EPIDEMIOLOGICAL STUDIES ON BOVINE PARASITIC OTITIS

BY

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ABSTRACT

Experimental infection with *Rhabditis bovis*, *Pseudomonas aeruginosa* and *Actinomyces pyogenes* was carried out on fifty-three male weaner calves. The calves were inoculated into their ears with seven different combinations of suspensions of *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*, the eighth group (control) was inoculated with phosphate buffered saline (PBS). Twenty-five calves (47.2%) developed mild to severe clinical manifestations in-groups inoculated with the nematode and the bacteria. Calves inoculated with the bacteria alone and the control did not develop clinical manifestations. There was significant difference ($P<0.05$) in responses between groups one, two, three and four which were inoculated with bacteria and nematode and those inoculated with bacteria alone and the control group. The responses were not significantly different ($P\geq0.05$) between groups five, six, and seven which were inoculated with bacteria alone and the control group. Histopathological studies revealed polymorphonuclear cell infiltration, thickening and desquamation of the epithelium of the aural canal. In this study, *Rh. bovis* was shown to be a primary pathogen although histologically there was no evidence of the involvement of the nematode. Bacteria exacerbated the clinical effects only in the presence of the nematode.

Studies on the roles of dips and spray races in the transmission of the nematode *Rh. bovis* were carried out by subjecting 20 healthy cattle to dipping, 20 to spraying using Steladone®, and 20 to a topical application with Ectopor® SA 020 (Ciba - Geigy Limited, Switzerland) as controls. No animal in all three groups contracted the disease. The dip wash and mud from the footbath and in the collecting pens were examined for

the presence of nematodes. Nematodes were isolated from manure from night pens in only one farm and one dip and none from the footbath and collecting pens. Dip tanks were established as temporary reservoir of the nematode only during dipping and nematodes were introduced mainly through ears of infected animals.

The prevalence of *P. aeruginosa* and *A. pyogenes* in the ears of clinically healthy cattle and those clinically affected with bovine parasitic otitis was established by bacteriological examination of 652 samples from diseased animals and 410 from non-diseased animals. Prevalence rates of 1.5% in diseased ears and 5.1% in non-diseased ears for *P. aeruginosa* and 4.9% and 2.5% in diseased and non-diseased ears for *A. pyogenes* respectively were determined. From this study, it has been found that severe clinical disease of bovine parasitic otitis is significantly associated with *A pyogenes* (OR=1.27) while *P. aeruginosa* has a "sparing" effect (OR=0.27) Further studies on the role of flies on the transmission of bovine parasitic otitis are required.

DECLARATION

I, ELIZABETH TABU ELIKANA SHAYO, do hereby declare to the Senate of the Sokoine University of Agriculture that this is my own original work and has not been submitted for a degree award in any other University. Some of the work embodied in this thesis has been presented in the following publications:

1. Shayo, E.T.; Msolla, P.M.; Kassuku, A.A. and Semuguruka, W.D. (1992). The prevalence of *Pseudomonas aeruginosa* and *Actinomyces (Corynebacterium) pyogenes* in clinically normal bovine ears and those infected with *Rhabditis bovis*. In: *Proceedings of the Tenth Tanzania Veterinary Association Scientific Conference. Arusha, Tanzania.* (Edited by Msolla, P.M. and S.I.) **1,** 148-157.

2.Shayo, E.T.; Msolla, P.M.; Kassuku, A.A. and Semuguruka, W.D. (1994). The role of *Rhabditis bovis*, *Pseudomonas aeruginosa* and *Actinomyces pyogenes* in the pathogenesis of bovine parasitic otitis. *Tanzania Veterinary Journal.* **10,** 141-147.

3.Msolla, P.M.; Kassuku, A.A.; Shayo, E.T.; Mhairwa, A.P. (1994). The efficacy of 1% moxidectin in the treatment of bovine parasitic otitis. *Tanzania Veterinary Journal.* **10,** 109-112.

Signature.....

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DEDICATION

To my husband Dr. Mungubariki Elirehema Shayo, my children Apollo, Flora, Helen, Grace, and Frida.

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ABBREVIATIONS AND SYMBOLS

BA	Blood agar
BHC	Benzene Hexachloride
BPO	Bovine parasitic otitis.
CFU	Colony Forming Units.
CNS	Central Nervous System
CV	Coefficient of variation
DAFCO	Dairy Farming Company
DDT	Diethyldimethyltetracetate
DF	Degree of Freedom
DPX	diestrene (a polystyrene) a plasticizer and xylene
ECF	East Coast Fever
EF	Elongation factor
HE	Haematoxylin and Eosin
HMU	Heifer Multiplication Unit
GLM	General Linear Model
LB22	Local Breed 22
LSD	Least Standard Deviation
MODECO	Morogoro Development Cooperation
MR	Methyl red
MSE	Mean Standard Error
NAD	Nicotinamide adenine dinucleotide
NARCO	National Ranching Company

OR	Odds ratio.
PBS	Phosphate buffered saline.
PMNs	Polymorphonuclear cells
RBC	Red Blood cell(s)
RR	Relative Risk
SAREC	Swedish Agency for Research in Developing Countries
SAS	Statistical Analysis System.
SE	standard error
SUA	Sokoine University of Agriculture
TSHZ	Tanganyika Shorthorn Zebu
UV	Ultraviolet
VP	Voges Proskeur
v\v	volume by volume
w\v	weight by volume
g	gram(s)
kg	kilogram(s)
ml	millilitre (s)
mm	millimetre (s)
h	hour(s)
°C	degrees Celsius
Mc	MacConkey
Ltd	Limited
pH	Hydrogen ion concentration

μ	micron
α	alpha
ß	beta
≥	greater than or equal
>	more than
<	less than
%	percent
spp	species (plural)
P	probability
E	east
S	south
a.m	ante meridian
e.g	example given
i.e	that is

CHAPTER ONE

1.0 INTRODUCTION

Bovine parasitic otitis (BPO) is predominantly a chronic and occasionally an acute ear infection of cattle and rarely of goats (Jibbo, 1966; Odongo and D'Souza, 1989). The disease is primarily characterized by a chronic dark brown foul smelling aural discharge, wasting and in the terminal stages by central nervous system (CNS) symptoms. Staggering gait and walking with the head tilted towards the most affected ear manifests the central nervous symptoms. The disease is more prevalent in mature animals where the chronic form is the main feature than in immature which manifest the acute form of the disease (Msolla *et al.*, 1987). The disease in both age groups results in lowered productivity in terms of growth rate, thus increasing age at slaughter, lowered milk production in lactating animals as well as deaths and hence its economic importance.

The disease was first reported in 1959 by Malloy in a ranch in the northern eastern coast of the Tanzania (Jibbo, 1966). The incidence of the disease when first reported was low and confined to the hot and humid coastal areas of Tanzania (Lweno *et al.*, 1983a). Later the disease has spread to upcountry regions due to the free movement of animals where in some areas it has caused serious losses (Msolla *et al.*, 1985). To date the disease is well established in most parts of the country affecting individual herds in ranches and dairy farms and more so in the central and coastal regions of

Tanzania. Apart from Tanzania the disease has also been reported from Kenya (Round, 1962), Brazil (Walter, 1985), Botswana, Ethiopia, Sudan, and Zimbabwe (Msolla *et al.*, 1989).

The recent widespread nature of the disease within the country and the region is an interesting development meriting detailed epidemiological studies. Epidemiological studies to be undertaken are specifically on pathogenesis of various isolates which have remained obscure or have little experimental evidence as to their role in the pathogenesis of the disease. Equally important is the study on the transmission of the disease for effective treatment and control programmes. The disease has been claimed to be caused by *Rhabditis bovis*, a free living nematode and whose morphological features were described by Kreis, (1964). Life cycle and pathology of field cases were described by Lweno *et al.*, (1983a and 1983b) while the clinical features and treatment by Msolla *et al.* (1985 and 1987). As to the mode of transmission, dips have been incriminated as the main reservoir of the nematodes as the disease has been observed to be more prevalent in dipped (>70%) than in sprayed (5%) cattle (Msolla *et al.*, 1986a). Flies mainly *Musca domestica* and *Stomoxys spp.* has been incriminated in the transmission of the disease, an aspect requiring further experimental evidence. Soil and manure are also known to harbour the nematodes (Msolla *et al.*, 1989), but how the nematodes get to the ears of cattle has remained obscure. In view of the inadequate epidemiological information on the disease, the objectives of this study were to:

(1) Establish the prevalence of *Pseudomonas aeruginosa* and *Actinomyces pyogenes*, hitherto known to be common isolates of clinically affected animals, so as to form a basis for associating them with the disease.

(2) Study the roles of *Rh. bovis*, *P. aeruginosa* and *A. pyogenes* in the pathogenesis of the disease.

(3) Study the roles of dips, spray races, soil and manure in the transmission of bovine parasitic otitis and the viability of *Rh. bovis* in two-fold serial dilution of 0.17% Steladone®.

The understanding of the above will be instrumental in the formulation of tangible treatment and control programmes of the disease.

CHAPTER TWO

2.0 LITERATURE REVIEW ON BOVINE PARASITIC OTITIS

This review places emphasis on; Bovine parasitic otitis, otitis in other animal species and the selected bacteria (i.e. *Pseudomonas aeruginosa* and *Actinomyces pyogenes*) for the epidemiological studies on bovine parasitic otitis.

2.1 Otitis in domestic animals

Inflammation of the ears (otitis) in domestic animals is common in small animal species in particular the dog, cat and rabbit (Farrag and Mahmoud, 1953; Fraser, 1965; Grono, 1969; Ozaki *et al.*, 1990; Gentilini *et al.*, 1991), as well as in man (Shanon *et al.*, 1972, Hubbard *et al.*, 1957). In the dog, otitis externa is one of the commonest conditions encountered in canine practice (Bedford, 1989). The etiologies are complex but several factors have been positively correlated with the disease. The presence of ectoparasites mainly *Otodectes cynotis* has been associated with otitis where they have been reported to be responsible for 5-10% of canine and 50% of feline cases of otitis (Griffin, 1990). Bacteria such as *Staphylococci spp, P. aeruginosa* and *Proteus spp.* have been the common isolates from clinical cases of otitis in dogs (Grono, 1969; Fraser *et al.*, 1961). *Staphylococcus intermedius* has been isolated from up to 47.6% from healthy canine ears and a common isolate in acute otitis externa (Griffin, 1990). However bacteria, have been shown to play a

significant role only in the presence of aural lesions even though no generalized skin infection exists (Doxey, 1983).

Among the predisposing factors which enhance aural lesions include the anatomical structures of the ear, which are important in the initiation of inflammation. For example, the hanging flap type of pinna of the Cocker Spaniel and the Basset Hound denies adequate ventilation of the ear canal thus leading to accumulation of ear wax and tissue debris. Similarly, the narrow lumen of the canal in the Miniature poodles and the tight aural folds do retain earwax readily. Excessive hair contributes to build up of wax and prevents adequate drainage and ventilation. All these factors cause moist and warm conditions suitable for bacterial multiplication. Others are foreign bodies such as grass awns or skin diseases set up the primary traumatic irritation which do allow bacterial invasion to occur. The infection is characterized by a discharge, which is determined by the type of bacteria present. For example, incase of *Proteus spp.* the pus is yellowish while with the *P. aeruginosa* infection, the pus is greenish in colour.

In rabbits, several studies have revealed that most cases are associated with *Psoroptes cuniculi* (Rai *et al.*, 1986; Kambarage *et al.*, 1995). A study in India revealed that the complicating bacteria were *Pasteurella multocida* and in some cases mixed with *P. aeruginosa*. Torticollis, circling, inability to take feed and water which resulted into dehydration and finally death (Rai *et al* 1986) characterized these cases. In another study in Tanzania, the disease was found to be characterized by

scab formation extending to involve the outer parts of the ears and purulent exudation in the severely affected animals (Kambarage *et al.*, 1995).

In cats, similar mites as those involved in dogs have been shown to cause otitis. The severe signs of the disease tend to be seen in kittens while the adults appear to tolerate even a heavy infestation (Doxey, 1983). However, in a dog of any age presence of mites is always accompanied by acute reaction characterized by much head shaking, traumatic ear scratching and scanty discharge.

In sheep and goats, otitis is occasionally associated with *Psoroptes cuniculi* infestation and characterized by haematomas, crackled ears and exudative processes (Kambarage *et al.*, personal communication, 1996). Parasitic otitis due the nematode *Rhabditis bovis* has been reported in goats (Odongo and D'Souza, 1989). Bacterial otitis alone is rare few reports have been in lambs as a subclinical infection has been described by MacLeod *et al.*, (1972) and those being dipped (Davies and Done, 1993). Otitis of parasitic or bacterial nature is uncommon in the large animal species such as cattle, horses and buffaloes (Siegmund and Fraser, 1975).

2.2 Bovine parasitic otitis

There is very scanty literature on bovine otitis be it bacterial or parasitic in nature. However bacterial otitis in cattle was first reported by Azizuddin and Chandrasekharan, (1954) during their studies in India of *Pseudomonas aeruginosa*

infections in animals where they were able to isolate the bacteria from six cases of bovine otitis. Bovine parasitic otitis on the other hand, involves both bacteria and parasites.

In Tanzania the parasites which have been incriminated in clinical bovine parasitic otitis are the free-living nematodes *Rh. bovis* (Kreis, 1964) and *Rh. blumi* (Sudhaus, 1974). *Rhabditis bovis* is considered to be more pathogenic *Rh. blumi* as it is usually associated with clinical cases of the disease (Msolla *et al.*, 1986a). This assumption is also supported by the fact that *Rh. blumi* is usually found in large numbers in the soil and manure indicating that it is more of a free living nematode. In Kenya a similar species as those occurring in Tanzania have been demonstrated (Odongo and D'Souza, 1989) while in Brazil, two other species have been identified as *Rh. freitas* and *Rh. costai* which also cause bovine parasitic otitis (Walter, 1985).

Along with the isolation of these nematodes from clinical cases of bovine otitis, several bacteria have also been isolated from such cases. The most frequent isolates have been *P. aeruginosa, Staphylococcus aureus, Staphylococcus albus, Actinomyces bovis, Corynebacterium bovis, Escherichia coli, Streptococci spp., Micrococci spp., Bacilli spp., Proteus spp., Aerobacter spp.* etc. (Lweno *et al.*, 1983a; Msolla *et al.*, 1987; 1989). However, the incidences of these bacterial isolates have not been clearly worked out.

The majority of these bacterial isolates are normal flora of the intestinal tract, mucous membranes and the skin of animals and man and become pathogenic when the defense mechanisms are breached (Merchant and Packer, 1967; Cruickshank *et al.*, 1975; Buxton and Frazer, 1977; Carter, 1973; Gyles, 1986). The nematodes which have been associated with bovine parasitic otitis belong to the genus *Rhabditis* (Dujardin, 1845) and are free living commonly found in water and soils of decomposing organic matter. There are about 200 species which belong to this genus (Thorne, 1961). Although the majority of these nematodes are free living, some have been regarded as accidental parasites of animals and man (Levine *et al.*, 1950; Thorne, 1961; Goodey, 1963; Faust *et al.*, 1970; Georgi, 1974; Walter, 1985) and associated with pathological lesions in plants (Elrod and Braun, 1942; Kabashnaya *et al.*, 1988) and others have been. In cattle several species of *Rhabditis* have been isolated from faecal material (Skyrabin, 1954), skin lesions (Levine *et al.*, 1950, and otitis cases (Jibbo, 1966; Lweno *et al.*, 1983; Walter, 1985).

Bovine parasitic otitis is characterized by inflammation of one or both ears (otitis externa) (Round, 1962; Lweno *et al.*, 1983a,b; Msolla *et al.*, 1985). The initial stages of otitis externa usually pass unnoticed unless close examination of individual animals is carried out (Jibbo, 1966). This is because at this time, the lesions are not established and the discharge is minimal. The discharge becomes visible when the epithelium has been eroded and bacterial invasion has occurred. Such discharge can be readily seen with the naked eye and the nematodes are present in large numbers and are readily seen by the naked eye. The early signs observed include dullness,

inappetence and occasional head shaking due to the irritation caused by the nematodes in the ears. In the acute cases, which are mainly seen in immature animals, there is inflammation of the aural canal resulting in a bloody discharge accompanied by swelling of the ear base which may be manifested by swelling of the local lymph nodes and the eyelids. The condition is painful and the animal grazes little. The inadequate feed intake causes the animal to loose weight gradually. In relatively long-standing acute cases, the discharge becomes dark brown (a mixture of blood and pus) (Msolla *et al.*, 1986b). This discharge has an offensive smell that can be perceived from a distance and attracts flies that can be seen zooming around the affected ear. As the disease progresses, the discharge oozes down the external auditory meatus to soil the area in front and below the ears (Fig. 1). When the discharge dries up, scabs are formed which if removed cause localized areas of alopecia. Later the affected ear becomes droopy due to undermining of the ear base and hangs down. If both ears are affected, the animals tend to move with their heads held low and towards the most affected ear. In some cases the base of the ear may be so undermined by ulceration that the pinna sloughs off. Occasionally, the ulceration of the ear base may be so extensive that the horn becomes loose and may fall off (Msolla *et al.*, 1993).

Extension of the infection into the pharynx from the middle ear has been observed in some animals (Jibbo, 1966; Msolla *et al.*, 1985). The infection may extend further into the inner ear and to the brain (Kreis, 1964; Jibbo, 1966; Msolla *et al.*, 1993). When this occurs, the neck becomes stiff and the head is held low with the affected

side (ear) facing downwards. When the animal moves, it displays a marked circling tendency towards the affected ear and the gait is sometimes unsteady. Animals with central nervous signs will drown in the dip tank during dipping (Mwangulu personal communication, 1992).

Figure 1: A naturally infected animal with acute clinical manifestations showing swelling of the ear base and soiling of the hair below and in front of the ear (arrow1). There is a small bolus of food retained in the mouth (arrow2). Source: Msolla *et al.*, (1993).

