CASE REPORTS

Article 31

Biological Control of Helminth Parasites by Predatory Fungi

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Biological control of animal parasites could become a strong arm for Integrated Parasite Control in the very near future. Though various nematode-destroying fungi received attention, predominantly on academic interest, from the 18th Century in Scandinavian countries, work on their application to control animal parasites gathered momentum from 1990's. The philosophy behind biological control is to utilise one or more of the natural enemies of the nematodes, making it possible to reduce the infection on pasture to a level where grazing animals can avoid both clinical and subclinical effects of the parasitic nematodes. The important requirement is the presence of the fungi in the faecal pats where the development of the pre-parasitic larvae takes place. Therefore, to be effective, the fungi should pass through the gastrointestinal tract of the host without loss of viability. The fungi, Duddingtonia flagrans and Verticillium chlamydosporium, which can be isolated from organic environment of India produces thick walled chlamydospores, the stage responsible for their survival during passage through the gut of ruminants following oral administration. The results had indicated survival of the fungus during gastrointestinal transit in grazing animals and successful reduction of numbers of parasitic nematode larvae on pasture. The dose of fungal spores to be given to an animal and the time of administration for effective parasite control has been standardised. The fungus behaves in density dependent manner and appears to be environment-friendly. The challenge lies ahead in its field application.

KEY WORDS
Biological control, Helminth, Predatory fungi.

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INTRODUCTION

During the last 10-15 years there has been an increasing emphasis on the need of development of new alternative to or supplements for chemical control of parasitic nematodes in grazing livestock. The background for this interest is multi-factorial but the major reason is the serious development of anthelmintic resistance in parasitic populations. Anthelmintic resistance involving particularly the gastrointestinal nematodes of small ruminants is escalating globally and is the single most important concern of parasitologists around the world since it threatens the survivability of small ruminant farming as well as the helminthologists. The problem is most severe in the countries of southern hemisphere like South Africa (Van Wyk et al., 1997), Australia (Waller et al., 1995a), New Zealand (Kettle et al., 1983) and many other Latin American countries (Waller et al., 1995b).

India is slowly and steadily emerging as the resistance epicenter of South Asia (Sanyal, 1998). The global tempo of development and extent of anthelmintic resistance in helminths of small ruminants in particular, indicates that the numerous anthelmintics and strategies developed and implemented over the period of last 40-50 years have been incorrectly applied (Van Wyk, 2001). Other reasons include handling of parasite problems in the organic livestock production, regulation of conventional drug use by legislation, political and consumer pressure for reduced

chemical residues in products. Sutherst (1986), among others, has talked about the importance of implementing integrated parasite control based upon host resistance, immunization and non-living vaccines to reduce the use of chemotherapy. To become sustainable, parasite control schemes need to be based on the principles of integrated pest management (Waller, 1993). Towards this objective, significant advances have recently been made in the development of worm vaccines (Emery, 1996; Smith, 1997), in the breeding of animals for parasite resistance (Woolaston and Baker, 1996) and biological control exploiting predacious fungi (FAO, 2002).

PARASITE CONTROL MEASURES

Gronvold et al. (1996) have suggested two major groups of control measures that are in use and would be tried in future.

Chemical Control	Non-Chemical Control
Chemotherapy	Biological Control
Spraying of Poison	Worm Vaccines
Use of Repellants	Selection for Host Resistance
Use of Pheromones	Grazing Management
	Nutritional Management
	Inter-Specific Competition
	Sterile Male Technique

PRESENT STATUS OF DIFFERENT CONTROL STRATEGIES

Chemical Control

Three crucial reasons for opting alternative parasite control strategies including biological control are drug resistance, residues on food and environmental degradation. Frequent and haphazard use, over-reliance on chemicals are the causes of the drug resistance. As resistance to newer anthelmintics develops, there is a need for control measures alternative to chemotherapy. Chemical residues in foods are now a major concern and a strong driving force for reduced chemical inputs in agriculture. Consumers increasingly demand that food supply should be free from contaminants of all kinds. Unlike organochlorine ectoparasiticides, residues are not a major problem for anthelmintics. The benzimidazoles and their prodrugs are subjected to close scrutiny because some are known teratogens. The environmental impact of anthelmintics has been generally regarded as un-important. Levamisole and the benzimidazoles pose little cause of concern, but there is greater worry regarding the avermectins. There is evidence of adverse effects on a variety of dung colonizing insects.

Worm vaccines

Within the last decade, much effort has been directed at the development of a vaccine especially against Haemonchus contortus, either based upon naturally exposed or hidden antigens (Smith, 1997). Despite promising results and mass appraisal over the years, a commercial product is still to be released. Dictol, based on infective Dictyocaulus viviparous larvae attenuated by irradiation, is the only marketed vaccine against GI nematodes but it has only a very limited distribution.

Grazing Management

Grazing management strategies have been demonstrated to be useful to alleviate the impact of GI nematodes in livestock (Barger, 1999; Stromberg & Averbeck, 1999). Unfortunately, these strategies have not been adopted to their full extent, perhaps due to the ease for the farmer to use drugs and secondly, the increased demand for land, which makes this proposition less likely in many intensive livestock systems. Where it is used it is often in combination with chemotherapy. In organic livestock production these strategies are widely used, but are primarily based upon the availability of herbage rather than an active measure to control problems with GI nematodes (Thamsborg et al 1999).

Breeding for Resistant Host

Breeding for resistance in host animals to GI nematodes has been attempted with some success (Kloosterman et al 1992; Woolaston & Baker, 1996; Gray, 1997), but although breeding programmes are promoted and adopted based

upon these principles (e.g. Nemesis in Australia, Worm FEC in New Zealand), they are far from widely implemented.

Nutritional Management

This is now a well-established fact that supplementation of the diet with additional protein does not appear to affect initial establishment of nematode infection but the patho-physiological consequences are generally more severe on lower planes of protein nutrition. The main effect of protein supplementation is to increase the rate of acquisition of immunity and increase resistance to re-infection and this has been associated with an enhanced cellular immune response in the gastro-intestinal mucosa. Studies on the influence of nutrition on the expression of genotype have shown that the benefits of a superior genotype are not lost on a low protein diet whereas a high protein diet can partially ameliorate the disadvantages of an inferior genotype (Coop and Holmes, 1996). Although many aspects of the interaction between nutrition and helminth parasites have been established many features remain to be examined. It has now largely been acknowledged that parasite control cannot be achieved by a single method. The available classical and alternate technologies should properly be integrated similar to those practiced in the integrated pest control in agriculture. Biological control seems to be one of such alternate control strategy.

CONCEPT OF BIOLOGICAL CONTROL

This area of research has attracted increasing attention, especially within the last 10-12 years. Biological control is defined as the action of natural enemies which maintain a host population at levels lower than would occur in the absence of enemies. This not only includes classical un-exploited organisms but also those that are genetically modified to enhance these properties (Waller and Faedo, 1996). Biological control is divided into two major categories, viz., natural and applied. Natural biological control is affected by native or co-evolved natural enemies in the environment without human intervention. Within the environment, the pre-parasitic stages of nematodes are subjected to a variety of both abiotic and biotic factors that can profoundly influence their development and survival. The most important abiotic factors are temperature; humidity and oxygen-extremes in these can be lethal on the free-living stages. In regard to biotic factors, there exists a vast assemblage of living organisms that can affect the success of worm eggs developing larvae. From these may emerge candidate(s) for biological control of worm parasites. By definition, biological does not assume to be a substitute for chemotherapy, where the expectation, if not the reality, is that parasites may be eradicated by frequent use of drugs with efficacies approaching 100%. Biological control agents really eliminate the target organism, but reduce the number of expectable levels and maintain a balance between the pathogen and the antagonist. In contrast to chemical control of nematode parasites, which is directed entirely at the parasitic stage within the host, biological control will almost certainly be focused on the free-living stages of parasites on pasture.

The intention of using biological control methods is to lower the density of pest population below the clinical level and perhaps below the economic threshold above which production losses are obvious owing to a high parasitic population density.

Agents for Biological Control

All gastro-intestinal nematode parasites of livestock have a life-cycle which involves not only the parasitic stage within the host, but also a free-living or pre-parasitic stage on pasture. The pre-parasitic stages on the pasture are potentially vulnerable to attack by biological control agents. A number of organisms have been identified to exploit the free-living stages of parasites as food source and are likely to be commercially exploited in the near future. These organisms include micro-arthropods, protozoa, predacious nematodes, virus, bacteria and fungi. Although the all are of intrinsic interest, it is from the last group of organism that breakthroughs in

biological control of nematode parasites of livestock are likely to emerge.

FUNGI AS BIOLOGICAL CONTROL TOOL

Fungi that exhibit anti-nematode properties have been known for a long time. They consist of a great variety of species characterized by their ability to capture and exploit nematodes either as the main source of nutrients or supplementary to a saprophytic existence. They are divided into three major groups based on their morphology and types of nematode-destroying apparatus (Barron, 1997; Nordbring-Hertz, 1988).

Predacious Fungi

They produce specialized nematode-trapping structures (adhesive knobs, networks, rings etc.) on the mycelium. The idea of possibly using predacious micro-fungi to control animal nematodes arose in the 1930's. It was not until the mid 1980s before thorough and systematic investigations were undertaken and since then, two lines of work can be clearly distinguished.

Trials performed with mainly with *Arthobotrys* spp. (*A. oligospora*) and *Monacrosporium* spp. as biological control agents. A group of Danish researchers, testing the effect of fungus *A. oligospora* primarily against parasitic nematodes in cattle but also in other livestock species. Testing different doses of spores mixed into faeces, 250 and 2500 conidia per gm of faeces was found to significantly lower the number (70 & 99% reduction, respectively) of developing *C. oncophora* larvae in faecal cultures (Gronvold et al. 1985). The trapping activity of the fungus was influenced by the motility of the infective larvae & there is no specificity for the parasitic species (Nansen et al. 1996). Unfortunately various trials performed to test *A. oligospora* mycelium and conidia failed due to the destruction of these structures in the GI tract of the host animals (reported in Gronvold et al. 1993 a, b). A high dose (between 470 & 680 gm of fungal material on millet) of one of the three different fungal species (*A. musiformis, A. tortur, Dactylaria candida*) was fed to housed lambs, harboring a mono infection of either *H. contortus* or *O. circumcincta*. This subsequently led to survival Of *A. tortur* through the GI tract at a level high enough to significantly reduce the number of *H. contortus* in faecal cultures.

