PEPCK catalyses the formation of oxaloacetate (OAA) from PEP. The assay mixture contained 1ml of 300 mM imidazole buffer (pH 6.2) [25], 0.4 ml of 300 mM MgSO4, 0.3 ml of 400 mM KCl, 0.3 ml of 70 mM sodium bicarbonate (NaHCO3), 0.3 ml of 20 mM ADP, 0.3 ml of 40 mM PEP, 0.3 ml of 2 mM NADH, 0.05 ml of 15 units of MDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance was read at 340 nm for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised / min / mg protein.

2.4.3. Lactate Dehydrogenase (LDH)

The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed according to the method of Yoshida and Freese [26]. LDH catalyses the oxidation of lactate and reduction of pyruvate. For oxidation of lithium lactate, 0.8 ml of 60 mM phosphate buffer (pH 7.5) [27], 0.1 ml of 0.5 M lithium lactate, 0.05 ml enzyme sample and 0.05 ml of 20 mM NAD were placed in 1 ml cuvette. The increase of absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. For the reduction of pyruvate, 0.05 ml of enzyme sample was added to 0.8 ml of 60 mM phosphate buffer (pH 6.5) [27],

0.01 ml of 1 mM NADH, 0.01 ml of 10 mM sodium pyruvate and final volume was adjusted to 1 ml by the addition of distilled water in 1 ml cuvette. The decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.4. Malate Dehydrogenase (MDH)

Malate dehydrogenase (MDH, EC 1.1.1.37) catalyses the oxidation of malate and reduction of OAA. The activity of this enzyme catalysing the malate oxidation and OAA reduction was assayed in both cytosolic and mitochondrial fractions following the procedure of Yoshida [28]. For the oxidation of malate, the reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.4 for cMDH and pH 7.2 for mMDH) [21] 0.1 ml of 100 mM sodium malate, 0.1 ml of 10 mM NAD, 1.7 ml of distilled water and 0.1 ml of enzyme sample. For MDH catalysing the reduction of OAA, the reaction mixture contained 2.5 ml of 100 mM Tris-HCl (pH 7.4 for both cMDH and mMDH) [21], 0.05 ml of 100 mM oxaloacetate, 0.05 ml of 10 mM NADH, 0.3 ml of distilled water and 0.1 ml of the enzyme sample. The activity of the enzyme catalysing oxidation and reduction reaction was measured at 340 nm for 3 min at an interval of 15 sec each. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.5. Fumarate Reductase (FR)

Fumarate reductase (FR, EC 1.3.1.6) catalyses the reduction of fumarate to succinate. The enzyme was assayed as detailed by Sanadi and Fluharty [29].The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.6) [21], 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.3 ml of 1 mM ethylene diamine tetra acetic acid (EDTA), 0.3 ml of 50 mM fumarate, 0.7 ml of distilled water, 0.1 ml of enzyme sample and 0.3 ml of 1.6 mM NADH in a 3 ml cuvette. After the addition of NADH, decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec. The enzyme activity was calculated by using the millimolar coefficient of 6.22 and expressed in n moles of NADH oxidised / min / mg protein.

2.4.6. Succinate Dehydrogenase (SDH)

The activity of succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed according to the method of Singer [30]. The reaction mixture included 0.5 ml of 300 mM phosphate buffer (pH 7.5) [21], 0.3 ml of 0.1 M succinate, 0.1 ml of enzyme, 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.1 ml of 0.75 mM calcium chloride and 1.3 ml of water. The enzyme was incubated for 5 - 7 min to permit full activation. After incubation, 0.1 ml DCPIP (0.05 %) (W/V) and 0.3 ml of PMS (0.33 %) were added to initiate the reaction and decrease in absorbance was recorded at 600 nm. The enzyme activity was calculated using millimolar extinction coefficient of 19.1 and expressed in n moles of dye reduced / min / mg protein.

Protein in the sample was determined by the method of Lowry *et al.* [31].