The animal hardly grazes at this time and due to lack of feeding, the animal looses condition, becomes very weak and assumes a lateral recumbency with stretched limbs. Sometimes the animal assumes a recumbency similar to that of second stage of milk fever with the head thrown back on the flank (Jibbo, 1966). This position may be maintained for several days before death occurs.

In some long-standing cases there is furunculosis of the external ear canal that often distorts or occludes the lumen of the meatus completely leading to deafness. When such animals recover some of the ears may permanently stay droopy (Figure 2). The morbidity rate is about 70% but rates as high as 90% have been reported (Jibbo, 1966; Msolla *et al.*, 1986b) while mortality rate does not exceed 10% (Msolla *et al.*, 1985). A small percentage of affected animals have been observed to recover spontaneously following a protracted illness (Msolla *et al.*, 1987).

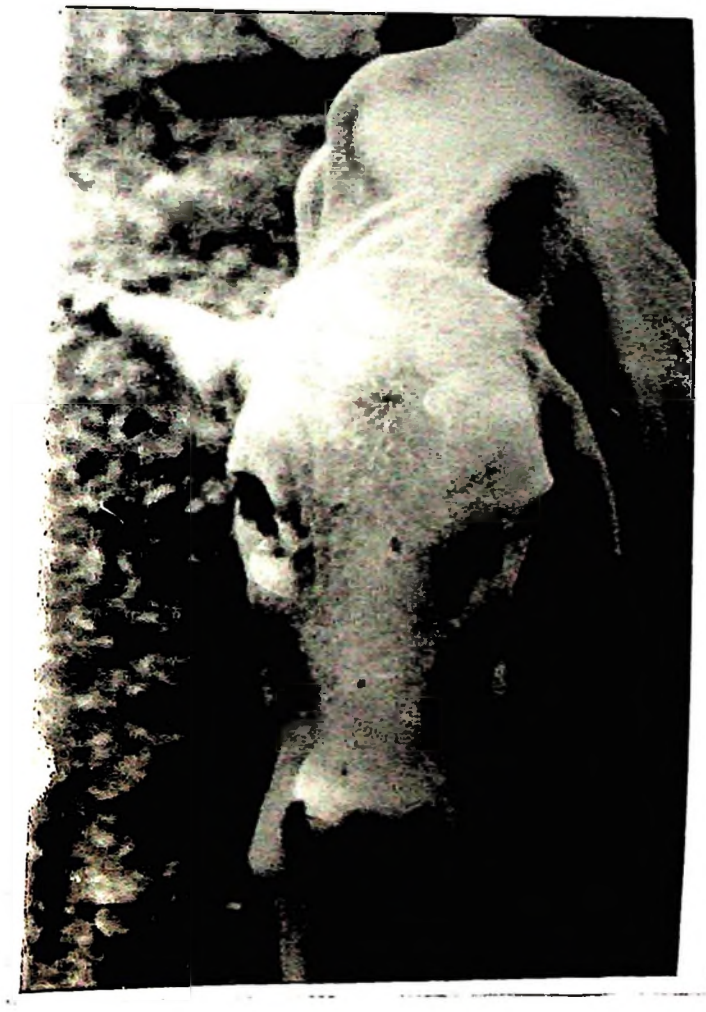

Figure 2: A recovered case of otitis that had the ear canal completely occluded (not shown) showing a left droopy ear. Source: (Mkata NARCO ranch, 1992).

2.3 *Rhabditis spp.* isolated from bovine parasitic otitis

2.3.1 Classification and taxonomy

The free-living nematodes of the genus *Rhabditis* were first described by Dujardin in 1845 (Thorne, 1961). The current classification according to Morgan and Phillips, (1949); Thorne, (1961); Goodey, (1963) and Soulsby (1982) is as follows: -

Phylum: Nemathelminthes (Vogt, Carus, 1863) alimentary canal with anus, separate sexes and unsegmented.

Class: Nematoda (Rudolphi, 1808)

Order: Rhabditida (Oerley, 1880; Chitwood, 1933) Non-spear-bearing secernentea with esophagus divided into corpus isthmus and bulbular regions.

Superfamily: Rhabditoidea (Oerley, 1880; Travassus; 1920) stoma consists of typical shallow cheilostom, elongated protostom and a glottoid apparatus.

Family: Rhabditidae (Oerley; 1880); Micoletzky, 1922) stoma an elongated chamber with short glottoid apparatus.

Subfamily: Rhabditinae (Oerley, 1880; Micoletzky, 1922) Lip region plain without elaborate cephalic appendages, prostom cylindroid strongly scleorotized, glottoid apparatus reduced but distinct.

Genera: *Rhabditis bovis* (Kreis, 1964)

Rhabditis blumi (Sudhaus, 1974)

2.3.2 Morphology

Rhabditis bovis was first described by Kreis in 1964 from ear discharges of a bull infected with nematode otitis in Mkwaja ranch Tanga region, Tanzania. *Sudhaus described Rhabditis blumi isolated from Tanzania and Sudan* in 1974. Most of the features described by Kreis and Sudhaus are similar to those described by Skryabin (1954) and are common to the genera. Emphasis will be given to those features that aid in the identification of *Rh. bovis* and *Rh. blumi* i. e shape and size, organs of digestion and the reproductive system.

2.3.2.1 Shape and size

Rhabditis bovis is a tiny nematode measuring 0.8-1.1 mm in length (Kreis, 1964), while larvae measure 0.35-0.61 mm (Soulsby, 1982). The males are shorter and plump (0.8-0.85 mm) while the females are longer and slender (1.0-1.1 mm). *Rhabditis blumi* is slightly smaller, the females are 0.71-1.2 mm and the males are 0.61-0.95 mm. The cephalic end in both species is narrow and the body gradually increases in diameter posteriorly reaching maximum (40-75μ) mostly in the region of the vulva in the females and midbody in males. Thereafter the body gradually tapers posteriorly and ends as a long and filamentous tail in females of *Rh. bovis* while in *Rh. blumi* the body tapers suddenly and ends in a shorter tail (Fig. 3a).

In males, the tail is either present or non-existence, *Rh. bovis* has no tail and the copulatory bursa is completely surrounded by the bursal bell (peloderan). Whereas in *Rh. blumi* there is a short tail which projects beyond the bursal bell (leptoderan). The bursa is a characteristic rhabditoidal that will be described under the reproductive system.

2.3.2.2 **Organs of digestion**

As with other *Rhabditis*, the cephalic end has six lips whose fusion gives the impression of just three lips. Each lip bears several papillae. The buccal cavity or stoma is divided into **cheilostom** or labial cavity, anterior stoma or **protostom**, and main stoma or **telostom** (Fig. 3b).

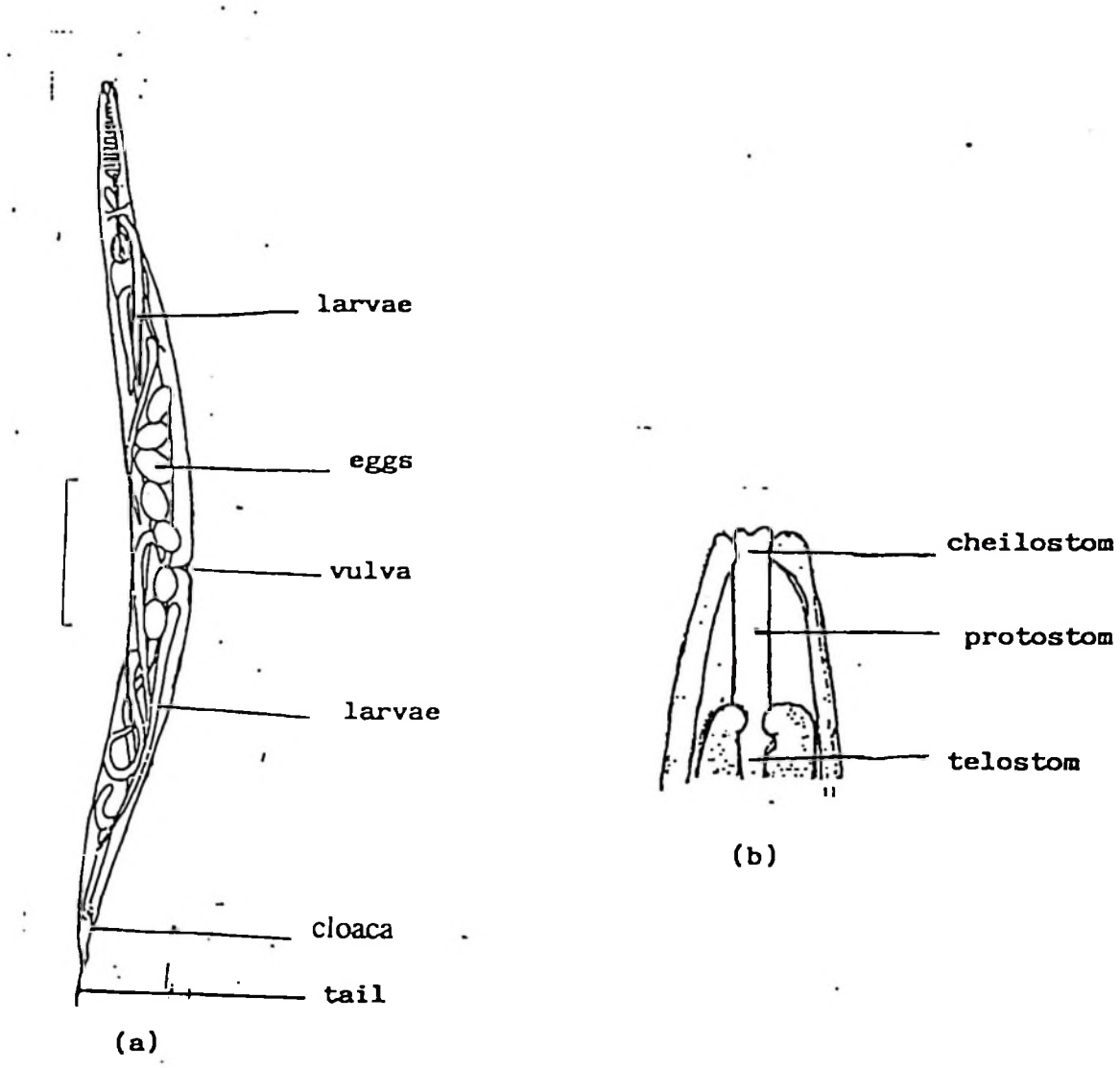

Figure 3: Sketch drawing of *Rhabditis blumi* (a) showing morphological features and *Rhabditis bovis* (b) showing the mouth-parts. Source: (Kreis, 1964; Sudhaus, 1974).

The oesophagus of *Rh. bovis* and *Rh. blumi* is typical of rhabditoidal oesophagus differentiated into three parts. The **corpus**, is the anterior part that covers the posterior half of the buccal capsule (beginning from mesostom). The **isthmus** is considerably narrower than the corpus and generally encircled with the nerve ring and **cardiac**, opens into the intestines and is separated from the later by the cardia (Fig. 4a). The cardia is like a valve, which opens in the lumen of the intestines. The most characteristic feature of the corpus is that the anterior part (metacorpus) is generally enlarged and develops a **median bulb** with a somewhat enlarged lumen in *Rh. bovis* (Fig. 4a).

The median bulb does not exist in *Rh. blumi* (Fig. 4b). The other important feature is the posterior section of the cardiac part, which also dilates into the cardiac bulb with the valvular apparatus. Well-developed muscle fibres characterize the entire oesophagus. There are also three glands located in the tissue of the oesophagus, one dorsal and two submedian. The glands however are masked by muscles and comparatively less developed and thus not distinctly visible in whole mounts.

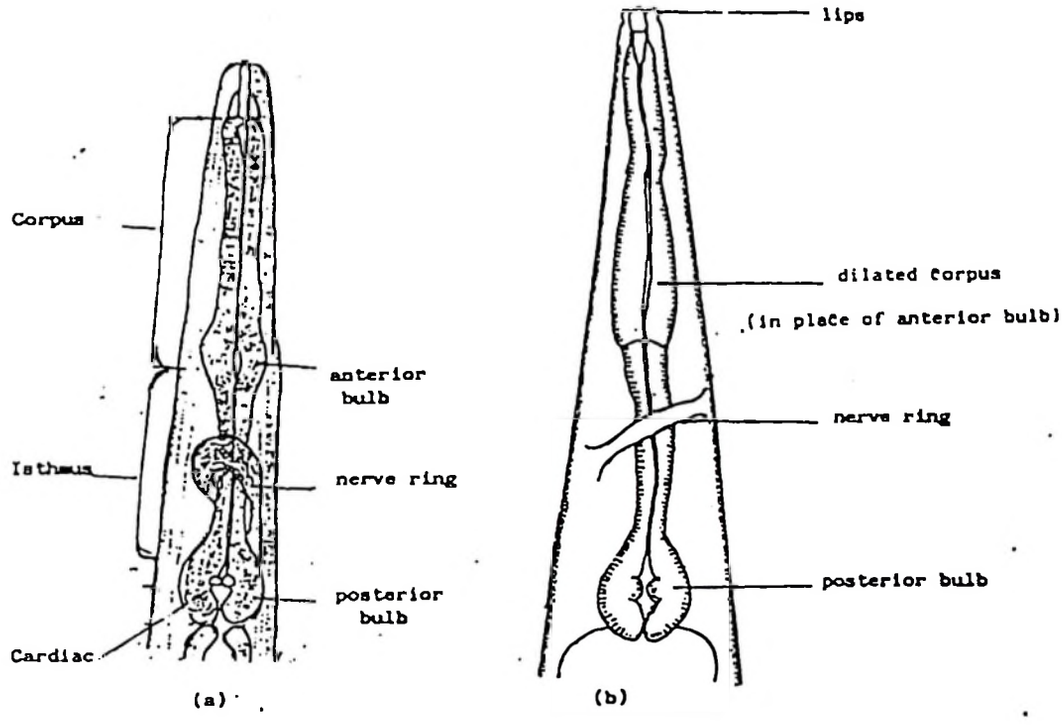

Figure 4: Sketch drawing of *Rhabditis bovis* (a) and *Rhabditis blumi* (b) showing the parts of the oesophagus. Source: Kreis, (1964); Sudhaus, (1974).

2.3.2.3 Reproductive system

Female reproductive system

The female reproductive system consists of two uteri tubes, one anterior and one posterior and the vulva is located at midbody in *Rh. bovis* and is in the caudal part in the anterior half in *Rh. blumi*. Two ovaries are present, one in the anterior and the second in the posterior position. The ovaries are flexed and oocytes arranged in many rows. The uterus is voluminous and contains 10-12 eggs in various stages of development. The nematodes are viviparous and therefore the fully developed larvae can be seen coiled in side the eggs or free in the uterus (Fig. 5).

Male reproductive system

In males the genital tube is divided into testis, seminal duct and ejaculatory duct. The testis is always reflected and contain spermatocytes arranged in rows. The seminal duct is thin walled consistently of columnar epithelium, the cells of which lie in a transverse row through out the entire duct. The ejaculatory duct is divided into two parts: the **glandular** proximal and much longer part; the **non-glandular** ejaculatory duct *per se*.

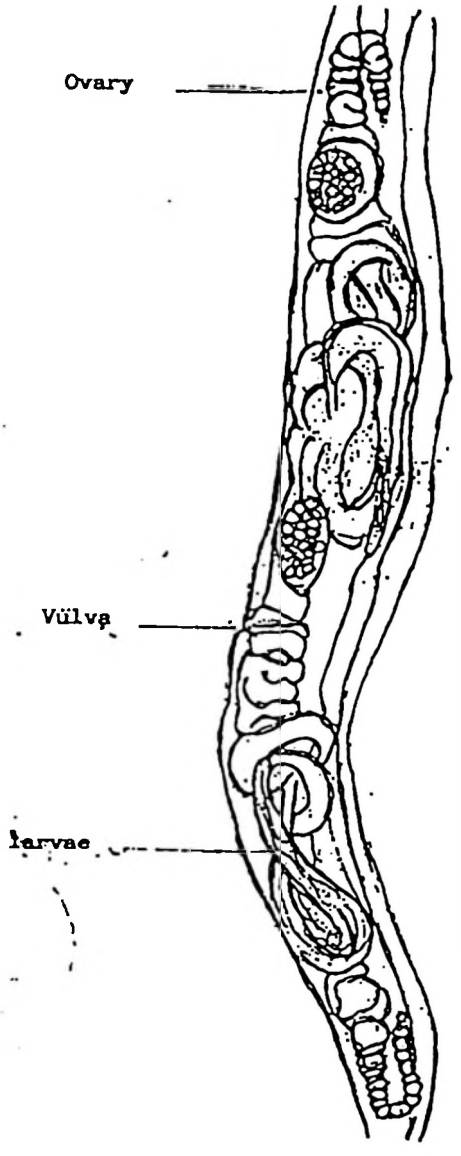

Figure 5: Sketch drawing of *Rhabditis bovis* showing the female reproductive organs.

Source: Kreis, (1964).

The structure of the copulatory bursa is that of rhabditoidal type characterized by high development of bursal alae. The development of the bursal alae and the bell completely and consistently correlate with the development of the tail of the male (Skryabin, 1954).

In *Rhabditis bovis* the tail does not project beyond the bursa i.e. peloderan (Fig. 6a), while in *Rh. blumi* the tail projects beyond the limits of the bursa i.e. leptoderan (Fig. 7a). The bursal rays, which are papillae like structures, are situated laterally on the tail. The rays are arranged radially with the cloaca as a focus, thus there are nine pairs in a bursal formula of 2-()4-3 in *Rh. bovis* (Fig. 6b) and eight pairs arranged 1-2()3-2 in *Rh. blumi* (Fig. 7b). The parenthesis indicate the position of the cloacal opening (anus). These bursal papillae are designated as follows;

(a) **Preanal papillae**-These are situated in front of the cloaca. They are usually shorter and small in size.