The other line of research is with *Duddingtonia flagrans*. This predacious fungus produces three dimensional, sticky networks on its growing hyphae. It also produces an abundance of intercalary thick walled resting spores, chlamydospores. This fungus is relatively slow growing and as with other predacious fungi growth is strongly influenced by temperature (Fernandez et al. 1999e). Many other species of predacious fungi are fast growing but the spores of these fungi are much more sensitive to the stress of the GI tract than that of the chlamydospores of *D. flagrans*. In plot trials *D. flagrans* have shown good reduction of free living larval stages of parasitic nematodes of cattle (Gronvold et al. 1993a, b), sheep (Peloille, 1991) and horses (Fernandez et al. 1997, 1999a; Baudena et al. 1999). These field trials have shown that daily feeding of fungal spores to grazing animals for 3-4 months prevents build-up of dangerous levels of infective larvae on the pasture. In an Australian study Knox and Faedo (2001) found that sheep feed supplement containing *D. flagrans* chlamydospores had lower egg counts and improved liveweight gains compared to untreated animals.

Endo-parasitic Fungi

These invade nematodes either by penetration of cuticle from sticky spores adhering to the cuticle or following ingestion of spores which lodged in the gut. This type of fungi is obligate parasite of nematodes, with very limited capacity to develop outside the prey and density dependent (Jaffe et al, 1993). This has led researchers to believe that there might be better or stronger BC candidate against pest nematodes. *Drechmeria coniospora* is a fungus producing sticky drops on very small conidia, which adhere to the cuticle of the nematode, penetrate the cuticle and destroy the

victim. By applying a very high dose (108 conidia per gm of faeces) to faecal cultures, Santos & Charles (1995) found that only infective third stage parasite larvae stripped of the protective extra (second stage) cuticle, became infected by the fungus. Another endoparasitic fungus, *Harposporium anguillulae* produce very small, half moon shaped conidia which lodge in the digestive tract of the feeding nematode and after germination totally digest the victim before finally breaking through the cuticle to produce new conidia on the short conidiophores. In a laboratory study it was found that at a dose of 3 lakh conidia/ gm faeces, the number of *H. contortus* larvae recovered was significantly reduced (Charles et al, 1996). The requirement of spore dispersion or infection to be more or less directly from one infected individual to the next, severely limits or almost excludes the use of this group of fungi as practical BC agents.

Egg-parasitic Fungi

These have the ability to attack the egg stage and may have a role in the control of animal parasites which have a long development and/or survival time in the egg stage in the environment outside host, e.g., *Ascaris*, *Fasciola* spp., amphistomes etc.

Eggs of *Ascaris lumbricoides* collected from naturally infected pigs were used to test the effect of mainly the fungus *Verticillium chlamydosporium* but also other *Verticillium* spp. the fungus was shown to be able to degrade the egg shell enzymatically and infect the eggs (Lysek & Krajci, 1987; Lysek & Sterba, 1991; Kunert, 1992). Short exposure to high temperature or UV- irradiation rendered the eggs more susceptible to fungal attack (Lysek & Bacovsky, 1979). In the USA, Chien and colleagues have shown that *V. chlamydosporium* attacked and destroyed eggs of *Ascaridia galli* and *Parascaris equorum* but only rarely invaded *Trichuris suis*. In Denmark works were done on the predacious fungi *Arthobotrys* spp. and egg parasitic fungi *Paecilomyces lilacinus* for activity against eggs of *T. canis*. *P. lilacinus* showed some activity (16% eggs infected after 7 days) but the predacious species did not attack the eggs.

PRESENT STATUS

Present Global Status

As a result of renewed interest and intensified research in biological control during the last 15 years, a convincing amount of evidence on the potential of this principle has been gathered (Larsen, 2002). Most of this work has been carried out in Europe (Denmark, Sweden, UK and France) and Australia and recently initiated in USA, Latin America, Africa, South-east Asia and Far East. Among the nematode trapping fungi, *Duddingtonia flagrans* has displayed superior abilities with respect to survival through gastro-intestinal transit as well as subsequent destruction of parasitic larvae in faecal pats (Larsen, 2002). Danish scientists first demonstrated through laboratory and field trials, that biological control against pre-parasitic stages of nematodes could be achieved by feeding chlamydospores of this fungus to cattle (Gronvold et al., 1993), horses (Fernandez et al., 1997), pigs (Nansen et al., 1996) and sheep (Githigia et al., 1997). Successful pilot scale trials with *D. flagrans* chlamydospores through feed supplement (Knox and Faedo, 2001), feed blocks (Waller et al., 2001a) and slow release devices (Waller et al., 2001b) provide sufficient euphoria for commercial exploitation of fungal delivery devices in future integrated parasite control programmes. Besides being safe for animals and man, it is imperative that new technologies dealing with BC need to be of no negative impact to the grazing environment. Short time impact studies have shown no negative effect of the fungus on earthworms (Gronvold et al., 2000) and on soil nematodes (Yeates et al.1997; Faedo, 2001). The future appears to be very promising in early outcome of a bio-control product to control nematode parasite of livestock.

Present Indian Status

India initiated the work on biological control of animal nematode parasites using mycological means in 1998 and two species of nematode-

trapping fungi, viz., *Arthrobotrys oligospora* and *D. flagrans* and two species of egg parasitic fungi, viz., *Paecilomyces lilacinus* and *Verticillium chlamydosporium* were isolated from organic environment of Gujarat and Chhattisgarh (Sanyal, 2005; Sanyal et al., in press). They were subjected to stringent screening for their suitability as biocontrol agents against nematode parasites of ruminants using growth assay, predatory activity, germination potential and ability to survive ruminant gut passage. The study indicated that the isolates of *D. flagrans* and *V. chlamydosporium* fulfilled all the possible criteria. A strategy is formulated for application of nematode-trapping fungi to control gastrointestinal nematodosis of ruminants (Sanyal et al., 2005). he available classical and alternate technologies should properly be integrated similar to those practiced in the integrated pest control in agriculture. Biological control seems to be one of such alternate control strategy.

CONCLUSION

There is an increasing awareness that in future the parasitic control programme should reduce reliance on chemical anthelmintics. Compared to the other non-chemotherapeutic approaches to parasitic control in ruminant livestock, use of nematode trapping fungi has shown promising results in Denmark, Australia & India. This has been well exemplified through both in vitro and in vivo studies resulting in reduced translation of larvae to the herbage from faecal pats and reduced worm burdens in livestock. As integrated sustainable control strategies would be the modus operandi to control the parasitic gastro-enteritis in livestock both in conventional and organic farming system, mycological control would be arm for integration with both non-chemical and chemical means.

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Article 32

Therapeutic and Cosmetic uses of Botulinum Toxin

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KEY WORDS
Botulinum toxin, *Clostridium botulinum*.

INTRODUCTION

From times unknown man has greatly been benefited from uncovering and utilizing the chemicals from the natural world. Living organisms, such as plants, animals, microorganisms, offer a huge source of pharmaceutically useful medicine and toxins. Depending upon their source, the toxins are categorized as phytotoxins, mycotoxins and, zootoxins including venoms and bacterial toxins. Botulinum toxin is neurotoxic protein produced by the gram-positive, rod shaped, spore forming, strictly anaerobic bacterium *Clostridium botulinum*. These bacteria are widely distributed in soil and water (Dowell, 1984). Botulinum toxin is one of the most acutely toxic naturally occurring substances in the world with a lethal dose of about 200-300 pg/kg (100g could kill every human on earth. Botulinum toxin is odorless and tasteless, and shares many properties with the other bacterial toxins such as tetanospasmin and diphtheria toxin (Davis, 1993). Thousands of people in the world each year continue to be poisoned with botulinum toxin food-borne, infantile, or wound botulism but the neurotoxin is now sufficiently understood to allow it to be used as medicinal agent to paralyze specific muscles, giving temporary symptomatic relief from variety of neurologic disorders and for certain cosmetic purposes in minute doses. (Davis, 1993). The clostridia produce more protein toxins than any other bacterial genus and are a rich reservoir of toxins for research and medicinal uses. Research is underway to use these clostridial exotoxins or their toxin domains for drug delivery, prevention of food poisoning, and the treatment of cancer and other diseases. The remarkable success of botulinum toxin as a therapeutic agent has created a new field of investigation in microbiology.

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BOTULINUM TOXIN

It constitutes a family of serologically closely related 7 neurotoxins i.e. A, B, C1, D, E, F, and G. The primary structure of most serotypes has been determined. Each toxin has a inhibitory chain (light chain) and binding chain (heavy chain) and the two chains are held together with a disulfide bond. These chains have three major types of domains, the binding and translocation domains present on heavy chain and the catalytic domain present on the light chain. Toxin types A, B, E and F are the main toxins that affect humans (Dowell, 1984) and toxin types C and D affects birds and mammals (Davis, 1993). For therapeutic purposes botulinum toxin A is preferred because of its long duration of action and ease of production. Botulinum toxin type A also has several advantages including relatively rare systemic side effects, lack of tissue destruction, graded therapeutic response by dosage adjustment and, above all, high patient acceptance (Dresseler et.al, 2000). The type A toxin is produced as a single chain polypeptide with a molecular weight

of 150000 Da and later on it is transformed into its active structure by protease which causes nicking of single chain polypeptide to di-chain polypeptide of about 100000 Da and 50000 Da molecular weight (Bandyopadhyay, et al.1987).
Botulinum toxin B (BTX-B) is preffered to type A toxin for the treatment of cervical dystonia. BoNT/B is stable in solution at an acidic pH and is available as a solution containing 5000 units/mL.

MECHANISM OF ACTION

At neuromuscular (N-M) junction release of acetylcholine (Ach) is mediated by assembly of synaptic fusion complex that allows membrane of synaptic vesicle to fuse with neuronal cell membrane. Synaptic fusion complex is a set of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which include synaptobrevin, SNAP-25 (synaptosomal-associated protein of 25 kd), syntaxin. After membrane fusion, acetylcholine is released into synaptic cleft and then bound by receptors on the muscle cell.
When there is exposure to botulinum toxin, it produces its action by involving several steps: systemic absorption, binding to the nerve terminal, internalization and synaptic poisoning. The toxin first reaches the lymphatic channels and then the blood stream either by absorption through the upper gastrointestinal tract (food borne and infantile botulism) or through tissue absorption (wound botulism) (Bonventre, 1979). Then the toxin circulates in the blood until it reaches cholinergic synapses in the peripheral nervous system. The toxin appears not to cross the blood brain barrier, so the cholinergic synapses of central nervous system are not involved (Sugiyama, 1980). The toxin binds with the help of binding domain to cholinergic neuronal cell membrane at nerve terminal and enters neuron by endocytosis (internalization). The light chain of botulinum toxin crosses the membrane of the endocytic vesicle and enters the cytoplasm of the pre synaptic terminal (Davis, 1993). Then it cleaves specific sites on SNARE proteins thus preventing complete assembly of synaptic fusion complex which in turn blocks docking, fusion and acetylcholine release. Blockage of acetylcholine release in turn blocks activation of muscarinic and nicotinic receptors due to which there will be decreased/no secretion in case of exocrine glands and paresis due to chemical denervation in case of a muscle.
Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin.