2.5. Statistical Analysis

All the data obtained in the present study were statistically analysed using the statistical software SPSS version 16.0. One-way Anova using Bonferroni test was applied to find out the significant difference between the different concentrations of plant extracts and periods of incubation.

3. Results and Discussion

Helminth parasites derive energy for their survival mainly through the degradation of carbohydrate. Several scientists [25, 32, 33, 34, 35] have studied the influence of anthelmintics on the carbohydrate metabolism of helminth parasites. A good understanding on different carbohydrate metabolic reactions forms solid basis for choosing appropriate targets for new chemotherapeutic agents. Investigations on the effect of ethanolic extracts of *S. aromaticum* on the cytosolic and mitochondrial fraction of *C. cotylophorum* revealed a significant inhibition of the key regulatory enzymes involved in carbohydrate metabolic pathway.

PK and PEPCK activity was found to be inhibited in *Sa*EE treated flukes (Table 1a). Inhibition of both PEPCK and PK activities arrests the PEP-succinate/PEP-lactate pathways. Consequently, the energy yielding process is impaired and deprives the parasite of its ATP production. Decreased generation of ATP proves fatal to the parasites [36].The inhibition of PK and PEPCK activities treated with anthelmintics has been observed in other helminths [37, 38, 39]. Also, Navaneetha Lakshmi and Veerakumari [40] reported the inhibitory effect on the PK and PEPCK activities in *Haemonchus contortus* treated with *Allium sativum*.

The action of PK on PEP results in the production of pyruvate. Pyruvate so formed comes under the influence of LDH, which catalyses, the reduction of pyruvate to lactate and the oxidation of lactate to pyruvate. It is evident from the present investigation that *Sa*EE inhibited the LDH catalysing both the lactate oxidation and pyruvate reduction (Table 1b). It is interesting to note that LDH exhibits a peculiar type of chemotherapeutic response. Inhibition of LDH activity catalyzing pyruvate reduction was found to be higher compared to LDH inhibition catalysing the oxidation of lactate. Similar findings were also reported by various workers [41, 42, 43]. Inhibitory effect of albendazole on LDH activity of *Fasciola hepatica* reported by Ozcelik [44]. Veerakumari and Munuswamy elucidated the inhibitory effect of PZQ and LEV on LDH activity of *C. cotylophorum* [27]. Similar inhibitory effect of *A. sativum* on the LDH activity catalysing both the oxidation and reduction reactions in *H. contortus* has been reported by Veerakumari and Lakshmi [45]. The inhibition of lactate dehydrogenase might arrest the carbon influx in the glycolytic pathway and the generation of the necessary energy through oxidative phosphorylation [36]. Consequently, production of malate, which serves as main substrate for mitochondrial phosphorylation is reduced, which leads to reduced production of ATP [39].

Malate dehydrogenase (MDH) has been a rate–limiting enzyme in the phosphoenolpyruvate metabolism. *Sa*EE significantly inhibited the cytoplasmic MDH (cMDH) and mitochondrial MDH (mMDH) catalysing both the oxidation and reduction reactions in *C. cotylophorum* (Table 1c & 1d). Inhibition of MDH activity of *Ascaris suum*, *F. hepatica* and *Moniezia expansa* by mebendazole, albendazole and parbendazole was reported by Tejada et al. [46]. Also, Oztop et al. [47] reported the alteration of MDH and LDH activities of *Trichuris saginata* by albendazole and niclosamide. Similar inhibitory effect of *Acacia concinna* on the cMDH and mMDH activity of *C. cotylophorum* was reported by Priya and Veerakumari [25]. Anthelmintics may disturb the transmembrane proton gradient severely, leading to drop in cellular ATP levels [48]. Reduction in the MDH activity of the flukes exposed to *Sa*EE suggest that, plants act transtegumentally to target vital tegumental enzymes and interfere with the energy generating pathways depriving the parasite in acquiring ATP, thereby leading to paralysis and death [49]. The inhibition of both cMDH and mMDH observed in the present study suggests the declined production of oxaloacetate (OAA) and malate. The inhibition of MDH might subsequently result in the inhibition of FR, as OAA is essential for production of fumarate [50, 51].