(b) **Postanal papillae**-These are situated beyond the anus. They are longer and increase in size towards the tip of the tail. In *Rh. bovis* the second ray posteriorly is shorter than rest of the group and is characteristic of the species (Kassuku, A.A. personal communication 1991).

(c) **Terminal papillae**-They are situated on the border between the proximal conical distal part of the tail, and are smaller than the postanal papillae.

The spicules are separate, wide proximally and pointed distally and are 39-42μ (40.5μ). The gubernaculum has a curve club-shaped appearance laterally while it appears digit like on ventral view. Its distal end broadens to give a plate like appearance.

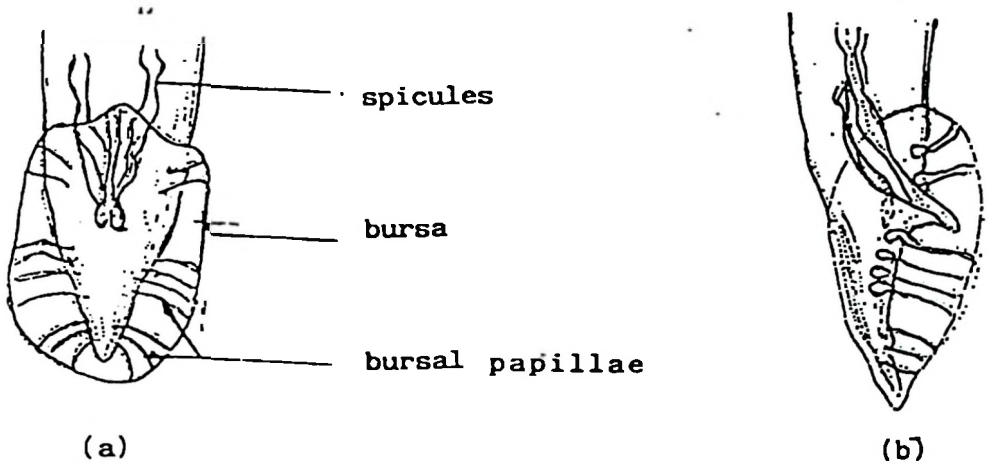

Figure 6: Sketch drawings of *Rhabditis bovis* showing the male reproductive organs, in the ventral view (a) and lateral view (b). Source: Kreis, (1964).

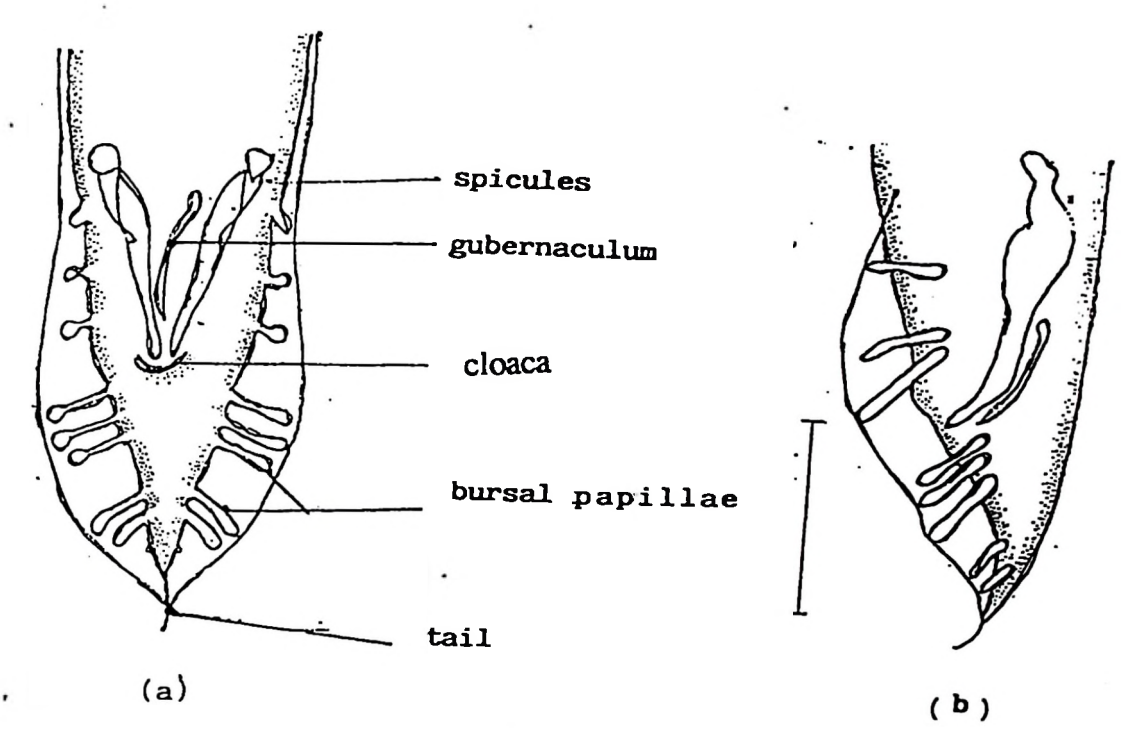

Figure 7: Sketch drawing of *Rhabditis blumi* showing the male reproductive organs. (a) ventral view (b) lateral view. Source: Sudhaus, 1974).

2.3.3 Life cycle

The nematodes of the genus *Rhabditis* are free-living nematodes which do have a direct life cycle with no prolonged resting periods (lethargies) at any stage (Thorne, 1961). The life cycle of *Rh. bovis* has been studied and that it is completed within 24-48 hours (Lweno *et al.*, 1983b). The life cycle of *Rh. blumi* has not been well studied, but it seems to be similar to that of *Rh. bovis* but possibly takes a shorter time (Msolla *et al.*, 1986b). The nematodes are viviparous and the females die in the process of releasing the larvae which range from 10-12 (Kreis, 1964; Msolla *et al.*, 1986a).

2.3.4 Growth on artificial media

The first report on the growth of *Rhabditis* nematodes on artificial media was by Chandler (1924). The nematodes of this genus grow very well on artificial media at most incorporated with bacteria and can be maintained by constant subcultures (Chandler, 1924; Glaser, 1940; Doughtery, 1953a, b, 1960; Nicholas, 1959). Very few species of this genus have successfully been grown in axenic (without bacteria) cultures (Doughtery, 1960).

Both *Rh. bovis* and *Rh. blumi* have been successfully grown on artificial media in the presence of bacteria (Lweno *et al.*, 1983b, Msolla *et al.*, 1986b, Kassuku *et al.*, 1989). Kassuku *et al.*, (1989) found that the abundance of growth depends on the

type of media, and that the nematodes flourished more on horse serum agar medium compared to blood or bovine and sheep serum agar media. Another observation on the growth of *Rh. bovis* and *Rh. blumi* on artificial media was that when these two nematodes are grown together, *Rh. blumi* seem to over grow *Rh. bovis* resulting in more population of the former (Msolla *et al.*, 1986b). This phenomenon has been a major constraint in establishing pure cultures or axenic cultivation of *Rh. bovis*. This observation implies that *Rh. blumi* is more of a free-living nematode than *Rh. bovis*.

2.3.5 Epidemiology

2.3.5.1 Prevalence and distribution

Bovine parasitic otitis was first described in the north east coast of Tanzania at Mkwaja Ranch in Tanga region in 1959 (Kreis, 1964; Jibbo, 1966). At around the same time a similar condition was described in Kenya by Round (1962). These areas have a hot and humid type of climate which favours the development of *Rh. bovis* (Jibbo, 1966). Later on the disease was found to exist in the whole of the coastal area of the country which has the same type of climate (Lweno *et al.*, 1983a). Recently the disease has been spreading fast to other upcountry areas where some serious outbreaks have been reported. The closure of the Uvinza NARCO ranch in Kigoma region due to a severe outbreak of bovine parasitic otitis is a case in point (Msolla, P.; Makundi, A.1991 unpublished report).

In Morogoro and Coast regions where most of the work on the disease has been carried out; the disease is prevalent in almost all of the ranches and government farms including the individual livestock owners (Msolla *et al.*, 1986a, 1986b, 1987, 1989). The areas sited in Morogoro region include Dakawa, Mkata, Morogoro Development Corporation (MODECO), and Wami ranches and individual livestock owners. In the Coast region, these areas include Lugoba in Bagamoyo, Kibaha, Ngerengere Dairy Farming Company (DAFCO) and Ruvu ranch (Lweno *et al.*, 1983a; Msolla *et al.*, 1989). In all these areas, the prevalence of the disease is estimated to be 70% while in some ranches, prevalence has been reported to be as high as 90% (Msolla *et al.*, 1989).

Apart from Tanzania, the disease has also been reported in Kenya (Round, 1962; Odongo and D'Souza, 1989), Uganda, Sudan, Ethiopia, Zimbabwe, and Botswana (Msolla *et al.*, 1989). Outside Africa the disease has also been reported in Brazil, where it is caused by different *Rhabditis spp.* (Walter, 1985).

The nematodes which belong to the genus *Rhabditis* are free-living nematodes found in fresh and salty water, soils of decaying organic matter, and have been associated with pathological lesions of plants and as accidental parasites in animals including human beings (Levine *et al.*, 1950; Thorne, 1961; Goodey, 1963; Kreis, 1964; Faust *et al.*, 1970; Zukerman *et al.*, 1971; Georgi, 1974; Lweno *et al.*, 1983a; Walter, 1985; Msolla *et al.*, 1985; 1986a; 1986b; 1987; 1989). Some of *Rhabditis spp.* are also found in invertebrate animals such as earthworms and dung beetles as temporary

hosts (Smyth, 1962; Lee, 1965; Bird, 1971; Edwards and Lofty, 1972), and in certain types of snails (Brockelman and Jackson, 1978).

2.3.5.2 Survival mechanisms

The survival mechanisms of *Rh. bovis* and *Rh. blumi* are not clearly known. Msolla *et al.*, (1989), in their epidemiological work found that there was a positive correlation between nematode populations and moisture content of manure\soil. This could explain the high incidence of the disease during the rainy season as compared to the dry season. During the dry season, the nematodes possibly survive by burrowing deeper into the soil as do other *Rhabditis spp.* (Thorne, 1961). The nematodes are also maintained through the seasons in the ears of chronically infected cattle which in turn shed the nematodes into the environment (Msolla *et al.*, 1989).

2.3.5.3 Hosts

Both *Rh. bovis* and *Rh. blumi* have been reported in cattle (Lweno *et al.*, 1983a; Msolla *et al.*, 1985, Odongo and D'Souza, 1989). However, *Rh. bovis* has also been reported in goats as a cause of otitis in Kajiado district of Kenya (Odongo and D'Souza, 1989).

2.3.5.4 Age and breed prevalence

Jibbo in 1966 observed that the disease was more prevalent in Zebu than exotic cattle and attributed this to be due to the anatomical differences of the external acoustic meatus. The assumption was that the acoustic meatus of Zebu is longer and more torturous than that of exotic cattle. This type of anatomical morphology creates a favourable environment for reproduction of the nematodes because of the retention of warmth and moisture. This hypothesis however, seem circumstantial than data derived (Msolla *et al.*, 1985) and would require additional work for justification. Round (1962) in Kenya also observed that the disease was more common in boran cattle that are of zebu in origin.

Age has also been described to influence the incidence and severity of the disease. The disease has been observed to affect all age groups but the immature animals are more severely affected with the acute form of the disease (Msolla *et al.*, 1989). The adults do suffer the chronic form of the disease.

2.3.5.5 Reservoir of infection

Rhabditis worms are found in soil especially of decaying organic matter e.g. manure and decomposing plants (Thorne, 1961; Lee, 1965; Nicholas, 1959). They are also found in salty and fresh water (Goodey, 1963) and are also associated with pathological lesions in plants (Elrod and Braun, 1942; Thorne, 1961).

Rhabditis bovis and *Rh. blumi* so far have been isolated from soil and manure in areas where the disease has been reported to occur (Msolla *et al.*, 1989). The nematodes are found in large numbers where animals spend most of their time especially in night holdings and collecting pens. The infected animals especially the chronically affected ones, are the usual source of infection to healthy animals (Msolla *et al.*, 1989). Dip wash has been incriminated as a reservoir of the nematodes as it has been shown that nematodes do survive in normal strengths (0.25%) of Toxaphene® (chlorinated camphene)(Msolla *et al.*, 1986a).

2.3.5.6 Mode of transmission

The nematodes, *Rh. bovis* and *Rh. blumi* have been found in the soil, mud and manure as well as in the ears of infected animals (Jibbo, 1966; Msolla *et al.*, 1989). The mechanism by which the nematodes reach the ears of healthy animals is not clearly known. However, there are several hypotheses on the mode of transmission which have been put forward as a result of field observations. Jibbo, (1966) hypothesized that when animals scratch altered and irritating ears with their hooves as a result of trauma or excessive secretions or tick bite, they introduce the nematodes from the manure carried in between their hooves into the ears. In the ears the nematodes find a warm environment where they multiply to reach a critical population and ultimately cause disease. Animals are also probably infected by lying on damp bedding, herbage or debris in which the nematodes breed (Dunn, 1978).

Another possible mode of transmission is the flies belonging mainly to the genus *Musca domestica* (Msolla *et al.*, 1989). This hypothesis has been put forward due to the observation that flies are usually attracted to the foul smelling aural discharges which contain a lot of nematodes. They have also been incriminated due to the fact that a high disease incidence coincides with the large fly population that occurs during the hot and humid season. As a result of the above two observations flies possibly play a role in further spread of the infection as they have been demonstrated as mechanical carriers of infectious agents as well as of larvae of some nematodes carried on their bodies and hairy legs in the process of feeding (Bovien, 1937; Lee, 1965; Greenberg, 1971; Tod *et al.*, 1971).

The other possible mode of transmission is through the dip wash (Jibbo, 1966; Msolla *et al.*, 1985). This hypothesis was first put forward by Jibbo (1966). It was also observed later that the disease was more prevalent in dipped rather than sprayed animals. The prevalence of the disease in dipped animals was more than 70% while sprayed animals had a prevalence of less than 5% (Msolla *et al.*, 1986b). This observation led to some field trials the objective of which was to isolate the nematodes from the dipwash. Samples taken from four ranches, that is Dakawa, Mkata, Wami and Morogoro Development Corporation (MODECO) in Morogoro region revealed the presence of as many as 20 live worms from 100 ml of dip wash. The live worms were observed to survive in used dip wash of toxaphene for at least 15 days and 28 days in freshly prepared 0.25% toxaphene (normal strength for dipping cattle)(Msolla and Hansen, 1983 unpublished report).

2.3.6 Pathogenesis

The infection of the ear with or without the involvement of the nematodes is uncommon in the large animal species (Siegmund and Fraser, 1975). There are however, several factors such as trauma, excessive secretions, tick bites, mites and moisture which have been incriminated in the initiation of the disease (Jibbo, 1966; Siegmund and Fraser, 1975). The anatomical structure of the ears could be of importance in small animals (Bedford, 1989). However, the pathogenesis of bovine parasitic otitis is not clearly understood. Lweno *et al.*, (1983b) believed that the role of the nematode was initially to cause loss of epithelial integrity. However, in view of the fact that the mouth parts are not the biting type (Skryabin, 1954), the mechanism of loss of aural epithelium could probably be due to other factors such as maceration of the epithelium due to the constant wetness or the nematodes produce some products which act on the epithelium to soften it. When the epithelium is lost, the bacteria, some of which are normal flora of the ear, gain access to the lower epidermis and set an inflammatory reaction.

2.3.7 Pathology

2.3.7.1 Macroscopic appearance

During field studies of the disease in some farms along the coastal areas by Lweno *et al.*, (1983b), some pathological studies of some cases were undertaken. The affected

animals were emaciated and the ears had a dirty brown aural discharge in which there were detectable motions of the nematodes. This motion was due to movement of the worms. In severe cases the aural discharge soiled the side of the head and extending to the neck region. On removing the dried up discharges from the external ear, red raw ulcerations were evident. In severe cases the ulceration involved large parts of the visible ear canal while they were patchy in less severely affected cases. According to Jibbo (1966), apart from general emaciation encountered in chronic cases, lesions were confined to the head region. There was reddening of the lining of the auditory meatal canal. Excrescence did develop and these obliterated the external auditory meatus completely. Pharyngitis and lymphadenitis of regional lymph nodes were present when the infection involved the eustachian tube from the middle ear. If the brain was involved, there was gangrene of the lateral side of the medulla oblongata and the cerebellum. The cranial side bordering these lesions was necrotic and thickened with as lightly yellowish tinge.

2.3.7.2 **Microscopic appearance**

Examination of tissue sections from the external ear of field cases by Lweno *et al.*, (1983b) revealed epithelial cell desquamation in some cases but in other cases there was severe loss of epithelium as well as the sub-epithelium. The exudate overlaying the epithelium consisted of necrotic epithelial cells, some lymphocytes, plasma cells, macrophages and neutrophils invariable degrees of disintegration. Seen in the exudate too were worm sections. In the sub-epithelium, the few left epithelial cell

layers had cellular infiltrate of both mononuclear and polymorphonuclear cells. In a few cases the mononuclear cell infiltration was predominant. Vascular engorgement in the subepithelium was also evident.

2.3.8 Diagnosis

The diagnosis of BPO has been based on the clinical manifestations in particular the presence of foul dark brown aural discharges confined in the ear canal or overflow and soiling the area below the ear which in most cases dries up. The epithelial debris inside the ear canal when removed leaves hyperemic raw areas. *Rhabditis* nematodes can be demonstrated in the aural discharge under the stereo microscope (Jibbo, 1966; Soulsby, 1982). However, some field studies have revealed some cases where the animals showed clinical disease but no nematodes were isolated (Msolla *et al.* 1986b). Such cases could be difficult to differentiate BPO from otitis in cattle due to bacteria alone as described by Azizuddin and Chandrasekharan (1954). Other diagnostic features include loss of condition, and central nervous signs mainly tilting of the head towards the affected ear. The ears may have a distorted outline of the pinna or become droopy. The presence of the bolus of the card retained in cheeks in some cases should also be differentiated from foreign bodies or space occupying lesions such as abscesses. The typical dark brown foul smelling aural discharges are pathognomonic.