HISTORY AND FDA (FOOD AND DRUG ADMINISTRATION) APPROVALS

In 1944, Edward Schantz cultured *Clostridium botulinum* and isolated the toxin, and, in 1949, Burgen's group discovered that botulinum toxin blocks neuromuscular transmission thus blocks the release of acetylcholine at N-effector junctions. By 1973, Alan B Scott, MD, of Smith-Kettlewell Institute used botulinum toxin type A (BTX-A) in monkey experiments, and, in 1980, he officially used BTX-A for the first time in humans to treat strabismus. In December 1989, BTX-A (BOTOX) was approved by the US Food and Drug Administration (FDA) for the treatment of strabismus, blepharospasm, and hemifacial spasm in patients over 12 years old. (Dresseler, 2000) observed that the effects of botulinum toxin are fully reversible.
Botulinum Toxin Type B (BTX-B) received FDA approval for treatment of cervical dystonia on December 21, 2000. On April 15, 2002, the FDA announced the approval of botulinum toxin type A to temporarily improve the appearance of moderate-to-severe frown lines between the eyebrows (glabellar lines). BTX-A has also been approved for the treatment of excessive underarm sweating. The acceptance of BTX-A use for the treatment of spasticity and muscle pain disorders is growing, with approvals pending in many European countries and studies on headaches (including migraine), prostatic symptoms, asthma, obesity and many other possible indications are ongoing. Botox is the only FDA-approved botulinum toxin A, but a potential competitor,

Dysport, has an FDA approval application pending.

THERAPEUTIC USES

As the mechanism of action of the botulinum toxin was better understood, it was recognized that the toxin could be used selectively to paralyze muscles. Researchers discovered in the 1950s that injecting minute quantities of botulinum toxin type A in overactive muscles resulted in decreased muscle activity by blocking the release of acetylcholine at the neuromuscular junction, thereby rendering the muscle unable to contract for a period of 4-6 months. At that time, the botulinal toxin is often re-administered to the same muscles. Repeated muscle injection may provide relief for shorter periods than the initial administration. It is not yet clear if botulinum toxin can be re-administered indefinitely or whether the effectiveness eventually wears off. Clinical effect of the serotype A and B toxins begins within 24-48 hours, peaks at 2-3 weeks and lasts for 3-4 months (Brin, 2002). FDA has recommended uses of botulinal toxins for:

1) Migraine Headaches: Botulinum toxin in headache prophylaxis was found by Serendipity whereby patients receiving BoNT/A for frown lines reported reduced frequency of their headaches. BoNT/A at the doses range between 50-100 U is injected in the tender muscles of the frontal, temporal and cervical regions for the relief from tension-type and chronic daily headache. Studies have proven its efficacy in migraine prophylaxis (Silberstein et al., 2000). Though the exact mechanism is unknown, it has been postulated that BoNT/A reduces the afferent volley of pain impulses. The exact doses are still being worked out and it is best to begin with smaller doses and, if necessary, gradually increases the dose.

2) Dystonia: In general, more than 90% of treated cases of blepharospasm and laryngeal dystonia show a satisfactory result. Type B toxin, which has been carefully studied to date only in cervical dystonia, shows similar results as type A toxins. More than 75% of patients with cervical dystonia and jaw-closing oromandibular dystonias benefit significantly from type A toxins. The response in upper limb dystonias is less.

a) Benign Essential Blepharospasm: Dystonic hyperactivity in the orbicularis oculi muscles causes blepharospasm. Orbicularis oculi, corrugator supercilii and procerus are targeted with botulinum toxin at the dose rate of 25 U for each side. Complications, namely ptosis, dryness of eyes, lateral rectus palsy, facial muscle paralysis and hematoma formation, are transient and reversible (Brin, 1998). Considerable improvement occurs in the quality of life measures in patients with blepharospasm.

b) Meige's syndrome: Patients have dystonia of both upper and lower halves of face. The lower face muscles have a very narrow therapeutic window and thus, have substantially higher chances of functional impairment. The dose used varies between 50 to 100 units.

c) Oromandibular Dystonia: In jaw closure dystonia, masseters are commonly targeted bilaterally by BoNT/A at the dose rate of 30U for each side. In jaw opening dystonia, lateral pterygoids together with anterior belly of omohyoid are targeted but the response is variable. It is advisable to avoid injecting tongue muscles in lingual dystonia as a weak tongue can choke a patient, requiring intubation (Brin, 1998). Oromandibular dystonias are difficult to treat without causing dysphagia and should only be done by well-trained and experienced clinicians with adequate experience in use of neurotoxins.

d) Cervical dystonias: Botulinum toxin is the treatment of choice for cervical dystonias and they significantly improve the quality of life measures (Brin, 1998). Identification of the muscles responsible for torticollis, laterocollis, anterocollis and retrocollis is essential. Muscles are targeted specifically after observing the pattern of shift, tilt and rotation of the neck and through EMG assessment. Approximately 20-60 U are needed for a muscle. Anterocollis with head protrusion has a poor response. Side effects include reduced head control and dysphagia. Injections into the sternocleidomastoid and

scalenes have a higher risk of dysphagia. Dysphagia can be minimized by avoiding bilateral sternocleidomastoid injections, targeting its insertion and ensuring that the muscle bulk is not penetrated. Patients in whom dysphagia might pose increased risk for aspiration pneumonia should be carefully assessed prior to injection. The effect of injections lasts for around 3-4 months.

e) Pharyngolaryngeal dystonias: Botulinum toxin therapy is the treatment of choice for both abductor and adductor forms of pharyngolaryngeal dystonias. Amongst all the indications for BoNT/A therapy, spasmodic dysphonia shows the highest success rate (Dresseler, 2000). Botulinum toxin type A is injected either per orally under laryngoscopic guidance or transcutaneously through the cricothyroid membrane under EMG guidance. The vocalis muscle is targeted in the adductor form and posterior cricoarytenoid muscle in the abductor form. Approximately 2.5-10 U are used and the effect begins within 2-3 days and lasts for 2-9 months. Complications include transient dysphagia, weak cough, hoarseness and hypophonia. Dyspnea and stridor can occur after treating the abductor form (Brin, 1998) and)(Dresseler, 2000). Quality of life measures improve significantly after BoNT/A injections.

f) Writer's cramp and occupational cramps: Dystonic hyperactivity of forearm, hand and, occasionally, proximal arm muscles while writing occurs in writer's cramp. It is the most common occupational cramp. Response to BoNT/A is better if dystonia is limited to isolated muscles and if the initial hyperactive muscle trigger can be identified. Limitations to therapy include narrow therapeutic window of the wrist and finger flexors, large requirement for BoNT/A due to large number of muscles involved and difficulty to distinguish dystonic action from physiologic and compensatory action (Brin, 1998 and Dresseler, 2000).

g) Non-action induced limb dystonias: Botulinum toxin type A is useful in pain reduction and improvement in function.

3) Hemifacial Spasms: Unilateral, involuntary, recurrent twitches of the eyelids and other muscles of face characterize hemifacial spasms. Periocular muscles, risorius, depressor anguli oris, depressor labii inferioris, zygomaticus and mentalis are targeted. Doses ranging from 25-50 U are used. Therapy with BoNT/A has a high success rate and the effect is longer than for blepharospasm. Presently, BoNT is the first line treatment for hemifacial spasms and only those with a poor response may need surgical decompression of the facial nerve (Jankovic et al., 1997).

4) Strabismus or crossed eyes

5) Exocrine gland hyperactivity

a) Focal hyperhidrosis: It is defined as excessive sweating of the palms, soles, axilla or face. The iodine-starch test delineates areas of hyperhidrosis and 0.5-0.8 U/cm2 BoNT/A are injected intradermally. Approximately 30-80 U are used at 15-25 sites. The benefits last for 3-4 months and increased doses may extend this up to a year or more (Naumann et al., 1999).

b) Relative sialorrhoea: Botulinum toxin type A injection into the parotid gland is effective for controlling drooling in conditions such as Parkinson's disease, motor neuron disease and bulbar/pseudobulbar palsy without causing xerostomia (Dresseler, 2000).

c) Frey's syndrome: Areas of skin are targeted that show gustatory sweating due to aberrant innervation of facial nerve secretomotor fibers to sweat glands following parotidectomy (Dresseler, 2000).

d) Crocodile tears syndrome: Lacrimal glands are targeted in gustatory lacrimation due to aberrant innervation of facial nerve secretomotor fibers (Dresseler, 2000).

Other uses of botulinum toxin type A that are widely known but not specifically approved by FDA include treatment of:

A) Prostate Hyperplasia: BTX- A injection induces prostate apotosis in dogs and relieves BOO (Bladder outlet obstruction) due to benign prostatic hyperplasia (BPH) in humans. Intraprostate BTX-A injection may be a

promising, reversible and alternative treatment for refractory BOO due to BPH. Furthermore, previous studies in the rats have shown that intraprostatic injection of BTX-A induces selective denervation and subsequent atrophy of the glands.

B) Smooth Muscle Disorder: Direct injection of botulinum toxin is disclosed as an effective, safe and simple method of treatment for disorders of gastrointestinal muscle or smooth muscles elsewhere in the body, with results that appear to be sustained for several months. Muscle disorders which are suitable for such treatment include achalasia, isolated disorders of the lower esophageal sphincter, gastroparesis, hypertrophic pyloric stenosis, sphincter of Oddi dysfunction, short-segment Hirschsprung's, anal fissure, hemorrhoids, proctalgia fugax, irritable bowel syndrome, disorders of the upper esophageal sphincter, vasospastic disorders, and disorders of uterine and bladder spasm. Devices suitable for delivering this therapy are also disclosed.

C) Overactive Bladder Syndrome with or without incontinence.

D) Spastic Disorders associated with injury or disease of the central nervous system including trauma, stroke, multiple sclerosis, or cerebral palsy.