Fumarate is reduced to succinate using NADH as reducing equivalent and succinate formation is the final step of the glycolytic pathway [52]. In the present study, *Sa*EE inhibited the FR and SDH activity of *C. cotylophorum* (Table 1e). Priya and Veeerakumari [25] reported similar inhibition of FR in *A.*

concinna treated *C. cotylophorum*. The FR activity of *H. contortus* was also inhibited by other drugs such as tetramisole, thiabendazole, cambendazole, mebendazole, morantel tartrate and disophenol [53, 54, 55]. Barrowman *et al.* [56] demonstrated the inhibitory effect of benzimidazole and albendazole sulphoxide on the FR activity of *Ascaris suum*. Antiparasitic drugs, inhibit fumarate binding to FR, slowdown the synthesis of body constituents, curtail the energy production in the parasites [57], uncouple oxidative phosphorylation, hamper ATP production [58] and present an excellent biochemical target in the treatment of helminthic infections [59]. The SDH activity of *Heterakis, Trichuris, Ascardia, Chabertia, Bunostomum* and *Nematodirus* was inhibited by tetramisole has been reported by Van den Bossche and Janssen [60]. SDH has the ability to transfer electrons to the respiratory chain by catalysing the formation of fumarate and succinate [61]. SDH inhibition by anthelmintics could prevent the utilization of the chemical energy derived from electron transport for the net phosphorylation of ADP to ATP and deprive the parasite of its normal source of energy [62]. In addition, anthelmintics, affect tubulins bound in mitochondrial membrane of the parasites by influencing SDH–FR complex negatively inhibit succinate metabolism and diminish ATP–synthesis [63, 64]. Hence, SDH could potentially be an important target for anthelmintics against the gastrointestinal parasites of livestock [65].

The inhibition of enzymes of carbohydrate metabolism of *C. cotylophorum* by *Acacia concinna* was also reported by Priya and Veerakumari [25]. Impairment of carbohydrate metabolism in parasitic helminths may be disastrous since they depend almost entirely on it for their energy supply [66]. Present study manifested that PK, PEPCK, LDH, MDH, FR and SDH provide biochemical target for *Sa*EE which disrupt energy generation process in *C. cotylophorum*, resulting in decreased production of ATP. Consequently, the energy deprived parasite unable to sustain themselves *in situ* may be expelled from the host. The results of the present study holds a potential promise in the future use of active principles of *S. aromaticum* as effective anthelmintics and may help in designing assimilated solutions for the control of paramphistomosis.

4. Conclusion

The present study elucidated the anthelmintic effect of *Sa*EE on *C. cotylophorum. Sa*EE blocked the energy metabolism of the parasites by inhibiting the enzymes PK, PEPCK, LDH, MDH, FR and SDH. *Sa*EE possesses a remarkable anthelmintic activity against *C. cotylophorum*. It may serve as an alternative for anthelmintic chemotherapeutic agents to avoid their toxic side effects and development of resistance in a safe and ecofriendly manner. In depth field trials of plant based anthelmintics along with best farm management practices can play a great role in parasite control strategies and in enhancing productivity of livestock farming.

Table 1: *In vitro effect of SaEE on the enzymes involved in Carbohydrate metabolism of C. cotylophorum*

Table 1a PK and PEPCK						
Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	PK			**PEPCK**		
	2h	**4h**	**8h**	**2h**	**4h**	**8h**
0.005	9.95±0.03	13.72±0.04	30.72±0.03	9.08±0.10	26.57±0.08	47.97±0.06
0.01	11.75±0.01	16.84±0.03	48.33±0.05	14.47±0.08	38.51±0.04	56.11±0.01
0.05	17.26±0.01	23.04±0.05	59.89±0.01	21.98±0.08	45.44±0.09	62.37±0.13
0.1	20.55±0.06	32.33±0.12	65.37±0.17	30.01±0.07	52.38±0.12	66.80±0.18
0.5	24.18±0.07	46.12±0.13	76.92±0.19	44.16±0.01	63.70±0.06	90.67±0.11
Table 1b LDH						
Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			**Reduction**		
	2h	**4h**	**8h**	**2h**	**4h**	**8h**
0.005	9.70±0.05	23.06±0.09	59.21±0.01	9.08±0.10	26.57±0.08	47.97±0.06