2.3.9 Treatment and control

Treatment of BPO has been instituted with various compounds/chemicals with varying degrees of success (Jibbo, 1966; Lweno *et al.*, 1983b; Msolla *et al.*, 1985; 1987). Topical application of a combination of 3.9% w/v benzene hexachloride (BHC) (Coopers sheep nasal remedy) with sulphadimidine powder and Nuvan (an organophosphorus compound) loraxene terramycin mixture were instituted by Jibbo, (1966). Copper sulphate 1% w/v has also been used in the treatment of bovine parasitic otitis with little or no success (Lweno *et al.*, 1983b). In Kenya, a mixture of tannic and salicylic acids and a combination of trichlorfon (Neguvon® Bayer) and oxytetracycline have been used. The latter combination showed an efficacy of 75% (Odongo and D'Souza, 1989).

The more promising drug was a 1% w/v solution of ivermectin which was used parenterally at a dose of 10mg/50 kg live body weight resulting into a recovery rate of over 95.3% within 5-21 days. However, with this treatment regime, some of the recovered cases became reinfected after about 60 days possibly through dipping (Msolla *et al.*, 1985). Ivermectin is a broad-spectrum anthelmintic which has been used in various animals for treatment of endo and ectoparasites (Barth, 1983; Meleney *et al.*, 1982; Campbell, 1985). The limiting factor on the use of this drug is that it is very expensive.

Nicotine extract used at 2.08 ppm in 0.25% toxaphene has been shown to be over 95% effective in both the treatment and control of bovine parasitic otitis (Msolla *et al.*, 1987). Nicotine is an extract from tobacco waste, which is an alkaloid. Nicotine has been used in the control of ectoparasites and *Hypoderma* larvae. In South Africa, nicotine has also been used as nicotine sulphate at 0.04% to check against tick resistance in arsenic dips (Hansen, 1984 unpublished report).

2.4 *Pseudomonas aeruginosa*

2.4.1 Morphology and cultural characteristics

Pseudomonas aeruginosa is a gram-negative rod shaped bacterium measuring 1.5-3μ by 0.3-0.6μ. The organism may occur singly, in pairs, or in short chains, and is acid fast. The organism grows well on all common laboratory media at 5°C to 45°C as an obligate aerobe. Growth at 42°C or 45°C has been used as a characteristic feature to distinguish it from other fluorescent *Pseudomonads* (Seleen and Stark, 1943; Haynes, 1951; Rhodes, 1958). It is haemolytic on blood agar but there are variants that do not produce haemolysis (Seleen and Stark, 1943). It grows in a variety of colonial types, most strains produce round or ovoid, moist, and smooth colonies. A metallic sheen seen in cultures save to differentiate this organism from all other *Pseudomonads* (Wahba, 1974). Another feature is the characteristic odour developed by the cultures of this organism which resemble that of trimethylamine (Deubler and Cole, 1955; Ozaki *et al.*, 1990).

Pseudomonas aeruginosa produces three types of pigments into the growth medium. These are: **pyocyanin**; a greenish-blue pigment which is both water and chloroform soluble; fluorescein **pyoverdin** a yellow-green pigment soluble only in chloroform and fluoresces under ultraviolet (UV) light (Carter and Chengappa, 1991). There are occasional strains that produce **pyorun** a dark-red pigment or a brown-black pigment (Carter and Chengappa, 1991). These pigments are known to possess antibacterial

activities (Lusis and Soltys, 1971). Non pigmented (achromogenic) strains are rarely encountered but it is not known exactly to what extent they do occur (Kovacs, 1956; Haynes, 1961; Dourodoff and Paleroni, 1982).

2.4.2 Biochemical tests for identification

Pseudomonas aeruginosa is relatively inactive fermenter of carbohydrates although some strains produce acid from D-arabinose, L-arabinose, D-glucose, D-galactose, D-mannose, and D-xylose. It is Methyl-red (MR) and Voges Proskeur (VP) negative, catalase and oxidase positive. Although Kovacs (1956); Steel (1961) stated that oxidase test is a test of choice for *P. aeruginosa*, this does not hold true as several *Pseudomonads* and bacteria of other genera are oxidase positive such as *P. fluorescence* (Carter and Chengappa, 1991). Other features include indole negative and liquefaction of gelatin.

2.4.3 Epidemiology

2.4.4.1 Prevalence and distribution

Pseudomonas aeruginosa is ubiquitous. The organism is present as the normal flora of the small intestines of animals and man (Hoadley and McCoy, 1968; Matthews and Fitzsimmons, 1964; Mushin and Ashburney, 1964; Contrepois and Govet, 1977). It also inhabits mucosal surfaces of the reproductive tract of both female and male

animals (Hughes *et al.*, 1966a, 1966b; Hossain *et al.*, 1990). *Pseudomonas aeruginosa* is also found on the skin of clinically normal animals (Carter, 1973); Woolcock, 1991) and ears of healthy dogs (Grono, 1969). The organism is also found in sewage (Lusis and Soltys, 1971), in fresh water of lakes and marshes (Ringen and Drake, 1952; Aznar and Alcaide, 1992). The organism has been isolated in treated water where it has been incriminated as possible cause of diseases in animals (Beckenhaeur and Miner, 1960; Robert and Pinheiro, 1967; Hicks *et al.*, 1991). In the soil however because of its high temperature requirement, it is not able to compete successfully with other microorganisms (Hoadley and McCoy, 1968).

2.4.4.2 **Resistance and viability**

Pseudomonas aeruginosa resembles other vegetative bacteria except that it is more resistant to higher dilutions of quaternary compounds and phenolytic compounds (Carter and Chengappa, 1991; Hicks *et al.*, 1991). It also flourishes well in cetrimide which is used as a disinfectant (Bedford, 1989). Most strains of *P. aeruginosa* are susceptible to gentamycin, amikacin, tobramycin, colicillin, and polymyxin B. It is resistant to nitrofurans, trimethoprim-sulphonamide, ampicillin, methicillin, and kanamycin. Resistance to carbenicillin, sulfonamides, neomycin, streptomycin, tetracylines, and chloramphenicol varies from one isolate to another (Gyles, 1986; Anzai *et al.*, 1991).

2.4.5 Pathogenesis

Pseudomonas aeruginosa produces a number of extracellular products that play a role in its pathogenicity. When the bacterium lives as a saprophyte, extracellular toxins are not required for its survival. However, when the bacterium infects an animal, the four amino acids present in animal tissues in the greatest quantities (glutamine, glutamic acid, aspartic acid and alpha-alanine) do provide an ideal environment for the production of extracellular toxins (Lusis and Soltys, 1971) which are responsible for its pathogenicity. The toxins produced are:

i) Exotoxin A

This catalyzes the transfer of the adenosine diphosphate-ribosyl moiety from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF2), thereby inhibiting protein synthesis (Pollack, 1983).

ii) Proteases

The proteases are capable of causing tissue damage by their attack on elastin. They also degrade certain plasma proteins such as immunoglobulins, complement factors, and alpha-proteinase inhibitor (Gyles, 1986).

iii) **Haemolysins (phospholipase)**

Heat-stable and heat labile haemolysins act synergistically with phosphatase on lipids and lethinase to produce necrosis. They also break down pulmonary surfactant resulting in atelectasis (Carter and Chengappa, 1991).

iv) **Leukocidin**

Leukocidin is produced by certain strains and does cause damage of the cytoplasmic membrane of polymorphonuclear leukocytes (PMNs) which undergo lysis.

Disease develops as a result of establishment of infection followed by the production of toxic metabolites. Establishment of infection is accomplished by the pili of bacteria that are mediators of attachment of microorganisms to a variety of cells (Sato *et al.*, 1988). Local lesions caused by *P. aeruginosa* show inflammation and necrosis and may involve invasion of vascular walls.

Degenerative changes also occur in epithelial and endothelial cells as well as leukocytes (Gyles, 1986). Defects in the host resistance are usually a prerequisite for infection by *P. aeruginosa* to establish. The most important predisposing factor is a defect of integument of the skin as occurs in wound infections of various animal species.

2.4.6 Experimental production of otitis

Experimental work to reproduce otitis in various animal species has been carried out by several workers. The animals used in these studies were rabbits, dogs and cats. By instilling a pure culture of *P. aeruginosa* into eight ear canals of dogs, otitis developed in two of them; a mild otitis externa developed in three of six ear canals following trauma to the meatal epithelium (Grono, 1969). Farrag and Mahmoud, (1953) produced otitis externa in six dogs by instilling a saline suspension of *P. aeruginosa* into each ear canal and rubbing the ear vigorously. Salvin and Lewis (1946), were able to cause otitis externa in rabbits with the combination of trauma and infection with *P. aeruginosa*. They were unable to produce a clinical otitis externa with *P. aeruginosa* alone. Msolla *et al.*, 1986b produced otitis in rabbits by using *Rh. bovis* alone, and in combination with *P. aeruginosa* and *A. pyogenes*. No experimental transmission study has been carried out in cattle using *P. aeruginosa* alone or in combination with other bacteria.

The pathogenic ability of *P. aeruginosa* has been extensively reviewed and the literature contains numerous references as a cause of localized and generalized infections in a wide variety of animals, man and plants (Appendix 1).

2.5 *Actinomyces pyogenes*

2.5.1 Classification and taxonomy

Actinomyces pyogenes originally belonged to the family Corynebacteriaceae. However it differs from other animal and plant pathogenic Corynebacteria in that it is catalase negative, produces soluble haemolysin, has a very different cell wall composition and shares a cell polysaccharide antigen with streptococci of Lancefield group G (Morrison *et al.*, 1984). On that basis its classification has for long been in doubt (Jensen, 1952; Harrington 1966). In this respect therefore it has been reclassified into the family Actinomyceae and to the present genus Actinomyces (Carter and Chengappa, 1991).

2.5.2 Cellular morphology

Actinomyces pyogenes is a gram positive pleomorphic rod 0.2 by 0.3-2 μm varying from coccoid to bacillary, club-shaped, curved or in chains of coccal forms and resembles streptococci. The organism is non-motile and does not produce spores. The cellular morphology depends on the medium employed, conditions of growth and the strain (Lovell, 1937, Cobb, 1963). Lovell, (1937) found that when the organism is grown aerobically on blood or serum agar for 48 hours at 37°C, most of the bacteria appear cocco-bacilli, usually arranged in parallel bundles (palisades) or at an acute angle. When grown on blood or serum agar under anaerobic conditions

for two days, the organism tends to be longer and more bacillary and irregular in actual morphology and in staining reactions.

2.5.3 Cultural and biochemical characteristics

Actinomyces pyogenes is aerobe, anaerobe and facultative anaerobe. Growth is enhanced in media containing blood or serum though they are not essential for growth (Lovell, 1937). On blood agar, it produces a wide zone of ß haemolysis almost 2-3 times the size of the colony (Morrison *et al.*, 1984), and due to this characteristic it was at one time thought to be a mutant to *Corynebacterium haemolyticum* of which they share some of the characteristics (Barksdale *et al.*, 1957). The colonies are visible after 48 hours incubation and they are less than one millimetre in diameter. However grayish and glistening, large colonies do occur (Morrison *et al.*, 1984).

The organism is the only pathogenic Actinomyces that produces gelatinase and soluble haemolysin (Lovell, 1937; Cobb, 1963; Carter and Chengappa, 1991). An important biochemical feature of this organism is that it is catalase negative while all Corynebacteria are catalase positive. Other characteristics are fermentation of glucose and lactose, while mannitol is not fermented. In litmus milk the organism produces acid and clot after three days growth and there after the clot is digested.

2.5.4 Epidemiology

2.5.4.1 Prevalence and distribution

Actinomyces pyogenes has a worldwide distribution and is known to be a common commensal on the mucous membranes of the nasopharynx of cattle, sheep and swine and it is in these species that the organism is a frequent cause of suppurative lesions. It may be shed from apparently normal udders of cattle and is frequently recovered from tonsils and retropharyngeal lymph nodes (Carter and Chengappa, 1991). The organism has been isolated from a normal bovine uterus and in the urinary tract (Buxton and Frazer, 1977).

2.5.5 Pathogenicity and pathogenesis

Actinomyces pyogenes causes a wide variety of non-specific purulent infections in cattle, sheep, swine and occasionally in other species. It is a major opportunistic bacterium of cattle where it causes mastitis (Vogt, 1986; Madsen, 1987). Mastitis due *A. pyogenes* has also been reported in sheep (Al-Samarrae *et al.*, 1990) in the horse (Roberts, 1986) and in buffalo (Kapur *et al.*, 1990). Some rare infections caused by this bacterium are: calf diphtheria (Panciera *et al.*, 1989), nephritis in cattle (Buxton and Frazer, 1977), and endocarditis in cattle, horse and pig (Roussel and Kasari, 1989). It has also been isolated from pneumonic lungs in goats (Ugochukwu, 1985) and from sheep (Vyas *et al.*, 1984). Pyometra and endometritis

in cattle associated with *A. pyogenes* is an infection which develops as a result of predisposing factors related to calving, retention of the placenta, and to different stages in oestrus cycle (Buxton and Frazer, 1977). However it's role in otitis in any animal species has not been reported.

Actinomyces pyogenes has a tendency to disseminate from its normal habitat on mucosal surfaces, commonly resulting in abscesses at any site in the body. In abortion it is frequently isolated mixed with other bacterial infections, particularly with *Fusobacterium necrophorum* and *Peptostreptococcus indolicus* because of the production of stimulatory growth factors for these species. Infections also arise when the organisms gain entry into tissues as a result of various injuries and other infections including those caused by viruses, mycoplasmas, and other bacteria.

The factors that are responsible for the pathogenicity of A. pyogenes have remained obscure and little is known (Lovell, 1937, 1944). The possible virulent factors, which have been described, are:

i) **Haemolysin**

Actinomyces pyogenes produces a soluble **haemolysin** active against human, guinea pigs, horse and rabbit erythrocytes. Both the toxic and free extracts are neutralized by antitoxin (Lovell, 1937, 1941; Barksdale *et al.*, 1957). The haemolysin kills mice when given intravenously and produces skin necrosis (Carter and Chengappa, 1991).

ii) **Proteases**

Another virulent factor is **protease**, which is capable of causing tissue damage by its attack on elastin. It is able to degrade certain plasma proteins such as immunoglobulins, complement factors etc, thus contributing to the reduced immune system of the host (Carter and Chengappa, 1991).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The prevalence of *Pseudomonas aeruginosa* and *Actinomyces pyogenes* in clinically normal bovine ears and those infected with *Rhabditis bovis*

3.1.1 Introduction

As stated in the objectives, this field survey as part of the whole study was carried out to determine the prevalence of *P. aeruginosa* and *A. pyogenes* in clinically normal ears of cattle and those clinically affected with bovine parasitic otitis.

Pseudomonas aeruginosa and *Actinomyces pyogenes* were selected for this study because they had consistently been isolated from clinical cases of bovine parasitic otitis, but their prevalence rates had not been established (Lweno *et al.*, 1983b; Msolla *et al.*, 1989). As such it was felt necessary to establish the prevalence rates with which they were being isolated for the purpose of interpretation of results. *Pseudomonas.aeruginosa* has also been shown to be a common cause of otitis in small animal species as well as in human beings (Azizuddin and Chandrasekharan , 1954; Fraser, 1965; Shanon *et al.*, 1972 ; Bush, 1984; Ozaki *et al.*, 1990), and hence the interest to establish it's role in bovine parasitic otitis. Bovine otitis caused by *P. aeruginosa* has been mentioned only once (Azizuddin and Chandrasekharan, 1954).

Although *Actinomyces pyogenes* has not been associated with otitis in any animal species, it has been isolated from BPO on occasions (Lweno *et al.*, 1983b; Msolla *et al.*, 1989). It was also selected for this study because of its common association with pyogenic infections (Prescott and AnneMukle, 1986).

The prevalence of other bacteria in the ears of diseased and non-diseased cattle was also studied to look into the possibility of them being of any significance in the pathogenesis of the disease.

3.1.2 **Study area**

The study was carried out in four regions of Tanzania namely Morogoro, Coast, Tanga and Dodoma. These regions are situated along and/or close to the eastern coast of the country except Dodoma region which is more in the central part of the country. These regions are located between latitudes 4° and 10°S, and longitudes 35°5' and 39°E. The altitude ranges from sea level at the coast to 2000 metres above sea level. There are mountainous areas in Morogoro region which go up to 2646 metres. The average annual rainfall per region is 200-800 mm in Dodoma, 200-1200 mm for coastal and Tanga, and 600-1600 mm for Morogoro region. The vegetation varies from thin thicket to savanna in Dodoma and Coast regions while in Morogoro it ranges from steppes to forest type of vegetation. These areas were selected on the basis of three factors; (i) their vicinity to the University; (ii) the previous knowledge of the existence of the disease in these areas; (iii) the large number of animals which

were managed both traditionally and under ranch conditions where handling facilities and management were better.

In Morogoro region, areas surveyed were government and private ranches, farms and traditional livestock herds. The ranches visited were Mkata and Dakawa National Ranching Companies; the farms were, Kingolwira prison, Sokoine University, Taji Mohammed farms and small-holder farmers around Morogoro municipality. In the coast region the areas surveyed were Ngerengere Dairy Farming Company (DAFCO), Kibaha Heifer Multiplication Unit (HMU), Kibaha Education centre dairy farm, Ruvu ranch, Ruvu DAFCO, Ubena prison farm and traditional livestock herds in Lugoba, Chalinze ward in Bagamoyo district. In Tanga region the area surveyed was Mkwaja ranch, a private owned ranch by Amboni Limited, and in Dodoma region, Kongwa National Ranching Companies (Table 1).

Table 1: Areas sampled for the determination of the prevalence of *P. aeruginosa* and *A. pyogenes* from the ears of clinically healthy cattle and those clinically infected with bovine parasitic otitis.