E) Anal Fissure

F) Diabetic neuropathy

G) Wound healing

H) Excessive salivation

I) Parkinson Disease

J) Depression

COSMETIC USES OF BOTULINUM TOXIN A (BTX-A)

The cosmetic effect of BTX-A was initially described by the Carruthers, a dermatologist/ophthalmologist husband and wife team working in Vancouver, Canada, although the effect had been observed by a number of independent groups. Cosmetically desirable effects of Botox were quickly discovered thereafter when the frown lines between the eyebrows were observed to soften following treatment for eye muscle disorders, leading to clinical trials and subsequent FDA approval for cosmetic use in April 2002. As of 2006, Botox injection is the most common cosmetic operation in the United States.

1) Hyperkinetic Facial Lines: The main application of botulinum toxin in facial plastic surgery is in the effacement of dynamic or hyperkinetic facial lines i.e. Frown/glabellar lines, squint lines /eye crows feet lines, forehead lines and the muscle bands on platysma muscles often visible on the neck, commonly known as "turkey neck" or platysma banding. The granting of US Food and Drug Administration approval for the use of Botulinum Toxin type A in the treatment of glabellar lines for people with age 18–65 years marks a major milestone for the more widespread usage of this product in cosmetic settings. The use of botulinum toxin in treatment of hyperkinetic conditions is well established and enjoys an excellent safety profile (Batniji, 2004). Either local injection or creams are available for local use and typically, no anesthetic is necessary. The “muscle relaxant” effect lasts about three to four months and can be repeated as needed.

2) Eyebrow Lifting: A study has been carried out on 22 patients desiring a cosmetic enhancement and injection of botulinum toxin A (7-10 units) directed to brow depressor muscle (lateral orbicularis oculi) bilaterally. No patients withdrew for adverse effects. All patients were evaluated 2 weeks after treatment. The average brow elevation from the mid-pupil observed after selected injection of brow depressors with botulinum toxin A was 1.02 mm. The average brow elevation from the lateral canthus observed after selected injection of brow depressors with botulinum toxin A was 4.83 mm. Significant temporal brow elevation occurs as the result of paralysis of brow depressors by using botulinum toxin A injection. This procedure may be considered an alternative to surgical brow elevation.

3) Frontalis muscle hyperactivity.

4) Dimpling of the chin from overactive muscles.

5) Raise drooping of the corners of the mouth.

6) Fine lines around the lips.
7) Fine wrinkles under the eye.

TREATMENT FAILURE

Treatment failure can be either primary, where failure occurs in a BoNT-naïve patient, or secondary, where failure occurs after an initial successful use. Treatment failure may result from misplaced toxin, sub-optimal dosing, or administration of toxin that has been inactivated by improper storage or handling. Antibodies to the toxin are presumed to be responsible for most remaining cases of resistance. Antibodies against the toxin have developed in patients receiving large doses of the toxin, and these patients do not benefit from repeated toxin injections. Treatment failure for antigenicity is assessed when there is either absent or reduced response to BoNT. It is assessed clinically using the Frontalis type A antibody test (FTAT) wherein 15-20 U of BoNT/A are injected into two sites of the muscle and ability to raise the brow at 2 weeks is studied. If eyebrows can be raised, it indicates resistance (Brin, 1998). Persistence of motor activity with ability to raise the eyebrow during electromyography (EMG) of injected muscles gives electrophysiological evidence of resistance. Antibodies are also detectable using ELISA, sphere-linked immunodiagnostic assay, western blot assay and combined fluorescein- and enzyme-linked assays (Brin, 1998). Unfortunately, correlation between clinical resistance and detection of antibodies by assays, especially ELISA, has not been established. Mouse neutralization assay /mouse protection assay is considered the gold-standard assay, whereby injected mice will not die if antibodies are present in the test serum, though the meaning of the test in clinical practice is controversial. Early reports indicated that neutralizing antibodies occurred in 3-10% of cervical dystonia patients treated with BoNT/A. The more recent preparations (available after 1997) have lower protein content, andare believed not to produce neutralizing antibodies possibly due to lower protein load.

Patients with resistance to BoNT/A may benefit from botulinum toxins B or F (commercially BoNT/F is not available presently), as their immunogenicity profiles are different (Dresseler, 2000). Care must be taken when the toxin is administered because it can diffuse from the inoculation site through tissue to paralyze neuromuscular junctions of adjacent muscles, causing unwanted side effects such as muscle weakness and dysphagia. Immune responses to BoNT/A and presumably other BoNT preparations can be minimized by using the least required dose and avoiding frequent repeat injections. Current labeling in most countries suggests 12-week separation between doses of BoNT.

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Article 33

Prevalence of Coccidiosis in Cattle in Kashmir valley

B. A. Pandit

Nine hundred seventy one cattle calves under two different managemental practices were screened for different eimerian oocysts, out of which 711 were found to be positive for Eimeria parasites. 70.7% and 75.8% of calves from organized and un-organized managements were found to harbour Eimeria infection, respectively. The species identified included: Eimeria bovis, E. zuernii, E. bukidnonensis, E. subsphrica, E. auburnensis, E. ellipsodalis, E. Canadensis and E. cylindrica among which. Eimeria zuernii and E. bovis were most predominant species.

KEYWORDS
Eimeria, Calf, Kashmir.

INTRODUCTION

Coccidiosis is one of the most alarming problem for calf rearing industry and is responsible for morbidity and mortality. The most common clinical manifestations include inappetance, weakness, loss of weight, diarrhoea, depression and anaemia (Levine, 1985; Soulsby, 1982). In view of the lack of authentic information available regarding the prevalence of *Eimeria* sp. affecting cattle calves in Kashmir valley, the present study was undertaken to find out the prevalence and identify various species of *Eimeria* affecting cattle in the area.

MATERIALS AND METHODS

Calves maintained under two managemental conditions viz. organized (university & military farm) and unorganized (locally reared calves) were used in this experiment. A total of 971 faecal samples were collected from rectum and were kept individually in polythene bags and labelled as per the management groups. The samples were kept at 4˚C till examination. The oocysts were concentrated for examination by centrifugation with saturated common salt solution and were identified on the basis of morphological characters. The oocysts recovered were kept in two lots of 2.5% potassium dichromate solution ($K_2Cr_2O_7$). The material of one lot was poured in Petri dishes to a depth of 3-4 mm and kept in 'Biological Oxygen Demand' (BOD) incubator at a temperature of 30±2˚C for sporulation. The other lot of culture was kept at 4˚C. The culture of both the lots was examined and morphological characters were studied before and after sporulation (Pellerdy, 1974; Soulsby, 1982).
The data was analyzed by Logit Model (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The results of microscopic examination of 971 faecal samples are depicted in the Table 1. An overall occurrence of 73.2% infection recorded was mostly of a mixed type with two or more *Eimeria* sp. Among the two managemental practices, calves reared under organized farm management showed 70.7% infection while as the free range (un-organised) calves harboured 75.8% infection with *Eimeria* sp. The higher though non-significant rate of infection in free range calves may be attributed to access to oocyst infested grasses during grazing. The infection ranged between 54.7% (December) to 90.6% (March) under organized and 58.9% (June) to 90.2% (April) under un-organized managemental

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practices. The prevalence was the highest in spring from both managemental practices at 80.9 and 85.4% respectively. However, it ranged between 78.3 to 74.1; 70.9 to 78.7 and 58.3 to 68.6 percent during summer, autumn and winter seasons, respectively.

In Kashmir province the temperature starts declining from autumn season and falls to sub-zero levels during winter months as a result of which the environment becomes un-favourable for development of the oocysts. Also during the cold period animals too become stationed in houses due to snow fall. The infection starts increasing by spring season as environmental factors become conducive and more favourable through the summer season. Rajkhowa, et al., (2004) has however observed highest prevalence (84.6%) during monsoon, lowest (27.3%) in pre-monsoon and 41.7% in winter in mithun calves in Nagaland.

The sp. identified from pooled samples were: *Eimeria bovis, E. zuernii, E. bukidnonensis, E. subsphrica, E. auburnensis, E. ellipsodalis, E. Canadensis* and *E. cylindrica*. Out of these species *Eimeria zuernii* and *E. bovis* were most predominant. The predominance of *Eimeria bovis* was earlier reported in domestic animals by Deka et al., (1995), and in mithun calves by Rajkhowa, et al., (2004). Raote et al., (1989) examined 1114 animals of 3 cattle farms in Bombay region and encountered Eimerian sp. were: *Eimeria bivis, E. auburnensis, E. alabanensis, E. zuernii, E. bukidonensis, E. cylindrica, E. ellipsoidalis, E. subspherics* and *E. brasiliensis*.

There were no apparent clinical signs in most of the animals sampled for the study. However, among cases of diarrhoea presented at clinics 20.5% were found positive for one or the other mentioned sp. of *Eimeria*. Few cases of one month old calves passing frank blood (Fig. 1) instead of faecal material were also seen and *Eimeria zuernii* oocysts obtained (full field filled with oocysts, Fig. 2).

This study also aimed to investigate the effects of season on flaring-up of the disease in calves and to suggest some concrete measures in reducing the infection to a good extent. Though there was non-significant ($P>0.05$) variation in the incidence of coccidiosis among various seasons and months under both organized and un-organized system of rearing (Table 1), however, higher incidence during spring and summer seasons due to very conducive atmosphere for development of Eimerian oocysts warrants organized anticoccidial prophylactic measures to be taken up from March to August so that the disease can be kept under control with a reduction in oocysts output as well.