0.01	21.12±0.01	48.85±0.06	61.03±0.06	14.47±0.08	38.51±0.04	56.11±0.01
0.05	24.63±0.02	53.26±0.04	72.62±0.07	21.98±0.08	45.44±0.09	62.37±0.13
0.1	28.76±0.04	56.08±0.06	76.51±0.09	30.01±0.07	52.38±0.12	66.80±0.18
0.5	46.84±0.02	66.81±0.03	80.28±0.13	44.16±0.01	63.70±0.06	90.67±0.11

Table 1c Cmdh

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			Reduction		
	2h	4h	8h	2h	4h	8h
0.005	19.59±0.07	45.40±0.24	61.12±0.08	7.50±0.17	40.49±0.22	61.09±0.18
0.01	31.44±0.11	49.27±0.01	74.44±0.01	12.58±0.28	43.73±0.14	75.94±0.40
0.05	40.00±0.03	55.26±0.10	83.71±0.04	23.68±0.13	56.23±0.24	79.69±0.17
0.1	46.23±0.13	64.21±0.13	87.72±0.14	33.62±0.22	63.85±1.21	88.46±0.23
0.5	53.22±0.14	72.18±0.16	98.77±0.18	59.03±0.35	75.06±0.70	92.91±0.39

Table 1d mMDH

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			Reduction		
	2h	4h	8h	2h	4h	8h
0.005	5.54 ± 0.03	32.25±0.06	62.96±0.17	15.40±0.14	35.12±0.01	54.00±0.07
0.01	15.25±0.04	40.45±0.06	69.46±0.24	19.22±0.07	44.05±0.06	58.51±0.05
0.05	22.96±0.03	43.05±0.14	78.34±0.19	23.48±0.04	51.87±0.05	65.73±0.06
0.1	35.79±0.01	54.49±0.03	81.77±0.16	27.38±0.03	55.55±0.03	72.68±0.07
0.5	53.40±0.06	64.67±0.12	93.41±0.04	42.66±0.11	60.22±0.13	84.29±0.18

Table 1e FR and SDH

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	FR			SDH		
	2h	4h	8h	2h	4h	8h
0.005	28.60±0.18	41.10±0.18	63.28±0.02	12.79±0.05	37.33±0.06	58.02±0.04
0.01	31.65±0.27	47.25±0.13	71.93±0.01	22.43±0.03	49.82±0.08	69.44±0.01
0.05	37.29±0.01	56.14±0.04	79.10±0.03	35.42±0.02	55.20±0.11	73.52±0.12
0.1	45.78±0.01	68.13±0.16	83.24±0.01	38.00±0.15	63.61±0.09	76.08±0.03
0.5	62.27±0.14	70.28±0.11	87.39±0.21	48.41±0.08	67.34±0.14	81.46±0.05

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (Bonferroni test; $P < 0.05$)

** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (Bonferroni test; $P < 0.01$)

References

[1] Sackett, D., Holmes, P., Abbott, K., Jephcott, S. and Barber, M. 2006: *Assessing the Economic Cost of Endemic Disease on the Profitability of Australian Beef Cattle and Sheep Producers.* Miscellaneous publication of Meat and Livestock Australia Limited. 087.

[2] Soliman, M.F. and Zalat, S.M. *Prevalence and Intensity of Nematodirus Sp. And Eimeria Sp. Infections in the Domestic Goats of St. Katherine's Protectorate (Sinai, Egypt): Relations with Some Ecological and Biological Factors.* Egyptian Journal of Biotechnology. 2003. 5; 78-85.