Region	Place/Owner	Samples collected		
		diseased	Non-diseased	Total
Morogoro	1.parastatal/government farms/ranches;			
	Mkata NARCO ranch	108	0	108
	Dakawa NARCO ranch	24	0	24
	Kingolwira prison farm	54	4	58
	Sokoine University farms;			
	Otitis project	56	168	224
	Animal Science	-	20	20
	University farm	-	25	25
	2.Private farms;			
	Taji Mohammed (Mlali)	20	15	35
	Gikas G (Municipal)	0	37	37
	Salum Mohammed (Kihonda)	11	18	29
	Mzee Christopher (Wami)	18	0	18
	Mzee Wacha (Melela)	18	0	18
	Mzee Kapuratu (Dakawa)	18	15	33
	Mzee Mambega (Melela)	12	0	12
	Total for Morogoro	**339**	**302**	**641**
Coast	Kibaha HMU.	67	-	67
	Kibaha dairy farm.	13	20	33
	Ngerengere HMU.	20	12	32
	Ubena prison farm.	28	3	31
	Ruvu NARCO ranch.	68	12	80
	Ruvu DAFCO.	27	9	36
	Lugoba traditional keepers	48	4	52
	Total for Coast	**271**	**60**	**331**
Tanga	Mkwaja ranch (private-Amboni Ltd)	41	-	41
Dodoma	Kongwa ranch	22	26	48
	Overall total	**674**	**388**	**1062**

3.1.3 Study design

The study design used in this prevalence study was the multistage sampling where the primary units were the regions, the secondary units being the ranches/farms, the tertiary units being the herds and the quartenary units being the animals within the herds. The animals in the herds to be sampled were selected on a purposeful basis i.e the majority of the diseased animals were sampled while the non diseased ones were randomly sampled since they were the majority compared to the diseased ones. Random sampling was achieved by picking up every third or fourth or fifth animal as they passed through the crush or cast down in the absence of one.

3.1.4 Sample size

The sample size for the study was determined by a formula derived from Martin *et al.*, 1987. The formula is as follows:

$$n=4PQ/L^2$$

where P= probability; Q=1-P; L= allowable error or required precision; n= required sample size; 4 is the approximate square of Z which is 1.96 a tabulated t-value which provides a 95% confidence level.

To use this formula one has to know the approximate probability of the disease/organism to be surveyed, but if the probability is not known, it is estimated at

P=0.5 (Kimera personal communication, 1994) at 95% confidence level and an allowable error of 6%. Therefore the sample size was calculated as follows:

$n=4 \times 0.5 \times 0.5/0.06^2$

$n=1/0.0036$

$n=277.78$

n=278 animals

This number of animals was just a guide as it could be doubled or even trebled according to the convenience of the survey or such as finances and time. In this study 1062 samples (ear swabs) were collected, which was equivalent to approximately 531 animals from the areas mentioned under 3.1.2.

3.1.5 Animals

Cattle from which samples were collected belonged to various breeds e.g Tanzania Shorthorn Zebu (THSZ), Boran, and crosses of various indigenous and exotic breeds as well as dairy breeds such as the Aryshire and Friesian. These represented the breeds in the areas surveyed. In sampling these animals there was no age or sex preference.

3.1.6 Equipment used in the preparation of media

The equipment used were those present in the laboratory at the Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro. In this case these were; a

hot air oven 30°C-200°C (Memmert, West Germany) for sterilizing the petri dishes, two incubators one (Memmert, West Germany) was set at 42°C for differential growth characteristics purposes, and the second (Heraeus, West Germany) at 37°C for normal cultivation of bacteria. Other equipment included Triple beam balance (Ohuas, West Germany) for weighing the media. Glassware used were; flat bottom flasks for boiling the media, measuring cylinders (500 ml and 100 ml), sedimentation flasks for sedimenting the nematodes, petri dishes for pouring the media for isolating the bacteria, and Bijou bottles for the media of preserving bacteria and transporting the specimen.

Media used for isolation, identification, and storage of bacteria

Before the media to be used were prepared, all the glassware viz petri dishes, universal and bijou bottles were sterilized. The petri dishes and other glassware were sterilized in the oven at 160°C for one hour, while the universal and bijou bottles were sterilized in an autoclave at 120°C for 15 minutes.

The media used for bacterial isolation were Blood and MacConkey agar media. Medium used for transport of specimen was Aimes Transport medium (Oxoid Ltd, England) while medium used for storage of bacteria was Nutrient Agar slant.

a) **Horse blood agar medium**

Collection of blood

The blood for preparation of media was collected from horses which are kept at the University. Horses were preferred for collection of blood because they were convenient to handle and a larger volume of blood could be obtained at a time compared to sheep. The procedure used was as follows:

(i) Blood was collected into sterile universal bottles into which 5 ml of trisodium citrate (3.8% w/v) (Oxoid Limited, England) was incooperated as an anticoagulant. The blood was collected aseptically from horses using a sterile 14 gauge needle from the jugular vein after mopping the site with a piece of cotton wool soaked in 70% alcohol.

(ii) Immediately after collection the blood and the anticoagulant were mixed very gently as to avoid clotting. This was accomplished by upward and downward movements with the cap tight. This blood was then stored in a refrigerator at 4°C until required keeping in mind that blood kept for over 2 weeks was not suitable as it haemolysed with slight heat.

Preparation of media

a. **Blood agar medium**

Blood agar medium was prepared using blood agar base (Oxoid Ltd Berkshire, England), a base for mixing with the horse blood at 7% v/v. The composition of blood agar base is in grams per litre of distilled water; extract from heart muscle 10.0g, tryptose 10.0g, sodium chloride 5.0g, Agar agar 15.0g, pH of the base medium at 37°C 6.8±0.2. The agar base was prepared at a rate of 40g per litre distilled water as follows:

(i) The agar base was dissolved by boiling in a flat bottomed conical flask, while agitating the flask carefully in circular movements from time to time so as to avoid the agar from sticking to the bottom of the flask and thus get burnt.

(ii) When the agar base was completely dissolved, the flask was plugged with cotton wool then covered with a piece of aluminium foil to avoid contamination during cooling.

(iii) The agar base was then autoclaved at 121°C for 15 minutes. Following sterilization the agar base was removed from the autoclave after being allowed to cool down to 90°C as recommended by Cowan and Steel (1974).

(iv) The agar base was allowed to cool further to 50°C in a water bath.

(v) The blood which had been stored as described under 3.1.6 (a) (ii), was removed from the refrigerator, mixed gently in upward and downward movements then aseptically added to the agar base in the flask after a quick sterilization of the mouth of the universal bottle over a bunsen burner flame.

(vi) The agar base and blood were mixed gently in circular movements then poured into the petri dishes arranged in rows on the working bench. A bunsen burner flame was used to sterilize the mouth of the flask during the process of pouring the media into the petri dishes and for removing air bubbles which could have formed on the medium.

(vii) About 15-20 ml of the medium was poured in each petri dish according to the size while shaking the flask gently from time to time to ensure uniform mixture was maintained all the time.

(viii) The medium was then left to solidify for half an hour then the plates (petri dishes) incubated in an inverted position for 24 hours at 37°C for sterility check-up. After incubation the plates were stored in a refrigerator until required.

This medium, because of its nutritious nature was used for primary isolation of bacteria which grow easily on this medium. The medium was also ideal for observing features such as haemolysis, and pigment production of some bacteria.

b. **MacConkey medium (Mc)**

MacConkey agar No.3 (Mast Laboratories Ltd. Merseyside, UK.) is a differential and selective medium . Differential in that it can differentiate bacteria into two groups of lactose fermenters (e.g. *Escherichia coli*) and non lactose fermenters (e.g *Pseudomonas spp.* and *Proteus spp.*). It is selective in that it inhibits the growth of some bacteria for example *Bacillus spp., Corynebacterium spp.* and *Staphylococci spp.* It's composition in grams per litre of distilled water is: selected peptone mixture 14.8g, lactose 10.0g, sodium chloride 5.0g, bile salts (RM 25) 0.6g neutral red 0.03g, crystal violet 0.001g, and agar A (RM 10) 14.0g and the pH approximately 7.2. This medium was prepared as described by the manufacturer as follows:

(i) The concentration at which it was used was 44.2g per litre of distilled water dissolved in a flat-bottomed flask.

(ii) The mixture was left for about five minutes to dissolve while shaking slowly in circular movements. The flask was plugged with cotton wool and the mixture sterilized in an autoclave at 121°C for 15 minutes, cooled and poured into petri dishes as described under 3.1.6.2.

(iii) The medium was not incubated for checking sterility as for blood agar because this was a selective medium and contamination was not easy.

(iv) After solidifying the medium was stored in a refrigerator at 4°C until required.

c. **Amies transport medium**

Amies Transport Medium (Oxoid Ltd. Hampshire, England) has a composition in grams per litre distilled water; charcoal, pharmaceutical neutral 10.0g, sodium chloride 3.0g, sodium hydrogen phosphate 1.15g, potassium dihydrogen phosphate 0.2g, potassium chloride 0.2g, sodium thioglycollate 1.0g, calcium chloride 0.1g, magnesium chloride 0.1g, agar 4.0, pH approximately 7.2. The charcoal prolongs the viability of pathogenic bacteria in the process of transporting the specimen (ear swabs) from the field to the laboratory. The medium was prepared as described by the manufacturer as follows:

(i) The medium was used at a concentration of 20g per litre of distilled water in a flat bottomed flask and dissolved by boiling as described under 3.1.6.2.

(ii) The medium was sterilized at 121°C for 15 minutes in an autoclave as described for other media above after dispensing it in bijou bottles up to the brim of the bottles and screwed with rubber lined caps. The medium was allowed to cool while bottles were in inverted position to distribute the charcoal evenly. When cooled the medium was stored in a refrigerator at 4°C until required.

d. **Nutrient agar slants**

Apart from being used for other bacteria, nutrient agar slants were also used for maintenance of *P. aeruginosa*. It is for maintenance due to its low nutritive value that does not allow the bacteria to grow fast but rather remain in a quiescence state.

Nutrient agar slants were prepared from Nutrient agar No.2 (Oxoid Ltd, England) which is composed per litre of distilled water; 'Lab-Lemco'powder (Oxoid L 29) 1.0g, yeast extract (Oxoid L 20) 2.0g, peptone (Oxoid L 37) 5.0g, sodium chloride 5.0g, agar No.3 (Oxoid L 13) 15g, pH approximately 7.4. The procedure was as follows:

(i) Nutrient agar was prepared by dissolving it at a concentration of 28g per litre of distilled water in a flat bottomed conical flask. The mixture was dissolved, dispensed in bijou bottles at four mls each.

(ii) The bijou bottles containing the medium were sterilized, while capped in an autoclave at 121°C for 15 minutes.

(iii) The medium was then cooled in a slanting position at an angle of approximately 30°.

(iv) When solid, the medium was stored in a refrigerator at 4°C until required.

e. **Nutrient broth**

Nutrient broth was used for growing bacteria which were used for carbohydrate fermentation and for motility tests. Nutrient broth contains per litre of distilled water 'Lab-Lemco' powder (Oxoid L 29) 10.0g, peptone (Oxoid L 37) 10.0g, sodium chloride 5.0g, pH approximately 7.5.

(i) Nutrient broth was prepared by dissolving it at the rate of 25g per litre of distilled water.

(ii) These contents were mixed well to dissolve by gently shaking the conical flask in circular movements. When dissolved, the contents were distributed in bijou bottles at 4 mls in each, capped slightly loose to avoid breakage of bottles under pressure.

(iii) The bijou bottles containing the medium were sterilized at 121°C for 15 minutes. When cool the bottles were tightly capped then stored in the refrigerator at 4°C until required.

3.1.7 Procedure for collecting samples

Samples for bacteriological examination were collected from the auricular canals with sterile cotton swabs. The swabs had been sterilized in 9-inch capped test tubes and three swabs were placed in each test tube. During swabbing, care was taken to extract one swab at a time from the test tubes without touching/contaminating the rest.

Swabbing was done carefully not touching the sides of the pinna or the hairs. After swabbing, the swabs were transported to the laboratory in Aimes transport medium (Aimes, Oxoid Ltd, England) in Bijou bottles with the medium filled to the brim as recommended by Cruinkshank *et al.* (1975). Swabs were broken to fit into the bottles and the cap tightly fitted.

3.1.8 Isolation.and identification of bacteria

3.1.8.1 Isolation

Procedure for isolation of bacteria was carried out as follows:

Before the medium was used, the petri dishes were dried in the incubator at 37°C in an inverted position for about an hour as storage in the refrigerator at 4°C had made them wet with water due to condensation. Inoculation was carried out by first inoculating the medium with the swab held by a thumb forceps, then spreading the inoculum with a wire loop as described by Cruinkshank, 1968). Inoculation was done on horse blood agar (7% v/v) and on MacConkey agar media and incubated at 37°C for 24-48 hours. Incubation was extended to 48 hours for obtaining readable sizes of the colonies and allowing time for expression of features such as pigment production for *P. aeruginosa* and for the growth of fastidious bacteria such *A. pyogenes* whose colonies appeared after 48 hours incubation. The plates were placed on a working table where the colonies were read or kept in the refrigerator at 4°C for further identification.

3.1.8.2 Identification

Identification of the bacteria was based on:

a. **Cultural characteristics**

Cultural characteristics used were:

(i) colonial morphology i.e. size, colour, texture and consistence.

(ii) smell and pigment production.

(iii)haemolysis production on blood medium.

(iv) inhibition of growth and lactose fermentation on MacConkey medium.

b. **Gram's staining**

Gram's staining was carried out as another method of identification as described by Carter (1973). This type of staining groups the bacteria into gram positive and gram negative. Gram positive organisms appear purplish because they retain the primary stain (Crystal violet) after being decolourized by acid alcohol. Gram negative bacteria appear reddish-pink, because they are decolourized by acid alcohol and thus pick the colour of the counterstain. There are several methods of performing Gram's staining but the one used in this study was as follows:

(i) A drop of tap water was placed on a clean microscopic slide using a wire loop.

(ii) A smear was made by emulsifying a colony from the plate using a wire loop. In case of *P. aeruginosa* only a portion of a colony was required as colonies were large while with *A. pyogenes* several colonies were required because the colonies were so fine that one colony couldn't make a visible smear. The colony was emulsified in circular movements to make a smear of one centimetre in diameter.

(iii) The smear was left to dry in air.

(iv) The smear was then fixed with heat by passing the slide quickly two to three times over a bunsen burner flame.

(v) When cooled the smear was flooded with crystal violet for 30 seconds, washed with tap water.

(vi) Then the smear was flooded with Lugol's iodine for another 30 seconds. The iodine was washed off with water.

(vii) The smear was decolourized by 10% (v/v) acetone alcohol for 1-2 seconds and quickly washed with tap water.

(viii) Finally the smear was counterstained by neutral red for 30 seconds.

(ix) The slide was then dried in air and examined over a compound microscope at 100x objective.

c. **Biochemical tests**

Carbohydrate fermentation

The sugars used for this test were glucose, lactose, mannitol, maltose, sucrose, xylose and salicin. These sugars were prepared at 1% w/v, Andrade's indicator being the solvent. Andrade's indicator contains per litre of distilled water acid fuschin 5g,N-sodium hydroxide 150-180 ml. The procedure for preparing sugar media was as follows:

(i) Andrade's indicator was dissolved in distilled water at 1%.

(ii) The sugars were added to it. The mixtures were then dispensed in bijou bottles 4 mls each and Durham tubes placed in lactose and glucose media.

(iii) The media was sterilized by steaming for 30 minutes. When cooled they were inoculated with bacteria grown on solid media.

(iv) The inoculated media were incubated at 37°C and observed daily for colour change of the media for up to 5 days. Those sugars into which *A. pyogenes* is grown, 1% horse serum was added to facilitate growth.

Oxidase test

This was carried out for identification of *P. aeruginosa*. It was an additional test in combination with above mentioned methods. The method used was as described by Kovâcs (1956).

(i) Two to three drops of 1% tetramethyl-*p*-phenylenediamine dihydrochloride were placed on a piece of filter paper in a petri dish.

(ii) While the drops were still wet the test organism was removed from the medium with a platinum wire and smeared on the impregnated paper.

(iii) After 10 seconds the area inoculated with the bacteria was observed for change of colour.

3.1.9 Preservation of bacteria

The field isolates of *Pseudomonas aeruginosa* and *Actinomyces. pyogenes* were preserved for further use in the experiment to determine the roles of the bacteria in the pathogenesis of bovine parasitic otitis. *Pseudomonas aeruginosa* was preserved on nutrient agar slants prepared as described under 3.1.6.4. The organism was stored in a normal refrigerator at approximately 4°C and subcultured every three months as

recommended by Cowan and Steel, (1974). *Actinomyces pyogenes* was preserved on blood agar plates and subcultured every three weeks.

3.1.10 Analysis of data

The data indicating the number of positive or negative samples for the study bacteria, was analyzed by the Epi Infor System (Version 6). This was for the determination of the association of *P. aeruginosa* and *A. pyogenes* and the disease in the diseased, and as well as in the non-diseased cattle by the Chi-square test. The choice of the test was based on the type of the study and numbers of groups as shown in Appendix 2.

The procedure produces a 2 x 2 table showing the relative frequency of the factors in each group. These frequencies were compared using the odds ratio(OR) and the relative risk(RR) at P= 0.05. The Odds ratio measures the strength of association while relative risk measures the biological association and P value measures the statistical difference (Martin *et al.*, 1987).

The prevalence rates of the *P. aeruginosa* and *A. pyogenes* in cattle infected with BPO were determined by the following formula derived from Martin *et al.*, (1987). At any particular point in time the prevalence of the factor is determined by the formula

p=N/E x 100. Where p is the prevalence, N is the number of animals with *P aeruginosa /A. pyogenes* and E the number of animals examined.

3.2 The roles of *Rhabditis bovis, Pseudomonas aeruginosa* and *Actinomyces pyogenes* in the pathogenesis of bovine parasitic otitis

3.2.1 Introduction

As the second objective of the whole study, to determine the roles of *Rh. bovis*, *P aeruginosa* and *A. pyogenes*, in the pathogenesis of bovine parasitic otitis, experimental infection using these agents was carried out in calves.