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Table 1

	Organized			**Un-organized**		
	Samples seen	**Samples +ve**	**%age prevalence**	**Samples seen**	**Samples +ve**	**%age prevalence**
March	32	29	90.62	49	39	79.59
April	57	41	71.92	41	37	90.24
May	42	36	85.71	33	29	87.87
Spring	**131**	**106**	**80.91**	**123**	**105**	**85.36**
June	35	26	74.28	39	23	58.97
July	42	33	78.57	40	31	77.50
August	38	31	81.57	37	32	86.48
Summer	**115**	**90**	**78.26**	**116**	**86**	**74.13**
September	30	21	70.00	36	29	80.55
October	32	23	71.87	39	30	76.92
Autumn	**62**	**44**	**70.96**	**75**	**59**	**78.66**
November	47	26	55.31	44	28	63.63
December	53	29	54.71	50	33	66.00
January	41	27	65.85	36	28	77.77
February	39	23	58.97	39	27	69.23
Winter	**180**	**105**	**58.33**	**169**	**116**	**68.63**
Overall	**488**	**345**	**70.69**	**483**	**366**	**75.77**

Fig. 1: Frank blood in fecal sample collected in polythene bag from a 28 day old calf

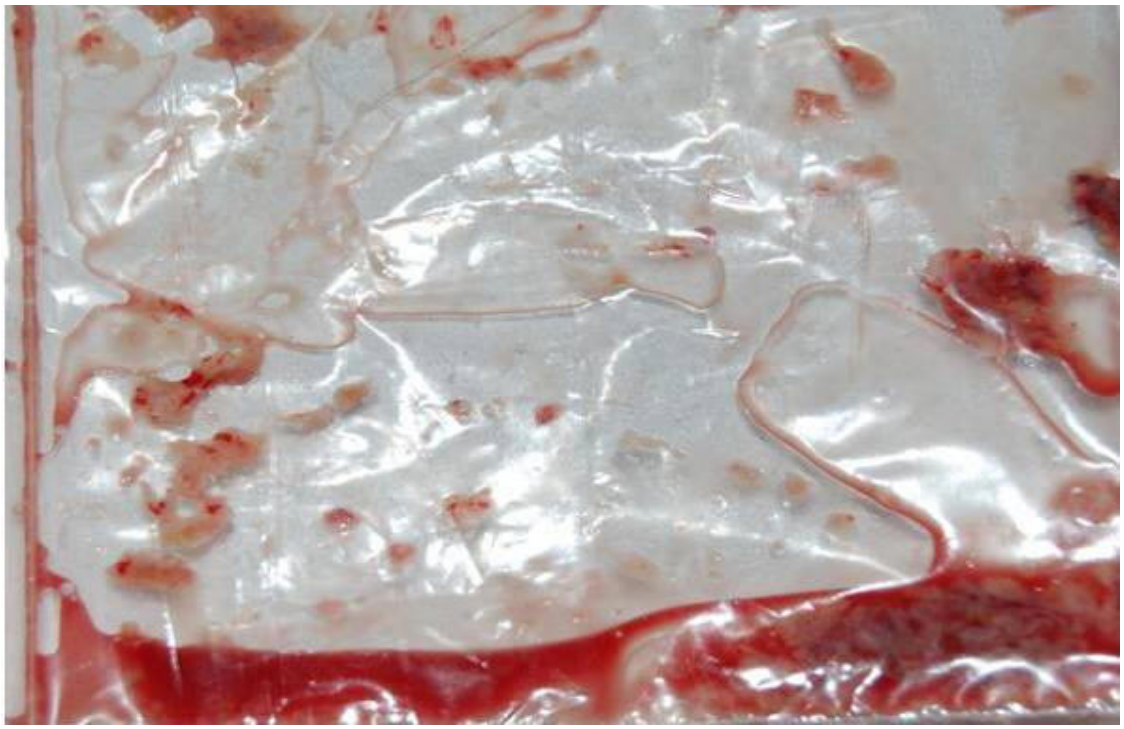
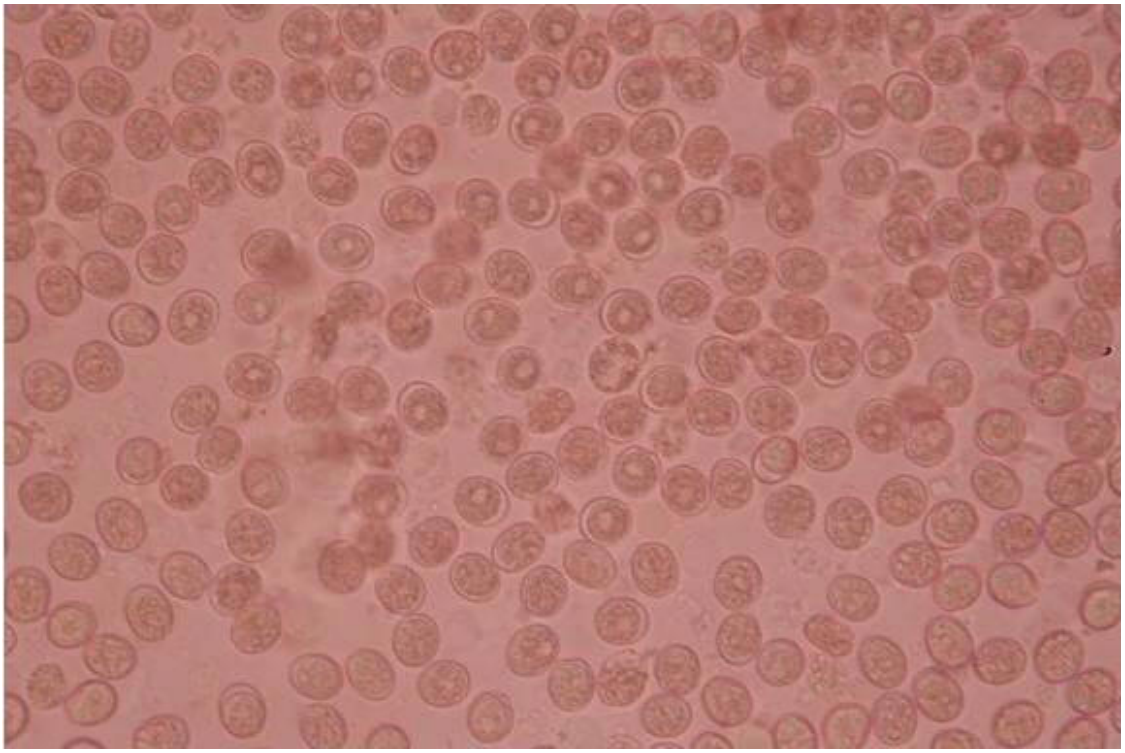


Fig. 2: Processed blood indicating full fields of oocysts of *E. zuernii*



Article 34

Comparison of Different Ketamine-Xylazine Combinations for Prolonged Anaesthesia in Budgerigars(*Melopsittacus undulatus*)

M. Javdani Gandomani[a]*, A. Tamadon[a], A. Mehdizadeh[b], H. R. Attaran[a]

Twenty eight budgerigars of either sex were randomly divided into four groups and injected with ketamine (20, 40, 60 & 80mg/kg respectively) along with xylazine (10mg/kg) intramuscularly. The combination of 80mg/kg of ketamine with xylazine (10mg/kg) was found to produce the longest period (159.43 ± 15.87 min.) of anaesthesia. However, some cataleptic effects of ketamine were also recorded in birds of this group.

KEYWORDS
Xylazine, ketamine, budgerigar, injectable anesthesia.

INTRODUCTION

Proper general anaesthesia is a prerequisite of all major and minor surgeries in birds. Although inhalation anesthesia (using isoflurane) is recommended for birds but non availability of a portable anesthesia unit, especially for the wild birds and its use in field conditions, due to several technical intricacies is impossible [3]. On the other hand, anesthesia induced and maintained by injectable agents is rapid, cheap and needs less equipment. Several injectable drugs are used in birds. They include barbiturates, chloral hydrate, phenothiazine derivatives, alpha 2-agonists, ketamine and propofol [1,9,10,13]. Ketamine is used only in combination with other agents like alpha 2-agonists to neutralize their individual side effects [2,4,12]. Reports regarding use of this combination in surgeries where anaesthesia is required for a protracted period are lacking. Therefore, an experiment was designed to study the stages and duration of anaesthesia using different dosages of ketamine with xylazine in budgerigars.

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MATERIALS AND METHODS

Twenty eight budgerigars aged four months belonging to both sexes and each weighing 30-35g, were randomly divided into four groups (seven per group). Before induction of anaesthesia all the birds were kept off-feed for one hour only. The induction was accomplished at room temperature in a semi-dark, calm environment. Xylazine (Alfazyne®, 2%, Woerden, Netherlands) @ 10mg/kg was mixed with Ketamine (10%, Woerden, Netherlands) @ 20, 40, 60 or 80mg/kg and injected into the brachial musculature of birds of group I to IV respectively. The time taken from injection to ataxia, falling and loss of pain reflex was recorded as the induction time. Intervening period till recovery of the pain reflexes was recorded as the maintenance period. The time required for regaining complete consciousness and standing without ataxia was considered as the recovery period. To confirm the loss of pain during the maintenance period, one centimeter longitudinal cutaneous incision was given at the cranial aspect of thigh (under aseptic conditions). Results and data were evaluated by adopting statistical methods of ANOVA (Tukey HSD).

RESULTS

The values of induction, maintenance and recovery periods from anaesthesia in budgerigars have been presented in table 1.

The time taken for induction in birds of group I was significantly ($P \leq 0.05$) more (85.57 ± 16.06 sec.) than any other group. Although in groups II to IV the values reduced from 44.57 ± 1.51 to 36.85 ± 1.21 sec. yet the differences were non-significant.

General anaesthesia was maintained for the maximum period (159.43 ± 15.87 min.) in budgerigars of group IV. This value was significantly ($P \leq 0.05$) higher when compared to those of the other groups.

The recovery period was significantly ($P \leq 0.05$) longer (60.14 ± 2.12 and 63.43 ± 1.90 min.) in the birds of groups III and IV when compared to those of groups I and II (39.43 ± 1.13 and 42.57 ± 1.40 min.) respectively.

Eye ball movements were noticed throughout in birds of all the groups. All the birds survived during the anaesthetic trial. However, muscular rigidity and convulsions were occasionally observed in birds of group IV.

DISCUSSIONS

In birds subjected to general anaesthesia endotracheal intubation is required to supply oxygen and institute positive pressure ventilation whenever cardiopulmonary emergencies arise. However, in this study tracheal intubation was not attempted in budgerigars as their dry mucosa could hinder the passage of tube and increased resistance to the air flow due to the narrow trachea of this bird was apprehended. The injection site chosen in this study was brachial musculature. Less bleeding was noticed at this site as compared to the femoral muscles in a previous pilot study.

In the birds of group IV high ketamine to xylazine ratio could have resulted in the predominance of ketamine effects (muscular rigidity and convulsions).

Ketamine is the most frequently used general anesthetic agent in birds and experimental animals [3,8].

It has good analgesic property [7,11] but muscle relaxation is poor. To overcome this disadvantage it is generally combined with drugs like xylazine [5,6].

From this study it is concluded that the ketamine (80mg/kg)-xylazine (10 mg/kg) combination can be used effectively and safely in budgerigars for induction and maintenance of prolonged anesthesia (up to 2.5 hours). However, the side effects of ketamine were not completely suppressed by xylazine (@ 10mg/kg).

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Table 1
Mean ± SD values of induction, maintenance and recovery from anaesthesia using various xylazine-ketamine combinations in the budgerigars.