[3] Tsotetsi, A.M. and Mbati, P.A. *Parasitic Helminthes of Veterinary Importance in Cattle, Sheep and Goats on Communal Farms in the Northeastern Free State, South Africa.* Journal of South African Veterinary Association. 2003. 74 (2) 45-48.

[4] Waller, P.J. and Prichard, R.K., 1985: *Drug Resistance in Nematodes.* In: Campbell, W.C., Rew, R.S. (Eds.), Chemotherapy of Parasitic Infections, Phenum, New York, USA. 339-362.

[5] Kaemmerer, K. and Butenkotter, S. *The Problem of Residues in Meat of Edible Domestic Animals after Application or Intake of Organophosphate Esters.* 1973. 46; 1.

[6] Satyavati, G.V., Raina M.K. and Sharma, M., 1976: *Medicinal Plants of India.* Vol. I. Indian Council of Medical Research, New Delhi. 201-206.

[7] Lewis, W.H. and Elvin-Lewis, M.P.H. 1977: *Medicinal Botany Plants Affecting Man's Health.* Wiley – Interscience. New York.

[8] Huang, Y., Ho, S.H., Lee, H.C. and Yap, Y.L. *Insecticidal Properties of Eugenol, Isoeugenol and Methyleugenol and Their Effects on Nutrition of Sitophilus zeamais Mostch. (Coleoptera: Curculionidae) and Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae).* Journal Stores Products Research. 2002. 38; 403-412.

[9] Pandey, R.C. and Dwivedi, B.K. *Comparative Study of Different Plant Extracts for their Nematicidal Potential.* Current Nematology. 2000. 11; 39-43.

[10] Srivastava, A.K., Srivastava, S.K. and Syamasundar, K.V. *Bud and Leaf Essential Oil Composition of Syzygium Aromaticum from India and Madagascar*. Flavour and Fragrance Journal. 2003. 20 (1) 51-53.

[11] Debjit bhowmik, Sampath Kumar, K.P., Akhilesh Yadav, S., Srivastava, Shravan Paswan and Amit sankar Dutta. *Recent Trends in Indian Traditional Herbs Syzygium aromaticum and its Health Benefits.* Journal of Pharmacognosy and Phytochemistry. 2012. 1 (1).

[12] Kumar and Singh. *Activity of Allium Sativum, Ferula Asafoetida and Syzygium Aromaticum against Fasciola Gigantica.* Journal of Biology and Earth Sciences. 2014. 4 (1) B57-B65.

[13] Manoj Dhanraj and Veerakumari. *In Vitro Effect of Syzygium Aromaticum on the Motility and Acetylcholinesterase of Cotylophoron Cotylophorum.* Indian Journal of Veterinary and Animal Sciences. 2014. 43 (3) 187-194.

[14] Martin, R.J. Modes of Action of Anthelmintic Drugs. Veterinary Journal. 1997. 154; 11-34.

[15] Tielens, A.G.M. *Energy Generation in Parasitic Helminths.* Parasitology. 1994. 10; 346-352.

[16] Skelly, P.J., Kim, J.W., Cunningham, J. and Shoemaker, C.B. *Cloning Characterization and Functional Expression of CDNA Encoding Glucose Transporter Proteins from the Human Parasite, Schistosoma Mansoni. The Journal of Biological Chemistry.* 1994. 269; 42-47.

[17] Thompson, D.P. and Geary, T.G. 1994: *The Structure and Function of Helminthes Surface. In: Biochemistry and Molecular Biology of Parasitres.* Eds. Marr, J.J. and Muller, M. Academic Press, London. 203.

[18] Barrett, J., 1981: *Biochemistry of Parasitic Helminths.* Macmillan Press, London. 308.

[19] Kaur, R. and Sood, M.L. *The Effects of DL-Tetramisole and Rafoxanide on Tricarboxylic Acid Cycle Enzymes of Haemonchus Contortus, In Vitro.* Veterinary Parasitology. 1983. 13; 333-340.