Where as there have been a number of bacterial and nematode isolates from clinical cases of bovine parasitic otitis, no detailed studies have been carried out to determine the roles of the most frequent isolates in the pathogenesis of the disease. However, the only pathogenicity studies carried out was by infecting calves with the nematodes *Rh. bovis,* and *Rhabditis blumi,* the bacteria *Staphylococcus albus, Staphylococcus aureus* and *Bacillus cereus* individually and in different combinations of two's (Lweno *et al.*, 1983b). Individual treatments were: *Bacillus cereus*; *S. aureus*; *Rh. bovis* and *S. albus.* The combinations used were: *Rh. bovis* and *B. cereus*; *S. aureus* and *Bacillus cereus.* The clinical manifestations developed by the calves following infection were very mild (Lweno *et al.*, 1983b).

In another study, rabbits were infected with *Rh. bovis* alone and a combination of *Rh. bovis* and *P. aeruginosa* and in both cases, severe clinical manifestations were apparent (Msolla *et al.*, 1986b). In the present study, the three commonest isolates, *Rh. bovis, P.*

aeruginosa and *A. pyogenes* were used to determine their role in the pathogenesis of the disease.

3.2.2 Experimental animals

Fifty-three bull calves were used in this experiment. They were bought from the University farm and smallholder farmers in Morogoro Municipality. They comprised of Tanganyika Shorthorn Zebu (TSHZ), Boran-Friesian crosses as well as Jersey-Aryshire crosses. Their age ranged from 6-12 months (immatures) and their weight from 37-120 kg at the time of purchase. Both sources of experimental calves had no history of bovine parasitic otitis. The calves were identified, clinically examined, checked for blood parasites and their aural bacterial flora determined.

The calves were housed in fly-proof pens and were divided in groups of seven (seven groups) and the eighth group (control) comprised of four calves, totalling 53 calves. The grouping was carried out according to the bacteriological aural findings thus calves with the test organisms were placed in the appropriate group.

The calves were fed green forage, hay, silage and maize bran enriched with mineral mix. The feeds were given *ad lib.* except for maize bran which was supplied at the rate of one kilogram per calf making a total of 7 kg per group in the mornings. Water was also made available *ad lib.* from a drinking trough in the pens.

3.2.3 Bacteriology of the aural flora

After the experimental calves were purchased bacteriological examination of the aural flora was carried out to facilitate grouping of the calves. If the test organisms, *P. aeruginosa* or *A. pyogenes* were present as part of the aural flora, the calves were placed in the group to be inoculated with the same bacteria. If the test organisms were absent, then the calves were allocated to any group. Swabs for bacteriology were taken in transport medium and then inoculated on 7% horse blood and MacConkey agar plates and incubated at 37°C for 24 to 48 hours for the isolation of bacteria. The isolates were identified as described under 3.1.8.2 and as described by Cowan and Steel (1974).

3.2.4 Preparation of nematode inoculum

Nematodes for the experiment were obtained from infected animals borrowed from Dakawa National Ranching Company and kept at the isolation unit at the Faculty of Veterinary Medicine. The procedure was as follows:

(i) The nematodes were collected from naturally infected animals using sterile swabs into 23-cm capped test tubes containing 5ml of sterile physiological saline.

(ii) In the laboratory the contents of the test tubes were poured through a tea bag paper suspended over a sedimentation flask filled with sterile physiological saline. The tea

bag paper allowed the nematodes to penetrate through and gradually sink to the bottom of the flask. The nematodes were left to sediment for 30 minutes.

(iii) The nematodes were then pipetted into a sterile universal bottle using a 20 ml pipette to which sterile physiological saline was added to obtain a 20 ml suspension.

(iv) The concentration of the suspension of nematodes was then estimated by pipetting 0.1 ml of the suspension into a ruled petri-dish onto which a few drops of absolute alcohol was added to inactivate/kill the nematodes for ease of counting.

(v) The nematodes were counted over a stereo-microscope using a tally-counter. The number obtained was multiplied by 10 to arrive at 1 ml the amount required for each inoculation.

The number of nematodes and the frequency required for the initiation of lesions had been determined in a preliminary experiment by using different numbers of nematodes per millilitre (600, 900, and 1 200) for a duration of twelve weeks.

3.2.5 Estimation of viable bacterial counts and preparation of inoculum for *Pseudomonas aeruginosa* and *Actinomyces pyogenes*

The bacteria used for the experiment were obtained from field cases and had been stored in the laboratory as described under section 3.1.9.

3.2.5.1 Recovery of bacteria from storage

Pseudomonas aeruginosa from storage medium (nutrient agar slant) was recovered by subculturing on to blood agar plate. *Actinomyces pyogenes* was also subcultured from storage medium (blood agar medium) on to blood agar medium in the same procedure as *P. aeruginosa*. Both plates were incubated at 37°C for 24 hours for *P. aeruginosa* and 48 hours for *A. pyogenes*. The colonies obtained were used for estimation of viable bacterial counts. The knowledge of viable bacterial count for these two bacteria was necessary for the determination of the dilution factor of the inoculum required.

3.2.5.2 Estimation of viable bacterial counts

Estimation of viable bacterial counts were carried out by the surface viable count by the spreading method for *P. aeruginosa* and pour plate method for *A. pyogenes*. The spreading method was used for *P. aeruginosa*, because the organism is a strict aerobe, thus colonies were able to grow only on the surface of the medium. The pour plate method on blood agar medium was used for *A. pyogenes* because of its ability to grow facultatively and of its haemolytic properties which made colonies growing under and with in the medium clearly discernible. These methods were used as described by Cruickshank *et al.* (1975) as follows:

(i) A bacterial suspension was prepared by inoculating an average of 1-4 colonies (several more for *A. pyogenes* due to its small size of the colonies) into 4 ml Oxoid Nutrient broth dispensed in Bijou bottles.

(ii) The media were incubated at 37°C for 24 hours.

(iii) Ten-fold dilutions of the bacterial suspension were made using phosphate buffered saline (Cruickshank *et al.* 1975; Shimoda *et al.*, 1991).

(iv) Then 0.1 ml of each dilution was inoculated onto blood agar plates, two plates per dilution were used as recommended (Cruickshank *et al.* 1975 described the use of three plates).

(v) At once the inoculum was spread evenly allover the medium with an L-shaped glass rod made by melting the tip of a pasteur pipette over a bunsen burner flame. The plates were then incubated at 37°C for 24 hours.

(vi) The colonies obtained were counted over a colony counter, the plates giving a bacterial count between 50 and 500 colonies per plate were considered for the calculation of colony forming units (CFU). The bacterial viable counts were calculated from the average colony count of the two plates and multiplied by the dilution factor and this provides the CFU/ml.

The bacterial suspension for *A. pyogenes* was prepared as described for *P. aeruginosa* except that the nutrient broth was enriched with a 5% (v\v) horse serum to facilitate growth and incubated at 37°C for 48 hours due to the fastidious nature of its growth (Cowan and Steel, 1974). After the ten-fold dilutions were made, 0.1 ml from each dilution was pipetted on to a petri dish on which 10 ml of cooled blood agar (about 45°C-50°C) was poured over. At once the two were mixed rapidly by moving the plates while flat on the bench in a combination of side to side and circular movements in different directions. The mixing was done for about ten seconds gently not to spill the contents. The medium was then allowed to set and then incubated for 48 hours at 37°C. The bacterial count and the CFU/ml were carried out as described for *P. aeruginosa* above.

3.2.5.3 Preparation of bacterial inoculum

The bacterial suspension for inoculating the calves was prepared by use of a 24 or 48 hours broth as described under 3.2.5.2 above. The bacterial inoculum used for inoculation of the experimental animals was obtained from the fourth dilution for *P. aeruginosa* and the third dilution for *A. pyogenes*.

3.2.6 Inoculation procedure

The calves were inoculated by instilling into their ears the inoculum containing the bacteria and/or nematodes after the animals had been sedated with Rompun (Xylazene

2% w/v Bayer, Levenshn, Germany) at a rate of 0.25 ml/50 kg live weight. The calves were sedated because in the preliminary studies, it was observed that when instilled with the inoculum without sedation, they shook their heads vigorously so that most of the inoculum spilled out. Other methods of minimizing loss of the inoculum such as plugging the ears with cotton wool did not work as the animals shook their heads even more and the inoculum got absorbed into the cotton wool.

After sedation, one millilitre syringes were used to instill one millilitre of the inoculum into each ear, and a separate syringe was used for each ear to avoid cross contamination. The ears of calves were held up immediately after inoculation and massaged gently for about 30 seconds to ensure proper lodgement of the inoculum. The inoculation was repeated weekly for three consecutive weeks (as shown in Table 2) and in all cases the same volume of one millilitre was used irrespective of the combinations of the agents which meant that when several agents were used, they were given in smaller volumes of high concentration. The dosage and the frequency of administration were calculated after a preliminary experiment and the dose arrived to was 1000-1200 nematodes per millilitre. Inoculating the nematodes once per week was based on the dipping regime of cattle during the dry period whereby they are dipped only once per week. In practice, cattle pick nematodes from infested dip tanks cumulatively whenever they get dipped.

3.2.7 Clinical examination

Clinical examination was done daily from day zero when calves were inoculated with the test organisms, and detailed aural examination was done using an otoscope for the detection of aural lesions. The aural lesions that developed were scored in the range of 0-3 according to the severity of the lesions as shown in Table 3.

Table 2: Inoculation regime adopted during infecting experimental calves. The calves in each group were inoculated for three consecutive weeks.

Group.	First inoculation	Second inoculation	Third inoculation	End of experiment
1.	25/05/1993	02/06/1993	10/06/1993	13/08/1993
2.	15/06/1993	22/06/1993	29/06/1993	30/08/1993
3.	08/07/1993	15/07/1993	22/07/1993	22/09/1993
4.	29/07/1993	05/08/1993	12/08/1993	13/10/1993
5.	27/08/1993	03/09/1993	10/09/1993	12/11/1993
6.	17/09/1993	24/09/1993	01/10/1993	03/12/1993
7.	29/10/1993	05/11/1993	12/11/1993	14/01/1994
8.	11/02/1994	18/02/1994	25/02/1994	29/04/1994

Table 3: Clinical manifestations and aural lesions used in scoring system.

Clinical sign/Lesion	Type of response	Score
1.No discharge or nematodes	none	0
2.Scanty discharge with nematodes confined inside the ear canal and only seen on close observation with an otoscope.	mild	1
3.Discharge with nematodes extending to the opening of the ear canal and could be seen easily. The underlying tissue slightly hyperemic. Other signs not evident	moderate	2
4.Plenty discharge oozing out and soiling the pinna and area below the ear. The underlying tissue hypereamic. Other signs more evident such as dullness, swelling of the ear base and the corresponding parotid lymph node, tilting of the head to the affected ear and retention of the bolus into the cheek.	severe	3

The otoscope head was then disinfected with absolute alcohol absorbed in cotton wool to ensure no transfer of nematodes/bacteria from one animal to another. Extra caution was taken for the otoscope not to make contact with the ear. Physiological parameters included during clinical examination were; temperature, pulse and respiration rates that were recorded daily for the whole observation period of eleven weeks. Body weight was determined on a weekly basis using an animal measuring tape (WE-BO, Denmark). If an animal developed severe clinical manifestations including pyrexia in the course of the experiment, blood slides were taken and examined to rule out tick borne and other haemoparasite infections that are most prevalent in the area. At the end of the experimental period, ear swabs were taken to determine the presence of nematodes and the type of bacteria.

Recording of pulse and respiration rates and body temperature

The above parametres were recorded once daily in the mornings between 8.00 to 9.00 a.m. This time was convenient as it was the time before the calves were fed.

3.2.8 Determination of the consistency of aural flora

During the course of bacteriological examination of the ear canals of healthy calves before the experiment, it was observed that the aural flora was not consistent from one examination to another. This was observed too in the experimentally infected calves. This observation necessitated the follow up on the nature of the changes in the bacterial

flora in some of the non-infected and the infected calves. The normal bacterial flora consists of three types of resident, the temporary bacterial flora, supplementary and permanent ones. It was necessary to establishing the type of bacterial flora for two reasons. First, the normal flora of the ears of cattle let alone that of the skin has not been extensively studied as compared to small animals (Woolcock, 1991). So it was found necessary to establish these bacteria to have a base for the interpretation of the results obtained. Secondly, the results could be used for further studies if the need arises.

Group two was taken as the representative of the non-infected calves, while group four represented the infected calves. Ear swabs were examined fortnightly under the procedures described in sections 3.1.8.1 and 3.1.8.2.

3.2.9 Analysis of data

The individual scores of the aural lesions, the body temperatures and the pulse rates were first entered in the DBase 4 System for determination of their group means. The means were then analyzed by Statistical Analysis System (SAS 604) using the General Linear Model (GLM) programme for comparison of the responses between groups.

3.2.10 Histopathological studies

The aim of this study was to observe the microscopic changes occurring as a result of infection with *Rhabditis bovis*, *Pseudomonas aeruginosa* and *Actinomyces pyogenes* in different combinations. Histopathological studies of naturally infected animals have been reported previously (Lweno *et al.*, 1983b). The main histopathological features included epithelial and subepithelial desquamation, cellular infiltration, and vascular engorgement. Histopathology of experimentally infected animals is lacking and thus this study will provide some knowledge which will be of use in further studies.

3.2.10.1 Source of specimen

Two heads obtained from calves sacrificed from each experimental group were used for histopathological studies after the observation period of eleven weeks.
Two heads from each group were used; one being from a calf that did not show clinical signs or showed mild response and the second was from the most severely affected calf in the group.

The specimen required for histopathological studies were taken from the pinna, the external ear, middle ear and the inner ear (bony part of the ear). To obtain these specimen, the head was first skinned, and the lower jaw was removed by carefully dislocating the masseter-mandibular joint. The remaining upper jaw and the head were cut through the midplane, resulting into two equal pieces. These portions were each cut

longitudinally infront of the eye, and behind the ear about 2.5 cm perpendicular to the head (Fig.8) while being careful not to disrupt the ear canal by using an electric saw. The resulting pieces were identified as left and right (Fig. 8) then fixed them in 10% formal saline for a minimum of 48 hours. The duration of fixing was not fixed, this depended on available time to do the trimming. After fixing the specimen were processed immediately or stored in the fixative until required for processing. From the large pieces, sections from the external ear were obtained by cutting with a butcher knife for the soft tissues i.e. the pinna and the external ear canal (Fig.9) and an electric saw for the bony part.

3.2.10.2 Preparation of specimen for histopathological studies

a. Decalcification

The sections resulting from the larger pieces (Fig. 10 and 11), were trimmed to smaller ones of about 3mm thick using a scalpel blade. These pieces were identified by attaching a number on a small piece of hard paper to a thread and then immersed in a beaker of 200 ml containing the decalcifying agent (RDO) (DUPAGEKINETIC Laboratories Inc. Illinois USA) of about 150ml. RDO is among the Proprietary decalcifiers whose name and formulae are secrets that cannot be revealed (Bancroft and Stevens 1990). The tissues were then left to decalcify for a maximum of twelve hours.

Figure 8: Processing specimen for histopathological studies: Portion of the longitudinal section of the head of a calf with mandibles and muzzle removed.

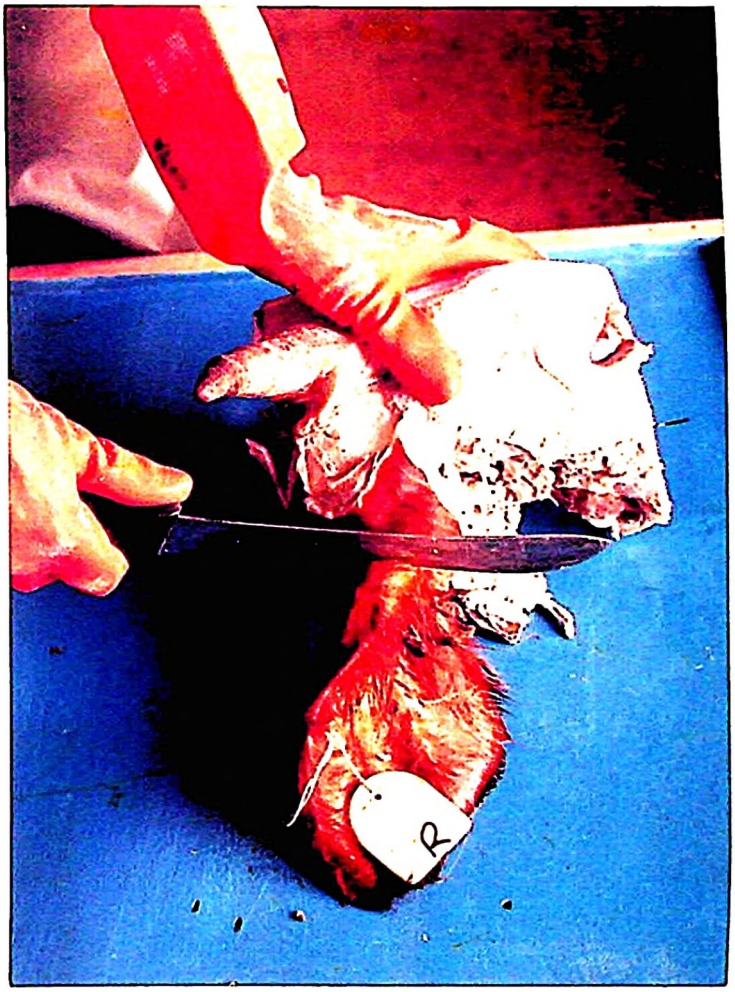

Figure 9: Excision of the ear for histopathological studies.

b. **Tissue processing**

After decalcification, the tissue sections as shown in Fig.11 were again trimmed using scalpel blade into even smaller pieces of 1 mm x 1 cm by removing excess tissue around the ear canal. This process was carried out under the hood so as to contain the toxic fumes released by the decalcifying agent. The resulting small pieces were placed into cassettes ready to be processed into histopathological sections. The cassets were then filled with wax, blocked then dehydrated through a Histokinet (2L Processor MkII, Shandon, England). During the process, the tissues were first dehydrated in a series of increasing concentrations of alcohol from 70%, 90%, 95%, absolute alcohol twice for two hours each.