Group	Xylazine (10 mg/kg) + Ketamine	Induction (sec)	Maintenance (min)	Recovery (min)
I	20 mg/kg	85.57 ± 16.06 [a]	32.71 ± 2.06 [a]	39.43 ± 1.13 [a]
II	40 mg/kg	44.57 ± 1.51 [b]	45.14 ± 5.37 [a]	42.57 ± 1.40 [a]
III	60 mg/kg	42.71 ± 1.38 [b]	51.57 ± 9.02 [a]	60.14 ± 2.12 [b]
IV	80 mg/kg	36.85 ± 1.21 [b]	159.43 ± 15.87 [b]	63.43 ± 1.90 [b]

[a, b] Values with different superscripts in the same column differ significantly ($P \leq 0.05$).

Article 35

Lacrimal apparatus of Iranian river Buffaloes (*Bubalus bubalis*): Anatomical study

A. S. Bigham[a]* and M. Shadkhast[b]

The gross anatomy of the nasolacrimal duct of ten buffalos (Bubalus bubalis) was studied. Anatomic casts were obtained after infusion of corrosion cast (Rodopas cast) material. The findings were similar to that of cattle described earlier. The present study would be useful for identification of congenital and clinical affections involving the lacrimal system of buffaloes.

KEYWORDS
Nasolacrimal duct, gross anatomical study, buffalo.

INTRODUCTION

Buffalo rearing has great economical importance in the Asian agriculture. They are an important source of draft power, milk, meat, and hide. As per current estimates, 150 million Asian buffaloes produce 77 million tons of milk and 3 million tons of meat. In several countries they also contribute up to 30% of the draft power for agricultural operations (10). Information on nasolacrimal apparatus system in buffaloes is limited. The lacrimal apparatus provides a passage for tear drainage from the eye to the nasal cavity. The system for each eye in most species consists of dorsal and ventral lacrimal puncta, paired canaliculi, lacrimal sac and the nasolacrimal duct (11). The nasolacrimal system of cattle has been studied in detail (6,15). Dacrocystorhinography, the radiographic visualization of the lacrimal apparatus using radiographic contrast media, has been used to study normal anatomy (4,14) as well as pathologic conditions of the nasolacrimal duct in human beings (7), dogs (3), horses (8), sheep (5), cattle (6,15), camels (14), cats (4) and llamas (9,13). The present study describes normal anatomical course of lacrimal duct in buffaloes and would be useful for identification of congenital or clinical affections.

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MATERIALS AND METHODS

Ten adult buffalo heads (separated proximal to the third cervical vertebra) were collected from a local slaughterhouse. On gross observation, the dorsal and ventral puncta were located in the medial canthus area of eyelids. They were sufficiently wide in diameter and could be catheterized without much difficulty (Fig 1 and 2). The distal opening of nasolacrimal duct was found in nasal cavity and was catheterized with a 4-F tube (Fig 3). Casting material was infused in retrograde direction. 4-6 milliliter of casting material was required to fill the entire length of naso lacrimal duct. The casting material was allowed to solidify. Nasal cavity was exposed from lateral side by removing segments of the lacrimal, zygomatic, maxillary & incisive bones. The lacrimal apparatus was then examined and its details recorded. Visualization of the medial side was facilitated by removal of the ethmoturbinates, ventral conchae and lacrimal bone.

RESULTS

On gross examination the lacrimal puncta appeared slit-like openings, 0.5-1.0 mm in diameter, 4.0-10.0 mm away from the medial

canthus located 1.0 mm from the mucocutaneous junction of the palpebral margin. The paired ventral and dorsal canaliculi were 7 to 9 mm long and converged into a small dilatation, the lacrimal sac located in the orbit on the fossa of the lacrimal bone. The nasolacrimal duct extended from the lacrimal sac to the nostril in the wall of the nasal cavity (fig 4). The proximal portion of the lacrimal duct (75 to 80 mm in length) was in the osseous lacrimal canal. The average total length of lacrimal duct was 260 mm. The osseous lacrimal canal with a slight curve at its origin was directed rostrally. It passed the lacrimal, zygomatic, and maxillary bones lateral to the lacrimal sinus before moving dorsal to the maxillary sinus. The middle portion of the nasolacrimal duct passed through the osseous lacrimal canal after crossing the ventral conchal crest. The duct covered only by nasal mucosa and a thin connective tissue membrane then traversed the nasal cavity in a curved (descending) fashion. The distal opening of the nasolacrimal duct was 1.5 to 2 mm in diameter located on the medial surface of the lateral nasal wall about 40 mm above the dorsal angle of the nostril.

DISCUSSION

The details of the nasolacrimal system of various domestic animal species have already been described (4,5,13,1,2,12). The findings of this study revealed the details of lacrimal apparatus in buffaloes. The lacrimal apparatus consisted of an orbital part and a nasal part. The orbital lacrimal apparatus consisted of a simple lacrimal sac and paired canaliculi with the dorsal and ventral puncta. All findings were similar to those of cattle (6,15). In pigs only one punctum and a duct are present (11). In dogs the punctum and the duct of the ventral eyelid is occasionally absent (4). The nasolacrimal duct coursed rostrally in a curved fashion. This finding was similar to that of cattle(6,15).However, in llamas the nasolacrimal duct courses rostrally in a sigmoid fashion (13). The nasolacrimal duct in the present study involving buffaloes was uniform in diameter throughout its length unlike horses (8).

ACKNOWLEDGMENT

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Fig. 1: Dorsal (a) and ventral (b) puncta