[20] Tielens, A.G.M. *Energy Generation in Parasitic Helminths.* Parasitology. 1994. 10; 346-352.

[21] Veerakumari, L., 1996: *In Vitro Studies on the Effect of Some Anthelmintics on Cotylophoron cotylophorum (Fischoeder, 1901) (Digenea: Paramphistomidae). A Structural and Biochemical Analysis.* Ph.D. thesis submitted to the University of Madras, Chennai.

[22] Harborne, J.B., 1998: *Phytochemical Methods a Guide to Modern Techniques of Plant Analysis*. 3rd Edition. Chapman & Hall, London. 302.

[23] Fry, M., Bazil, C. and Jenkins, D.C. *A Comparison of Mitochondrial Electron Transport in the Intestinal Parasitic Nematodes Nippostrongylus Brasiliensis and Ascardia Galli.* Comparative Biochemistry and Physiology. 1983. 75B; 451-453.

[24] McManus, D.P. and Smyth, J.D. *Intermediary Carbohydrate Metabolism in Protoscoleces of Echinococcus Granulosus (Horse and Sheep Strains) and E. Multiocularis.* Parasitology. 1982. 84; 351-366.

[25] Priya, P. and Veerakumari, L. *In Vitro Effect of Acacia Concinna on the Key Enzymes of Carbohydrate Metabolism of Cotylophoron cotylophorum* (Fischoeder, 1901). Biomedicine. 2011. 31 (3) 329-333.

[26] Yoshida, A. and Freese, E. *Lactate Dehydrogenase from Bacillus Subtilis.* In: Methods in Enzymology. Wood, W.A. (ed.), Vol. XLI. Academic Press, New York. 1975. 304.

[27] Veerakumari, L. and Munuswamy, N. *In Vitro Effect of Some Anthelmintics on Lactate Dehydrogenase Activity of Cotylophoron Cotylophorum* (Digenea: Paramphistomidae). Veterinary Parasitology. 2000. 91; 129-140.

[28] Yoshida, A. *L-Malate Dehydrogenase from Bacillus Subtilis.* In: Methods in Enzymology. Lownstein J.M. (Ed.), Vol. XIII, Academic Press, New York. 1969. 141-145.

[29] Sanadi, D.R. and Fluharty, A.L. *On The Mechanism of Oxidative Phosphorylation. VII- The Energy Requiring Reduction of Pyridine Nucleotide by Succinate and Energy Yielding Oxidation of Reduced Pyridine Nucleotide by Fumarate.* Biochemistry. 1963. 2; 523-528.

[30] Singer, T.P., 1974: *Determination of Activity of Succinate, NADH, Choline and?-Glycerophosphate Dehydrogenase.* In: Methods in Biochemical Analysis. Glick, D. (ed.), Vol. 22, Interscience Publications. John Wiley and Son, New York. 133-161.

[31] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. *Protein Measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry*. 1951. 193; 265-275.

[32] Bueding, E. and Fisher, J. *Biochemical Effects of Niridazole on Schistosoma Mansoni.* Molecular Pharmacology. 1970. 6 (5) 532-539.

[33] Schulman, M.D., Ostlind, D.A. and Valentino, D. *Mechanism of Action of MK-401 against Fasciola Hepatica: Inhibition of Phosphoglycerate Kinase.* Molecular Biochemistry and Parasitology. 1982. 5 (3) 133-45.

[34] Donahue, M.J., Masaracchia, R.A. and Harris, B.G. *The Role of Cyclic AMP–Mediated Regulation of Glycogen Metabolism in Levamisole– Perfused Ascaris Suum Muscle.* Molecular Pharmacology 1983. 23; 378-383.

[35] Veerakumari, L., Lalhmingchhuanmawii, K. and Ashwini, R. *Effect of Punica Granatum Ethanol Extract on the Carbohydrate Metabolism of Cotylophoron Cotylophorum.* International Journal of Biological Sciences. 2014.