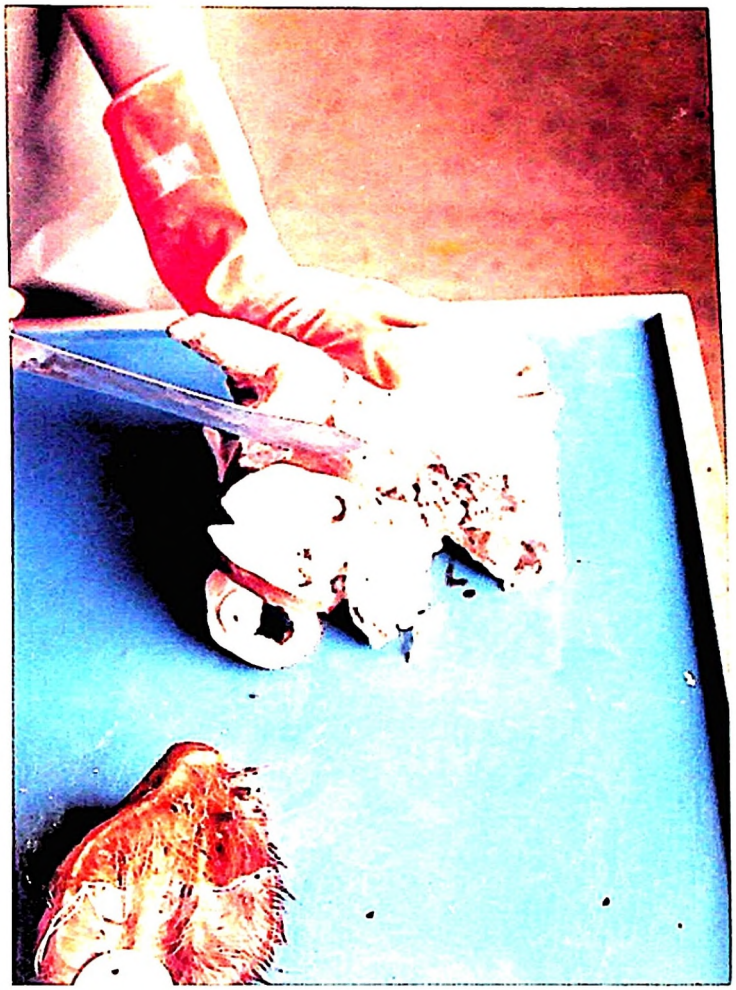

Figure 10: Slicing of the ear into sections for histopathological studies.

Figure 11: Sections from the soft part of the ear canal ready for decalcification.

The tissues were then cleared in (40%) chloroform for three consecutive times each for two hours and finally infiltrated and imbedded in paraffin wax twice for two hours. For production of wax blocks, Leuckharts 'L' pieces were used as molds, molten wax at 60°C i.e. 2°C above the melting point was dispensed into a mould to a depth adequate to cover the thickest tissue blocks. When a thin film of semi solid wax was formed on the base of the mould, the tissues were introduced with forceps, gently pressing the tissue in semi solid wax in the correctly oriented plane, i.e the side to be cut was oriented downwards. As soon as the film was solid on the surface, the whole block was submerged in cold water to hasten the solidification of the wax.

c. Cutting of sections

When the blocks were completely solidified, the paraffin wax above the tissue on the wax block was trimmed till a good part of tissue was exposed. A microtome was used to cut the tissue sections to 5μ thickness. The cut section was placed in a thermostatically controlled water bath at 56°C i.e 2°C below the melting point of paraffin wax. This process helped to flatten the tissue sections. The sections were picked from the water bath by a microscope slide at 60°C.

d. Staining

The tissue sections cut at 5μ thickness were stained with haematoxylin and eosin (H&E) stain. This involved first deparaffinizing in xylene for fifteen minute, then

dexylenating in a decreasing concentration of alcohol from absolute alcohol for 5 minutes, and again for 3 minutes, in 95% alcohol for 3 minutes, in 70% alcohol for 3 minutes and finally through running tap water for two minutes. During staining, the tissues were placed in a jar containing haematoxylin for 10 minutes, then washed briefly for 20 seconds in tap water before they were differentiated by passing them through 1% acid alcohol for 20 seconds. They were then washed in running tap water before they were counterstained in safranin lithium carbonate for 20 seconds followed by a quick rinse in tap water. Thereafter, the tissues were stained by 1% eosin for 2-3 minutes and rinsed in tap water for 20 seconds. The stained tissue sections were dehydrated by passing them through a series of increasing concentrations of alcohol, i.e. from 70%, to 95%, to absolute alcohol, and finally in absolute alcohol. The tissues were cleared by passing them through xylene for two minutes.

e. Mounting

After the alcohol in tissue sections had been removed, cover slips were mounted on to the stained tissue section on slides with DPX, (distrene a polystyrene, a plasticizer and xylene) a resinous mounting medium, and left to dry.

f. Examination

The slides were examined at 10x, 40x and 100x objectives and histopathological changes recorded.

3.3 The roles of dips, spray races and soil in the transmission of bovine parasitic otitis

3.3.1 Introduction

The third objective was to carry out field studies to determine the roles of diptanks, sprayraces, and soil/manure in the transmission of bovine parasitic otitis. A summary of the use of diptanks and sprayraces is given below.

The history of the use of plunge dips for the control of tickborne diseases mainly East Coast Fever (ECF), heartwater, anaplasmosis and babesiosis goes back to 1905 when the first dip tank was constructed in the country at Mpwapwa Livestock Research Station. From then on many other dip tanks were constructed in different parts of the country. Through the years various acaricide have been used including the chlorinated hydrocarbons such as Diethyldimethyltetracetate (DDT), Benzene Hexachloride (BHC) and Toxaphene® (chlorinated camphene) etc. Following development of tick resistance to these acaricide, synthetic pyrethroids such as decamethrin, cypermethrin, deltamethrin and the organophosphates were introduced for alternative use (Soulsby, 1982).

In the past dips were managed quite efficiently including the keeping of correct strength of dipwash, as well as emptying of the dips when it was necessary. With time the government switched these responsibilities to the district councils, which did not run

the dips efficiently. Many dips became functionless and those in use were in very poor condition with dip wash strengths not being checked.

The implication of dip wash as a method of transmission of bovine parasitic otitis was put for ward after some pertinent observations. The observations which led to this assumption were that the disease was of high prevalence in dipped (70%) than sprayed animals (5%) (Msolla *et al.*, 1987). Work done on the isolation of the nematodes from dip tanks revealed that up to 20 live worms were being isolated from one litre of dip wash (Msolla *et al.*, 1987). Despite of these findings, further studies were required in order to determine the dynamics of the spread of BPO through the dip wash.

In view of the above, the objective of this study was therefore to carry out a field study on the role of dips, spray races, soil and manure in the transmission of bovine parasitic otitis.

3.3.2 Location

The field study was carried out at Mkata NARCO ranch that is fifty kilometres north west of Morogoro municipality. It is situated approximately between latitudes 5°S and 6°S, longitude 35°E and 36°E. It stands at 1 000-1 500 m above sea level. The vegetation is characterized by thick thicket while the annual rainfall ranges between 600mm-1 200 mm divided into short rains in October and November and long rains in February to May.

3.3.3 Background information on tick control programme at Mkata ranch

The ranch has about 4 500 cattle of various breeds including Boran, Tanzania Shorthorn Zebu (TSHZ) and other crosses of Tanzania Shorthorn Zebu (TSHZ) and Bos taurus breeds which resulted from the LB22 (Local Breed 22) scheme. The scheme was established for upgrading local cattle using exotic bulls/semen.

The animals are kept at four different locations/stations in the ranch for the purpose of rearing different age/sex groups separately. Tick control is practiced by dipping and spraying. There is one dip and one spray race which are about three kilometres apart. About 400 cattle are dipped and the remaining 3 500 are sprayed. This second group is divided into two groups, one being sprayed using Steladone® 300 ES and the other sprayed with decartix as this group is more exposed to high tsetse fly challenge as compared to the other group. Decartix also acts as a fly repellant. Steladone® contains 300g chlorofenvinphos per litre (CIBA-GEIGY Limited, Basle, Switzerland). Dipping is carried out once per week in the dry season and twice during the rainy season. Spraying is twice per week due of the large number of animals and because of the use of two different types of acaricides as mentioned earlier. The strength of the acaricide is kept constant by replenishment before and during dipping. Testing of the strength of dip wash is rarely carried out because of the expensive testing fees for these acaricides (Mtae personal communication, 1995). The herds in different locations however are

not kept constant. Changes are made when necessary by moving animals from dipping to spray race and vice versa especially so during weaning.

3.3.4 Experimental animals

Animals used in this field study were selected from different herds in the ranch. Sixty animals were selected randomly from these herds. Twenty from the sprayed herd which were designated as group one. Forty from the dipped herd which was split into two groups, one group of twenty continued to be dipped which was designated as group two. The remaining twenty were topically protected against ticks by Ectopor® SA 020 (Ciba-Geigy Limited. Basle. Switzerland). Ectopor is a 2% Cypermethrin selected because of its long (up to three weeks) residual effect and its availability. This group was designated as group three. The first group were female Tanzania Shorthorn Zebu (TSHZ) cattle which had been purchased from local livestock keepers in Dodoma in February, 1995. The second group were also female boran crosses, one and a half to two years old which were allocated for Ectopor® application. Though the residual effect of this acaricide is up to three weeks, application was done on a weekly basis due to the study being carried out during the rainy season. Close supervision was necessary in case of development of tick borne diseases.

3.3.5 Experimental procedure

3.3.5.1 Preparation of the animals

After the animals had been grouped, they were identified by plastic ear tags so that they could be easily isolated from the rest of their groups when required as these animals were herded with the rest. On the first day of the study, ear swabs were collected to ascertain the absence or presence of the nematodes. Swabbing was considered to be more accurate than visual examination especially if animals were in their early stages of the disease. The swabs were transported in capped 9 inch (23 cm) test tubes into which were 5 ml of physiological saline as transport medium.

3.3.5.2 Application of acaricide

a. Dipping

The group that was selected for dipping was dipped along with the rest of the herd once per week. The acaricide used was Steladone® 300 EC. Replenishment was carried out before dipping at 3 litres of the concentrate per 1 000 litres of water. Replenishment was based on the amount of the dip wash lost during the previous dipping, and another replenishment was done during dipping by using 100 ml of the concentrate added to the dip tank for every 100 dipped animals. In the first replenishment, the amount of the dip wash lost was measured by a calibrated yardstick before dipping.

b. **Spraying**

Spraying was undertaken after preparing the spray race wash at an initial filling of 1 litre of Steladone® 300 EC per 600 litres of water (0.17%). The spray race tank (sump) which had a capacity of 2 000 litres was used to prepare the fresh wash at each spraying. Reinforcement/boosting was done with 100 ml of the concentrate for every 100 heads of cattle sprayed. After spraying, the spray race was cleaned the tank emptied and cleaned ready for the next use. Spraying was carried out once per week.

c. **Pour-on**

Topical application was carried out using Ectopor®. The acaricide was applied on the each animal body on predilection sites of the ticks. These are: between and around the horns; along the backbone from the head to the root of the tail and the switch; on the perineum; the area between the hindquarters (around the udder); the area between the forequarters (dewlap); and between the hooves. The amount of ectopor applied depended on the weight of the animal. The animals selected for the experiment were between 150 kg and 200 kg, and from the dose rate indicated each animal received 20 ml. On that basis about 450 ml of the acaricide was used per week. Although the acaricide could be applied at the maximum interval of three weeks, a weekly interval was preferred because of the wash down effect of the rains.

3.3.5.3 Examination of the animals for nematodes

Ear swabs were collected from the experimental animals fortnightly for examination of the nematodes. Sampling was carried out fortnightly because it was not possible to sample the animals on the same day as they were located at different stations.

The swabs collected were placed into 15cm capped test tube containing 5ml of normal saline as a medium of transport. The suspension formed from the immersion of the swab into the physiological saline was shaken gently and poured onto a ruled petri dish and examined under a stereo microscope at x2 objective.

3.3.5.4 Sampling of dipwash

Dip wash was sampled on dipping day and sampling was carried out from two sites, Mkata ranch (dip tank and spray race) and Taji Mohammed farm (dip tank) at Mlali. These two places have a history of having the disease for a long time. Sampling of the dip wash was accomplished by the use of a narrow mouthed one litre container fixed by a rubber band to one end of a long thick stick. The narrow mouthed container was appropriate because it enabled collection of dip wash from the deeper areas without the container being filled with the upper dip wash as it takes time to fill up compared to a wide mouth container.

Three litres were sampled at a time, one before dipping, this was done so to isolate nematodes which could survive from the last dipping an interval of three or six days according to the dipping regime. The second sample was taken at the middle of dipping so as to isolate any nematodes which could have been at the deeper parts of the dip as dipping many animals mix the dip wash thoroughly. The third sample was taken at the end of dipping to isolate those nematodes that could have been introduced into the dip tank by the affected animals or by the hooves. The samples were taken from the deepest part of the dip tank which is at the entrance.

3.3.5.5 Isolation and identification of the nematodes from the dip wash

Isolation

The method used for isolation from the dip wash was as described by Msolla *et al.* (1986b) and as follows.

(i) In the laboratory, the dip wash collected was first shaken to make a uniform suspension, then the suspension was poured fast through a tea bag paper folded over a sedimentation flask. The tea bag paper prevented the large particles to pass through and allowed only the small particle including the nematodes. This set up was allowed to stand for one hour so that the nematodes if present could penetrate the tea bag paper and sink to the bottom of the sedimentation flask. Because the sedimentation flask has a narrow bottom, they were easily collected after the supernatant had been poured out.

When the dip wash was dirty the process of sedimentation was repeated two to three times so as to obtain a much clearer sediment.

(ii) The sediment was poured in small amounts on petri dishes and examined over a stereo microscope.

(iii) The nematodes present were identified by sucking the nematodes with a pipette on to a microscopic slide and covered with a cover slip. The slide was then examined under a compound microscope at x4 for locating the nematode, and at x10 for visualizing morphology and at x40 for identifying fine structures such as rays. The criteria for identification are those as described by Kreis, (1964) under section 2.

3.3.5.6 Isolation of the nematodes from the soil, manure and mud from the footbath

Source of samples

The soil samples for the determination of the presence of the nematodes were collected from the study areas i.e. Mkata ranch, Kongwa ranch, Taji Mohammed farm, Ruvu ranch, Mianji farm and small-holder farmers at Mkata ranch and Morogoro municipality. The areas known to have the disease were Taji Mohammed farm, Kongwa ranch, Mkata ranch, Ruvu ranch and Lugoba, while those with no history of the disease were Mianji farm in Dakawa and from small-holder farmers in Morogoro

municipality. Samples were collected from the sleeping pens, collecting pens and foot baths at the dip tanks and spray race.

Sample collection

The soil manure mixture was collected using a hoe by digging to an approximately 10-20 cm depth into the ground because these nematodes of the genus *Rhabditis* have been observed to inhabit in such depths especially during the dry season (Thorne, 1961). During the rainy season, paddocks were so muddy that there was no need to use a hoe, the hand with gloves on was sufficient. About 200g of soil were collected, placed in 10 x 15 cm polythene bags which were carefully closed and kept in a cool box during transportation. The same amount of mud was collected from the first part of the foot bath because it contained more mud due to its proximity to the collecting pen.

Isolation and identification of the nematodes

The method used for isolation of nematodes from the soil was a modification of the method of isolating nematode larvae from the soil by Grφnvold (1984) as described by Msolla *et al.* 1989). The procedure was as follows:

(i) Two hundred grams of soil were weighed on a triple beam balance for isolation of the nematodes.

(ii) A plastic basin of about 45x30x20 cm was used (Fig. 12). A metal suspension rack was fitted in the basin. Over the suspension rack three tea bag papers were aligned in such a way that the sides were over lapping to protect the soil from falling into the basin.

(iii) About 200g of the soil were evenly spread over the tea bag papers.

(iv) Tap water was then gently poured into the basin at one of the sides by lifting up a small piece of the tea bag paper. The water was filled to the bream of the suspension rack thus just coming into contact with the tea bag papers aligned over it. This arrangement allowed water to seep to the soil osmosis, thus making it wet. By the soil becoming wet, the nematodes if present were able to wriggle out of the soil and penetrate the tea bag paper and into the water and settle at the bottom of the basin.

(v) This set-up was left for 18 hours after which the tea bag papers and the soil were removed carefully by folding over the edges together so that no soil could drop into the basin.

(vi) The rack was then lifted with the aid of thumb forceps. The suspension obtained was slowly poured into sedimentation flasks over which other tea bag papers were again folded so as to remove large particles that accidentally went through the basin. This set-up was left for 6 hours for the nematodes to settle to the bottom of the flask.

(vii) The supernatant was decanted until a small amount of the sediment of about 5 ml was left in the flask. When the sediment was too heavy and dark in colour, it was diluted with tap water so as to be visible under the microscope. By diluting the suspension, the amount became too much to be examined. As such a sample of 50 ml of the suspension was examined under a stereo microscope by taking 5 ml into a petri dish at a time.

(viii) The petri dish was marked with parallel lines at the bottom with a grease pencil to make counting possible.

(xi) The procedure for examination and criteria for identification of the nematodes was similar to that described under 3.3.5.5.