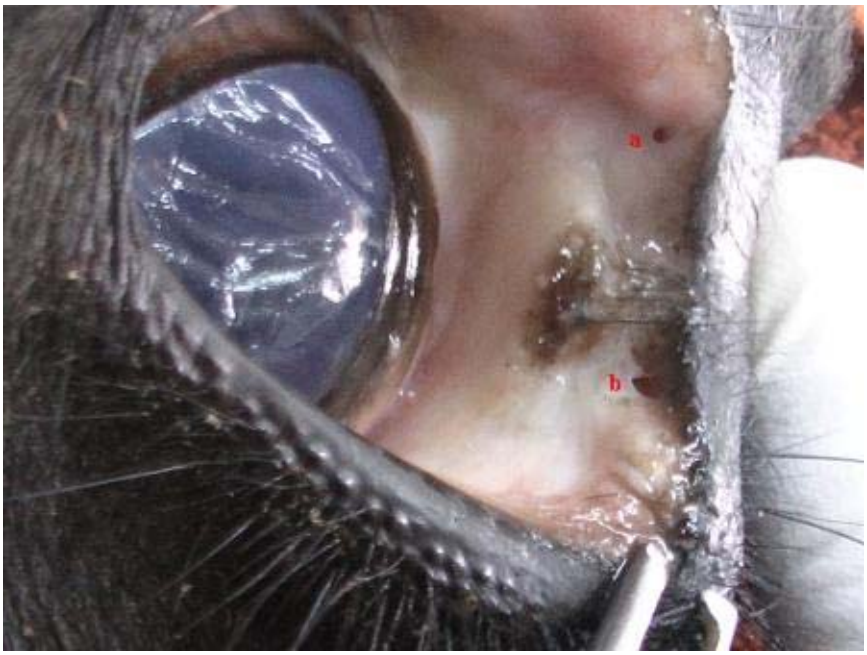


Fig. 2: Ventral puncta catheterization

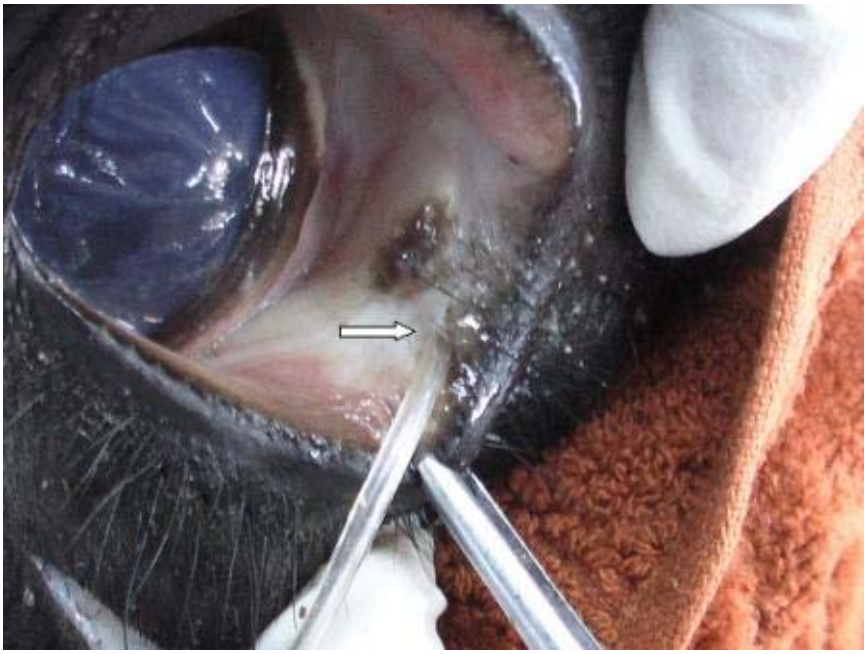


Fig. 3: Distal opening of nasolacrimal duct catheterization

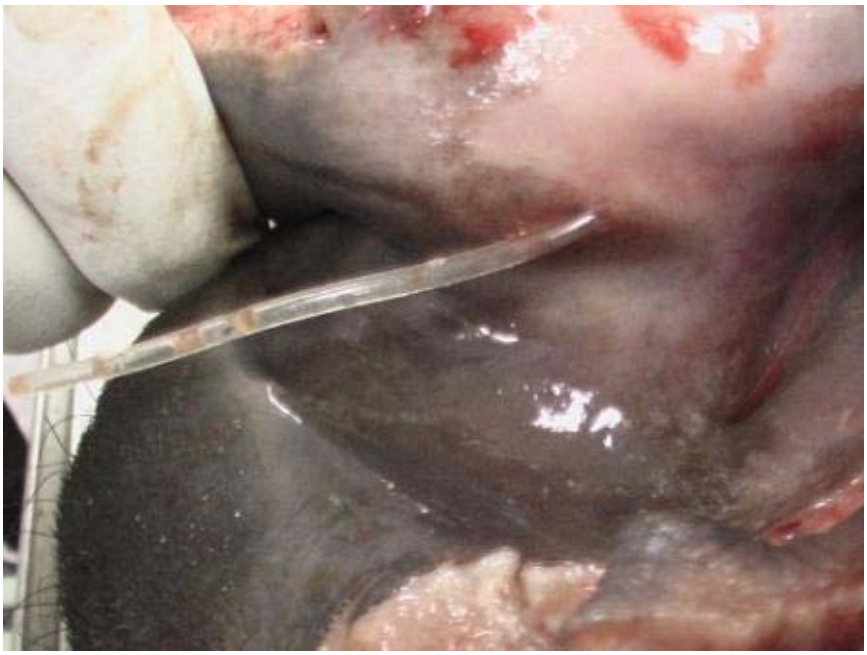
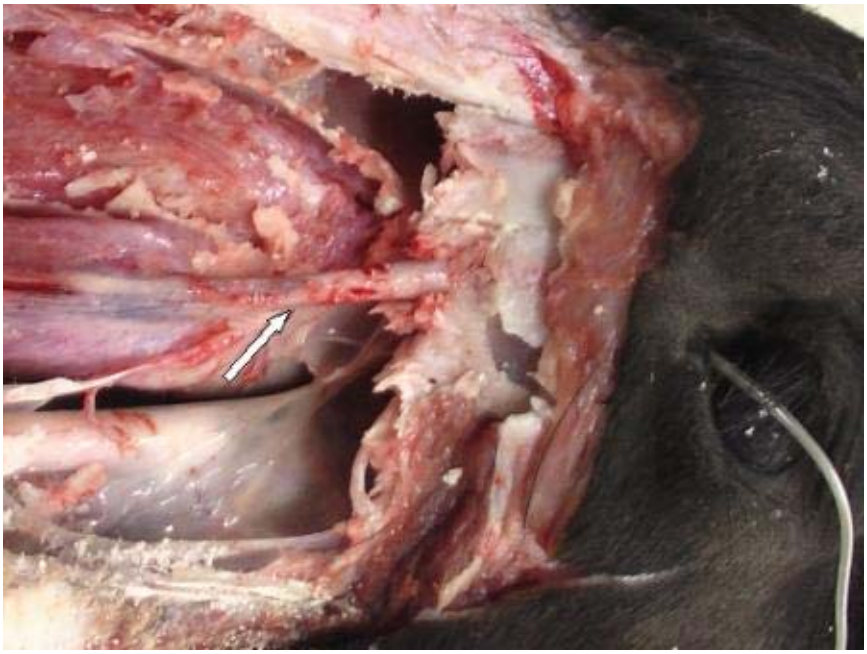


Fig. 4: Proximal part of lacrimal duct on the lateral surface of nasal cavity



Article 36

Differentation of closely related Vaccinal Strains of *Pasteurella multocida* using Polymerase Chain Reaction(PCR)

Waheed ullah[1], Muhammad Abubakar*[2], Muhammad Javed Arshed[2], Syed Muhammad Jamal[2], Najma Ayub[3] and Qurban Ali[2]

Nucleic acid based differentiation of closely related Pasteurella multocida vaccinal strains was performed. Morphological and biochemical characterization, HS-specific and species-specific PCR analysis of Pasteurella multocida vaccinal strains were demonstrated useful in distinguishing hemorrhagic septicemia-causing type B strains. The PCR assay performed for species specific P. multocida by using primer pair KMT1T7 and KMTISP6 resulted in amplification of all the strains. Another PCR analysis carried out for H.S. causing strain conformation by using primer pairs KTT72 and KTSP61 showed that only H.S. causing strains were amplified. It was also observed that PCR amplification performed directly on bacterial colonies or cultures was an extremely rapid, sensitive method of P. multocida identification.

KEYWORDS
Pasteurella multocida, Primers and PCR.

INTRODUCTION

Pasteurella multocida is a small, gram-negative, non–spore-forming cocco-bacillus with bipolar staining. It often exists as a commensal in the upper respiratory tract of many livestock, poultry, and domestic pet species and causes Haemorrhagic septicaemia (HS); a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity. The disease has been recorded in wild mammals in several Asian and European countries. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), corresponding to 6:B and 6:E (Namioka-carter system), are mainly responsible for the disease. In wild animals, serotype B:2,5 is predominantly present. The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (De-Alwis, 1990).

Vaccination against *P. multocida* can be achieved with whole-cell bacterins. However, efficacy is limited and restricted to the homologous serotype. Plain-bacterin, alum-type precipitated bacterin, and oil-adjuvant bacterin constitute the three widely used types of HS vaccine among which oil-adjuvant bacterin is the most effective.

A live intranasal vaccine prepared from a B:3,4 serotype of deer origin is also being used with reported success in southeast Asia (Muneer and Afzal, 1989).

Since the disease been reported even in the vaccinated animals and vaccination failure reports are common therefore the present study was undertaken with the objectives of; morphological and biochemical characterization of *P. multocida*, HS-specific and species-specific PCR analysis of *Pasteurella multocida* vaccinal strains to obtain a clear picture of HS vaccinal strains.

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MATERIALS AND METHODS

Analysis of *Pasteurella multocida*

Three bovine vaccinal strains of *P. multocida*, B:2, B:6 and B:3,4 were investigated in this study. The vaccinal strains were taken from the vaccine seed bank of vaccine quality control section of National Veterinary Laboratory (NVL), Islamabad, Pakistan. The strains were inoculated intra-peritoneal into mice of 3-4weeks age. The mice were slaughtered within 6-7 hours and the mice heart and liver were cultured to obtain pure growth. Staining and biochemical tests (Catalase Test, Oxidase Test, Urease Production Test, H2S Production Test, Nitrate Reduction Test and Motility test) were performed in bacteriology laboratory of NVL to confirm *P. multocida* before further investigation (Cheesbrough 1984).

Genomic DNA Analysis

Pure *P. multocida* culture was inoculated in brain heart infusion broth (BHI) at 37˚C for 24 hours. This culture was used for DNA extraction.

DNA Extraction Method (Antony et al., 2007)

The broth culture of *P. multocida* was transferred to an Eppendorff tube and centrifuged at 3000-x g for about 10 minutes. The pellet obtained after centrifugation was washed and re-suspended in PBS and then centrifuged again. The final pellet was re-suspended in 100 µl of distilled water. The mixture was boiled for 10 minutes in water bath and transferred immediately into ice and snap chilled for 30 minutes. The sample was then thawed and centrifuged at 3000 x g for 5 minutes. The supernatant was separated from pellet and used as template DNA.

PCR Analysis

1) Agarose Gel Running: 2% Agarose gel was prepared in Tris Boric EDTA (TBE) buffer. Ethidium bromide (0.1%) was added and was mixed gently. After polymerization of the gel, samples were added to fill the wells dip. The gel tank was filled with 1X TBE buffer up to the wells dip and run at a constant current of 80Volts. The final gel was viewed by UV trans-illumination.

2) PCR assay (Thermocycler, BioRed, PTA-200 with alpha engine) for analysis of both species-specific and HS-causing Type-B-specific *P. multocida* was performed as follow:

a) *Pasteurella multocida* specific PCR Assay

The primer pair, KMT1SP6 and KMT1T7 was used which specifically amplified a product of approximately 460 base pair (bp) in all subspecies of *P. multocida* (Townsend et al., 1998). The primer sequences were:

KMT1SP6 5'–GCTGTAAACGAACTCGCCAC- 3' &
KMT1T7 5' –ATCCGCTATTTACCCAGTGG- 3'

b) PCR Assay for HS-associated type B serotype of *Pasteurella multocida*

PCR analysis for HS-associated type B serotype *P. multocida* identification was performed. Primer pair KTSP61 and KTT72 were used which specifically amplified a product of approximately 560 base pair (bp) in all HS causing serotype of *P. multocida*.

The primer sequences were:

KTT72 5'-AGGCTCGTTTGGATTATGAAG-3' & KTSP61 5'-ATCCGCTAACACACTCTC-3'

The thermal cycling parameters included: The initial denaturation at 94˚C for 5 minutes; 30 cycles of 94˚C for 1 minutes, 53˚C for 1 minutes, and 72˚C for 1 minute; and final extension at 72˚C for 9 minutes.

RESULTS

Characterization of *Pasteurella multocida*

The study involved microbiological and molecular characterization of vaccinal strains namely B: 6, B: 2 and B: 3, 4. All the cultures showed luxuriant growth on blood agar (Oxoid) having translucent grayish or yellowish green colonies. All the microorganisms studied showed whitish gray rough opaque colonies on BHIA (Brain heart infusion Agar). The organisms when gram stained showed short rods that were gram-negative, coccobacilli, 0.2-0.4 mm in size. All the culture showing typical gram-negative coccobacilli were further processed through biochemical characterization (Table 1). The organisms when grown on TSA in the absence of CO_2 at 37˚C showed luxuriant growth. When inoculated in semisolid medium no motility was

observed. These results were in close agreement with findings of Kumar et. al. (2004).

DNA Extraction from *Pasteurella multocida*

The DNA extracted from all the three strains were run on agarose gel. All the samples gave positive bands for DNA presences. The first set of primer, KMT1T7 and KMT1SP6 was species specific and amplified all strains B:2, B:3,4 and B:6. of *Pasteurella multocida*, corroborating the findings of Townsend et al., (1997).

In another reaction, PCR was carried for HS-causing strain conformation by using primer pair, KTT72 and KTSP61. In this reaction only HS-Causing strains were amplified while Non-HS-Causing was not amplified (Figures 1 & 2). The PCR assay was specific and sensitive. The concordance of PCR results with the defined toxigenic status indicates 100% specificity and sensitivity as described by Carol et al., (1998).

DISCUSSION

Discrimination of the B:2 serotype with the clone KMT1 requires additional hybridization analysis. However, present study revealed that oligonucleotide primers designed during nucleotide sequencing analysis of the clone 6b can be used to identify type B *P. multocida* that causes HS (types B:2, B:5, and B:2,5). It is understood that this assay will not identify all HS-causing strains of *P. multocida*, as these primers do not amplify DNA from type E:2 strains that cause HS in Africa. Townsend et al, (2001), the same results were obtained during present trial as the type B specific primers didn't amplify the type B: 3, 4 (Figure 2).

The ability of the PCR assays described in this study to provide rapid identification of *P. multocida* and confirmation of the HS-causing serotype has the potential to reform HS diagnosis in Southeast Asia. This technique could be implemented to rapidly confirm a field diagnosis of HS without the need to obtain pure cultures and perform extensive biochemical tests. The development of DNA based techniques for differentiation of serotypes could provide best facility as compared to conventional serotyping systems, and has a potential to overcome the deficiencies associated with current serotyping techniques, which depends on inconsistent expression of phenotypic traits.