[36] Jasra, N., Sanyal, S.N. and Khera, S. *Effect of Thiabendazole and Fenbendazole on Glucose Uptake and Carbohydrate Metabolism in Trichuris Globulosa*. Veterinary Parasitology. 1990. 35; 201-209.

[37] Rahman, M.S. and Bryant, C. *Studies of Regulatory Metabolism in Moniezia Expansa: Effects of Cambendazole and Mebendazole*. International Journal of Parasitology. 1977. 7; 403-409.

[38] Lloyd, G.M. and Barrett, J. *Fasciola Hepatica: Inhibition of Phosphoenolpyruvate Carboxykinase, and End-Product Formation by Quinolinic Acid and 3-Mercaptopicolinic Acid.* Experimental Parasitology. 1983. 56; 259-265.

[39] Srivastava, J.K., Gupta, S. and Katiyar, J.C. *Effects of Methyl [5[[4-(2-Pyridinyl)-1-Piperazinyl [Carbonyl]-1H-Benzimidazol-2-Yl] Carbamate on Energy Metabolism of Ancylostoma Ceylanicum and Nippostrongylus Brasiliensis.* Indian Journal of Experimental Biology. 1989. 27; 735-738.

[40] Navaneetha, L.K. and Veerakumari, L. *Effect of Allium Sativum on the Phosphoenolpyruvate Carboxykinase and Pyruvate Kinase Activity of Haemonchus Contortus in Vitro.* Pharmacognosy Magazine. 2009. 5 (20) 430-432.

[41] Kumar, M., Pathak, K.M.L. and Pachauri, S.P. *Clinicopathological Studies on Naturally Occurring Bovine Fascioliasis in India.* British Veterinary Journal. 1982. 138; 241-246.

[42] Swarup, D., Pachauri, S.P., Sharma, B. and Bandhopadhyay, S.K. *Sero Diagnosis of Fasciola Gigantica in Buffaloes.* Veterinary Parasitology. 1987. 24; 67-74.

[43] Maiti, S.K., Rao, V.N., Ali, S.L., Dutta, G. and Mishra, A. *Haematobiochemical and Therapeutic Aspects of Biliary Amphistomiasis in Buffalo.* Indian Journal of Veterinary Medicine. 1999. 19; 49-51.

[44] Ozcelik, S., Bakir, S. and Bakir, M. *The in Vitro Effect of Albendazole on Fasciola Hepatica and Host Liver Lactate Dehydrogenase Enzymes.* Turkiye-Prazitoloji-Dergisi. 1992. 16; 6-10.

[45] Veerakumari, L. and Lakshmi, K.N. *In Vitro Effect of Allium Sativum on Lactate Dehydrogenase Activity of Haemonchus Contortus.* Journal of Veterinary Parasitology. 2006. 20; 93-96.

[46] Tejada, P., Sanchez–Moreno, M., Monteoliva, M. and Gomez–Banqueri, H. *Inhibition of Malate Dehydrogenase Enzymes by Benzimidazole Anthelmintics*. Veterinary Parasitology. 1987. 24; 269–274.

[47] Oztop, A.Y., Saygi, G. and Oztop, H.N. *In Vitro Effects of Some Anthelmintics on the Malate Dehydrogenase and Lactate Dehydrogenase Enzyme Activities of Taenia saginata.* Turkish Journal of Medical Sciences. 1999. 29; 365-370.

[48] McCracken, R.O. and Stillwell, W.H. *A Possible Biochemical Mode of Action for Benzimidazole Anthelmintics.* International Journal of Parasitology. 1991. 21 (1) 99-104.

[49] Swargiary, A., Roy, B., Giri, B.R. and Rohang, B. *A Comparative Study on the Anthelmintic Efficacy of Some Medicinal Plants of North–East India: Alteration in the Glycolytic Enzymes of Fasciola Buski, a Giant Intestinal Fluke.* Asian Pacific Journal of Tropical Medicine. 2013. 412-420.

[50] Lwin, T. and Probert, A.J. *Effect of Certain Fasciolicides on Malate Dehydrogenase Activity in Fasciola Hepatica: A Possible Biochemical Mode of Action of Hexachlorophene and Oxyclozanide.* Journal of Pesticide Science. 1975. 6; 121-128.

[51] Probert, A.J., Sharma, R.K., Singh, K. and Saxena, R. *The Effect of Five Fasciolicides on Malate Dehydrogenase Activity and Mortality of Fasciola Gigantica, Fasciolopsis Buski and Paramphistomum Explanatum.* Journal of Helminthology. 1981. 55; 115-122.

[52] Maule, A.G. and Marks, N.J. *Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology.* CABI. 2006. 448.

[53] Prichard, R.K. *Mode of Action of the Anthelmintic Thiabendazole in Haemonchus Contortus.* Nature. 1970. 228; 684-685.

[54] Malkin, M.F. and Camacho, R.M. *The Effect of Thiabendazole on Fumarate Reductase from Thiabendazole-Sensitive and Resistant Haemonchus Contortus*. Journal of Parasitology. 1972. 58; 845-846.

[55] Kaur, R. and Sood, M.L. *The Effects of DL-Tetramisole and Rafoxanide on Tricarboxylic Acid Cycle Enzymes of Haemonchus Contortus, In Vitro.* Veterinary Parasitology. 1972. 13; 333-340.

[56] Barrowman, M.M., Marriner, S.E. and Bogan, J.A. *The Fumarate Reductase System as a Site of Anthelmintic Attack in Ascaris suum.* Bioscience Reports. 1984. 4; 879-883.

[57] Kumari, Y.S. *Effest of Tolzan on Carbohydrate Metabolism and Protein Metabolism of an Acanthocephalan Parasite Pallisentis Nagpurensis Parasitising the Fresh Water Fish Channa striatus.*

[58] Lemke, T.L., Williams, D.A., Roche, V.F. and Zito, S.W. Principles of Medicinal Chemistry. Seventh Edition Copyright Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore. 2013. MD 21201.

[59] Turrens, J.F. *The Enzyme-NADH Fumarate Reductase in Trypanosomatids – A Potential Target against Parasitic Diseases.* Molecular and Cell Pharmacology. 2012. 4 (3) 117-122.

[60] Van den Bossche, H. and Janssen, P.A.J. *The Biochemical Mechanism of Action of the Antinematodal Drug Tetramisole.* Biochemical Pharmacology. 1969. 18; 35-42.

[61] Parvathi, J. and Aruna, K. *Correlation of Hyperglycemia and Succinate Dehydrogenase Activity during Hymenolepiasis in Mice and Treatment with Praziquantel.* IOSR. Journal of Pharmaceutical sciences. 2012. 1 (1) 022-028.

[62] Skuce, P.J. and Fairweather, I. *The Effect of the Hydrogen Ionophore Closantel upon the Pharmacology and Ultrastructure of the Adult Liver Fluke Fasciola Hepatica*. Parasitology. 1990. 76; 241-250.

[63] Boczoni, K., Uszynska, Z., Rodriguez-caabeiro, F., Criado-fornello, A. and De armas-serra, C. *The Effect of Some Anthelmintics on Trichinella Spiralis and Trichinella Pseudospiralis Mitochondrial Energy-Generating Pathways.* Research and Reviews in Parasitology. 1991. 51 (1-4) 61-63.

[64] Swan, G.E. *The Pharmacology of Halogenated Salicylanilides and Their Anthelmintic Use in Animals.* Journal of South African Veterinary Association. 1999. 70 (2) 61-70.

[65] Chen, M., Zhai, L., Christensen, S.B., Theander, T.G. and Kharazmi, A. *Inhibition of Fumarate Reductase in Leishmania Major and L. Donovani by Chalcones*. Antimicrobial Agents and Chemotherapy. 2001. 45 (7) 2023-2029.

[66] Saz, H.J. *Comparative Energy Metabolism of Some Parasitic Helminths*. Journal of Parasitology. 1970. 56; 634-642.