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Table 1
Microbiological and biochemical characterization of *Pasteurella multocida*

Microbiological/biochemical properties	**Reaction**
Gram stain	-ve
Catalase production	+ve
Haemolysis	-ve
Hydrogen sulphide production	+ve
Urease production	-ve
Oxidase production	+ve
Nitrate reduction	+ve
Indol reaction	+ve
Motility	-ve
Growth on MacConkey media	-ve
Growth in the absence of CO_2	+ve

Fig. 1: PCR Results of amplification of both Non-HS-Causing
and HS-Causing vaccinal strains of *P. multocida* but only HS-Causing strain is amplified

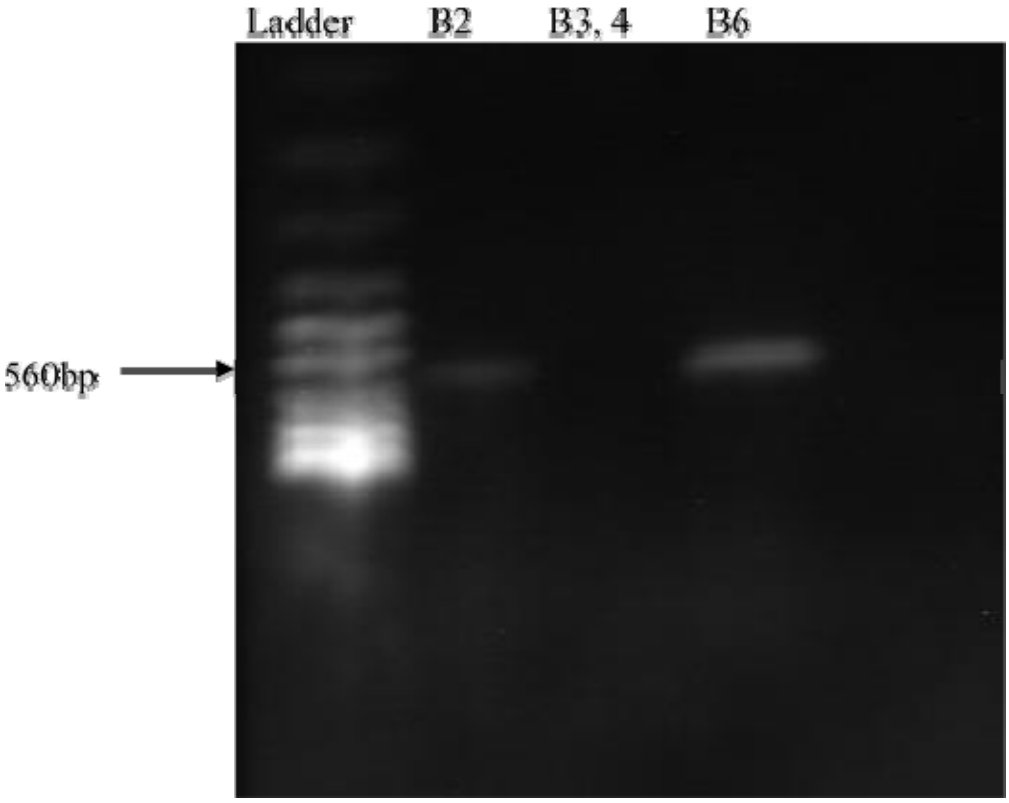
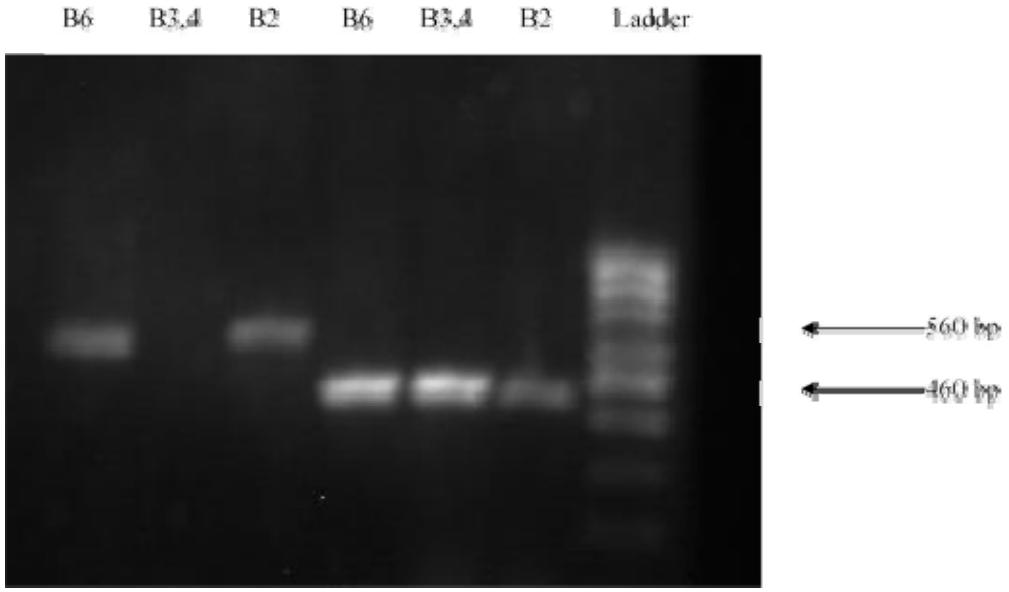


Fig. 2: Comparison of Band pattern of both Non-HS-Causing and HS-Causing vaccinal strains



Article 37

A note on Ivermectin and Clorsulon treatment of Cattle Infested with Subcutaneous Parasites

K. A. Shah[1]* and S. A. Andrabi[1]

Seven crossbred cattle infested with non-specific subcutaneous parasites were given a single subcutaneous injection of a parasiticidal preparation that provided a dose level of 0.2mg ivermectin and 2mg clorsulon per kg liveweight. Additionally, phenylbutazone @ 20 mg /kg liveweight was administered intramuscularly for four days. A complete recovery ensued within 2 weeks of treatment.

KEYWORDS
Ivermectin, Clorsulon, subcutaneous parasite.

INTRODUCTION

Subcutaneous parasitic infestation in cattle in India and abroad is caused by certain arthropods of the *Hypoderma* and *Dermatobia* species and filaroid nematodes of *Parafilaria*, *Stephanofilaria* and *Onchocerca* species which are transmitted by hematophagus vectors.

The affected animals do not show any marked clinical signs until larvae appear along the body coat and nodular or soft fluctuating painful swellings develop under skin at neck, brisket or shoulders and dorsal aspect of the body.

Subcutaneous parasitism inflicts great economic losses to cattle owners by causing reduction in milk production (up to 10 to 20%), loss of body condition, depreciation of value of meat and hide or even mortality in certain cases due to migration of larvae to vital organs (Soulsby, 1986). The present communication reports satisfactory treatment of non-specific subcutaneous parasitic infestation in cattle.

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MATERIALS AND METHODS

Seven crossbred cows, aged between 4 to 7 yrs, exhibited clinical signs suggestive of subcutaneous parasitic infestation. A single subcutaneous injection at a dose of 1ml/50 kg live weight was given aseptically by inserting a 16 gauge needle in loose skin in front of shoulder. This provided a dose level of 0.2mg ivermectin and 2mg clorsulon per kg bodyweight. Two days later, phenylbutazone @ 20mg/kg bodyweight was administered daily (IM) for four days. All animals were kept under close observation for two weeks.

RESULTS AND DISCUSSION

Clinically the affected animals were in poor body condition and yielded significantly low. Painful fluctuating or nodular swellings under skin were observed in brisket region or on shoulders and dorsal aspect of body (Fig 1-4) corroborating with the signs reported by Soulsby loc-cit. After treatment with ivermectin and clorsulon (supported with phenyl butazone), all the seven animals showed a marked clinical improvement during first week and a complete recovery with disappearance of local swellings (Fig 5) was achieved two weeks post treatment. Phenyl butazone was used to counteract effects of toxins liberated if any, by the dead parasites after treatment. These toxins often cause systemic reaction or local inflammatory oedematous swelling (Blood *et al*, 1995). Efficacy of ivermectin against sub-cutaneous parasites from *Onchocerca* and *Hypoderma* species was earlier reported by Klei *et al* (1980) and Soulsby (1986). Antiparasitic activity of ivermectin results from

an increased release of the neuro-transmitter gama amino butyric acid (GABA). Clorsulon , primarily a flukicide and nematocide inhibits enzyme system of the parasite and is reported to be 100% efficacious against helminths and flukes (Campbell and Benz, 2007) and (Nasreen *et al,* 2008). Injectables comprising of 1% w/v ivermectin and 10% w/v clorsulon were reported highly efficacious against subcutaneous and ecto-parasitism due to *Hypoderma*, *Bovicola*, *Sarcoptis* and *Psoroptes* species in goat (Marsy *et al,* 2001), cattle (OSU bulletin)and dog (Marsy *et al*, 2001)., respectively.

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Fig. 1-4: Swelling on skin of affected animal

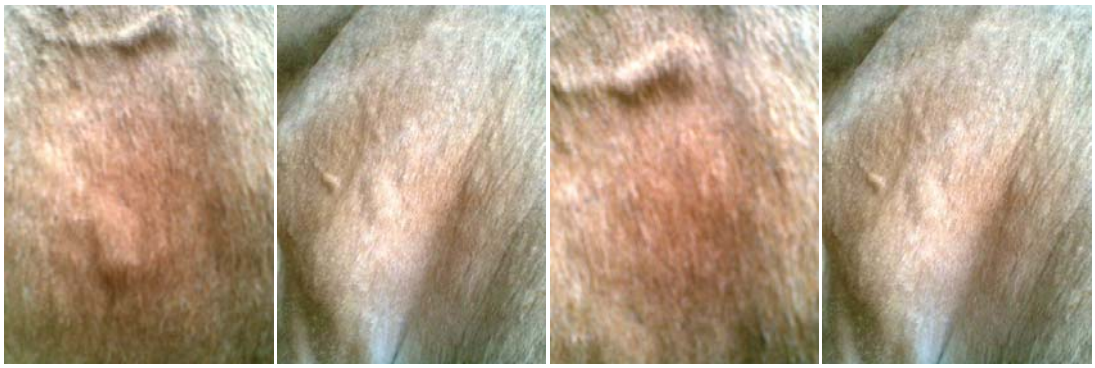
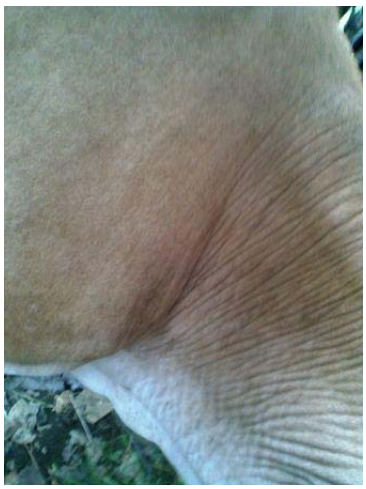


Fig. 5: Post recovery



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