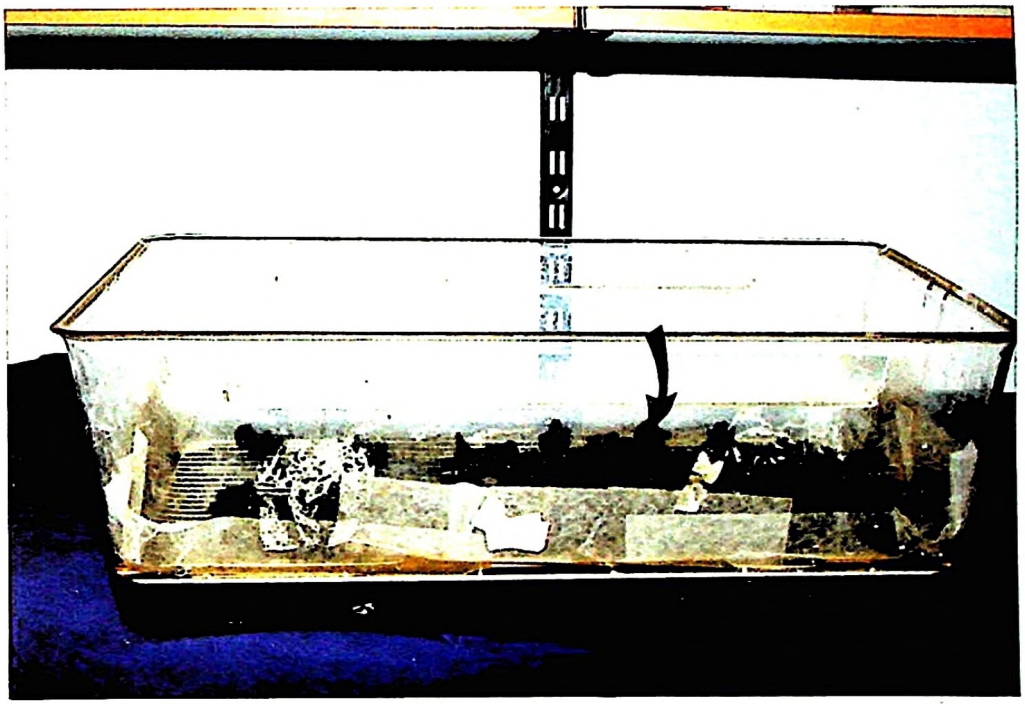

Figure 12: Isolation of nematodes from the soil: A plastic tray with a metal rack covered by tea bag papers on which soil was uniformly distributed (arrow).

3.3.5.7 An *in vitro* experiment on the viability of *Rhabditis bovis* in different dilutions of Steladone®

Introduction

This study was carried out as part of the third objective on the role of diptanks in the transmission of bovine parasitic otitis. The nematode *Rhabditis bovis* has been shown to survive in normal strength (0.25%) of Toxaphene® dip tanks by which healthy animals become infected in the course of dipping (Msolla *et al.*, 1987). This work was accompanied by *in vitro* tests to determine the viability of the nematodes in freshly prepared 0.25% Toxaphene® and other dilutions. The nematodes were found to survive for up to 15 days in freshly prepared 0.25% and 28 days in used dip wash.

Since other organophosphates and synthetic pyrethroids have been introduced in the country following the banning of Toxaphene®, the study on the survival of the nematode in these other acaricides was deemed essential. This work was carried out to study the viability of the nematode in Steladone® an organophosphate acaricide currently in use in the study areas.

Source of nematodes

The nematodes used for this study were isolated from ears of naturally infected cattle that were borrowed from Messes Taji Mohammed farm and being kept at the Faculty of

Veterinary Medicine experimental premises. The concentrate of Steladone® was obtained from Mkata NARCO ranch.

Experimental procedure

The nematodes were collected, sedimented as described under 3.3.5.5.

(i) The concentrate of Steladone® was first diluted to the strength which used for dipping i.e 0.17% by mixing one millilitre of the concentrate and 600 ml of tap water in a one litre flat bottomed flask.

(ii) A two fold serial dilution of a 0.17% solution of Steladone® (Table 4) was prepared in 9-inch (23cm) capped test tubes labeled one to ten and arranged serially on a metal test tube rack. Tap water was used as the diluent where by five millilitres was pipetted into each test tube using a 5 ml pipette fitted with a rubber teat.

(iii) Five millilitre of the suspension was added into the first test tube using a different 5 ml pipette, mixed well by using the rubber teat then transferred five ml into the next test tube and mixing with the rubber teat.

(iv) The same procedure was used for all tubes and the last five millilitres were discarded. Tap water was used as a control.

(v) Small plastic disposable petri dishes of 4.5cm diameter were arranged in two rows on a working bench, as a paired sample. Using a 1ml graduated pipette, 0.1 ml of the sedimented nematodes was withdrawn from the sedimentation flask and one drop was dispensed into each petri dish.

(vi) Three millilitres of the serially diluted acaricide were then added to each petri dish at three minutes interval, one at a time and recording the time for pouring of each dilution.

(vii) After the addition of the acaricide each petri dish was examined under the stereo microscope to determine the viability of the nematodes. Thereafter, each petri dish was examined every half an hour until all the nematodes were dead and the time recorded. The same procedure was carried out for the second row of petri dishes.

Table 4: Two fold serial dilutions of a 0.17% solution of Steladone®

Serial number	Concentration
1.	1:600
2.	1:1200
3.	1:2400
4.	1:4800
5.	1:9600
6.	1:19200
7.	1:38400
8.	1:76800
9.	1:153600
10.	1:307200

CHAPTER FOUR

4.0 RESULTS

4.1 The prevalence of *Pseudomonas aeruginosa* and *Actinomyces pyogenes* in clinically normal ears of cattle and those infected with bovine parasitic otitis(BPO)

4.1.1 Sample collection

A total of 1 062 samples were collected over a period of four years (1992-1996) from healthy bovine ears and those with BPO. Six hundred and fifty two samples were from diseased ears while four hundred and ten samples were from non-diseased ears(Table 5). Bacterial isolates from diseased and non-diseased animals are shown in tables 6a and 6b.

4.1.2 Identification of *P. aeruginosa* and *A. pyogenes*

4.1.2.1 Growth characteristics

i. *Pseudomonas aeruginosa*

After an incubation period of 24 hours on blood agar, the colonies appeared gray cream, convex, entire margin, with a smooth surface. There was haemolysis and

greenish colouration to the medium on confluent growth and individual colonies. After 48 hours, the colonies became grayish green, lost their convexity and became larger, about 3 mm, slightly flat and the margin irregular and were no longer smooth. The haemolysis and colouration deepened and a sweet odour became evident.

In most cases there was a metallic sheen on top of the colonies, a feature used for identification of *P. aeruginosa* (Wahba, 1964). With further incubation, at 72 hours, the colour of the colonies deepened to dirty green and the whole plate became haemolytic as most of these isolates were monocultures. The size of the colonies increased to about 4 mm and the colonies became more flat with irregular outline and odour increased in intensity. However, colonies which were not pigmented, oxidase positive with no characteristic odour but had a tendency of spreading, were classified as *Pseudomonas spp.* In few cases there were non-pigmented colonies amidst pigmented ones.

Table 5: Samples collected from various areas for the prevalence study.

Origin	Samples collected	Diseased	Non-diseased
Mkata NARCO	108	108	0
Dakawa NARCO	24	24	0
Ruvu NARCO	80	68	12
Mkwaja ranch (Amboni Ltd.)	41	33	8
Kongwa NARCO	48	22	26
Ruvu DAFCO	31	28	3
Ngerengere DAFCO	32	19	13
Kibaha HMU	67	61	6
Kibaha Education Centre	33	13	20
Kingolwira Prison Farm	58	54	4
Ubena Prison Farm	30	27	3
Sokoine University farms	286	60	226
Lugoba traditional stock keepers	52	48	4
Taji Mohamed farm	35	20	15
Gikas (Morogoro municipality)	37	0	37
Salum (Kihonda)	29	11	18
Christopher	18	18	0
Mzee Wacha	12	12	0
Mzee Kapuratu	33	18	15
Mzee Mambega	12	12	0
Total	**1062**	**652**	**410**

On MacConkey medium, the colonies were non-lactose fermenters, with blue green colouration of the colonies as well as the medium which was more evident than on blood agar. They were large, with irregular margin, mostly oval just after the first 24 hours incubation. The metallic sheen was found in most isolates and the characteristic odour was present in all isolates.

ii. ***Actinomyces pyogenes***

On blood agar medium, the colonies were not visible after 24 hours incubation. In case of monocultures, a faint haemolysis was noticed along the inoculation lines but not the colonies. After 48 hours incubation, very fine grayish colonies became visible and were about 0.5 mm. Individual colonies increased in size and the zone of ß-haemolysis became clearer with further incubation. These colonies did not grow on MacConkey medium. This was confirmed with monocultures and subcultures of such colonies on the media and this finding was in keeping with the observations made by other workers (Lovell, 1937).

4.1.2.2 Reaction to Grams staining of *P. aeruginosa* and *A. pyogenes*

i. ***Pseudomonas aeruginosa.***

Pseudomonas aeruginosa appeared as gram negative, medium slender rods with slight pleomorphism shown by the presence of short rods that were straight and slightly

curved. They were mostly in singles and twos (Fig. 13).

ii. *Actinomyces pyogenes.*

Actinomyces pyogenes appeared as gram positive, pleomorphic small rods which ranged from coccoids, coccobacilli to short rods. Some of the coccoids formed short chains and others were in singles or in twos. The short rods also varied in appearance; some formed chinese letters mostly v's and others had swollen ends either on both ends or one end. However, the cellular morphology is very much determined by the type of medium used (Lovell, 1937).

Other tests carried out for *P. aeruginosa* were the oxidase test and the growth at 42°C to which it was positive in both cases. The rest of the bacteria were not characterized as to species but only up to family or genera level (Table 6a).

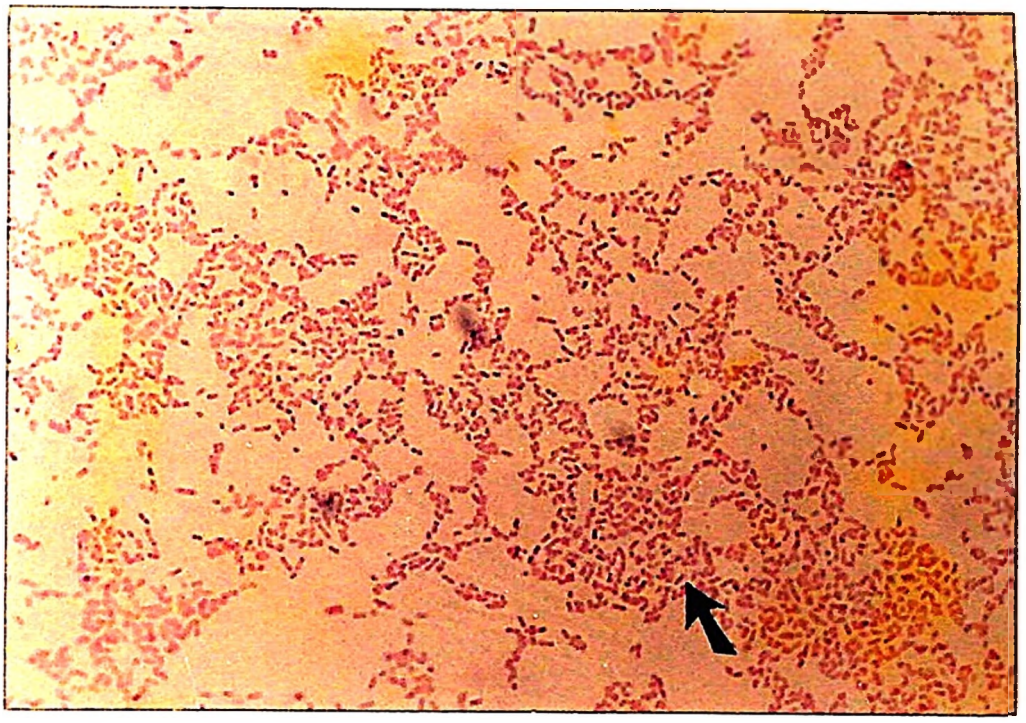

Figure 13: Gram negative rods of *P. aeruginosa* prepared from pure cultures, some rods are pointed by the arrow. The cultures were used for preparation of the inoculum for the experimental calves.

Table 6a. Bacterial isolates from samples collected from diseased and non-diseased cattle from various locations.

Origin	*Corynebacterium spp.*	*Actinomycetes spp.*	*Staphylococci spp.*	*Proteus spp.*	*Bacilli spp.*	*Klebsiella spp.*	*Escherichia coli*	*Actinomyecs pyogenes*	*Pseudomonas aeruginosa*	*Pseudomonas spp.*	*Streptococci spp.*	*Yeasts\Fungi*	No growth	Not identified
Mkata NARCO	41	18	12		11	4	8	8	2	5	1	1		9
Dakawa NARCO	5		8	6	1		4	4	2				1	
Ruvu NARCO	30	3	9		13		9	5		3	1		2	4
Mkwaja ranch	22	8	3		4	6		2		1				4
Kongwa ranch	20	6	13		4		5			2			4	3
Ruvu DAFCO	21	4	1	3	1		1	3						
Ngerengere DAFCO	17		5	2	7				1	1	1			
Kibaha HMU	35	17	10	6	7					3	5			
Kibaha EC	12	1	10	1	5	3	4		3	8		1	2	
Kingolwira prison farm	27	9	12	9	12		2	4	2		1		3	2
Ubena prison farm	20	3	8	1	1		3	1			1			3
Sokoine farms	108	25	117	75	23	9	21	9	28	12	3	10	8	13
Lugoba traditonal	30	7	6	3	3	2	5	1	2	9	1		1	4
Taji Mohamed	18	2	8	11	5	1	3	1	2	1			1	2
Gikas	27		18	5				1	2			1	1	4
Salum	16		8	2	3		1		5				1	1
Christopher	6	6	4		10		2						1	2
Wacha	8	4	1	1	2		3							
Kapuratu	22		2	9	3		1	2	2	3			1	3
Total	485	113	255	135	115	25	72	41	51	48	14	13	26	53

Table 6b. Bacterial isolates according to disease status.

	Diseased	Non-diseased
Corynebacterium spp	335	154
Actinomyces spp	87	26
Stapylococcus spp	114	141
Proteus spp	55	80
Bacillus spp	80	35
Klebsilla spp	9	16
Escherich a coli	51	21
Actinomyces pyogenes	34	7
Pseudom nas aeruginosa	16	35
Pseudomonas spp	36	12
Streptococci spp	4	10
Yeast/Fungi	3	10
No growth	17	9
Not identified	36	17

4.1.2.3 Cultural characteristics of other bacterial isolates.

i. *Actinomycetes*

On blood agar medium, this group of bacteria appeared as small, about 1 mm, gray to brown flat and others were wrinkled non-haemolytic colonies. They did not grow on MacConkey medium. By Gram's stain they appeared gram positive, branching rods which were either clubbed or not clubbed(Fig. 14).

ii. *Staphylococci spp.*

On blood agar medium, this group of bacteria appeared as small (1 mm) to large (3 mm) white, yellow orange colonies. In most cases they were smooth with an entire edge and in rare cases, especially for the small yellowish colonies, there were rough varieties with an irregular outline. On staining they appeared gram positive cocci in singles, twos, fours or clusters. Other gram positive cocci such as the *Micrococci spp.* were all included in this group.

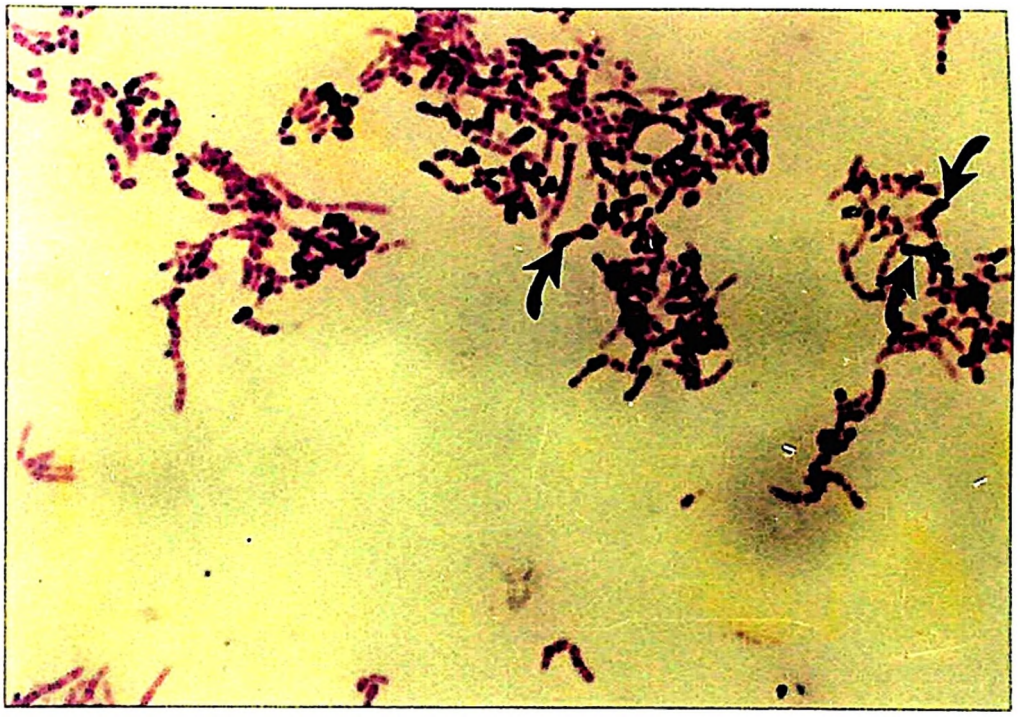

Figure 14: Gram positive branching clubbed or non-clubbed rods (arrows), a feature of the *Actinomycetes* bacteria.

iii. ***Proteus spp.***

On blood agar medium, this group of bacteria was extensive spreading colonies (swarming) with the typical so called sickly odour. On MacConkey medium, they were small to large non-lactose fermenter colonies. Some were smooth, slightly flat with entire edge while others, especially the large ones, had a tendency to spreading, becoming flat and their margin irregular. On staining they were gram negative, medium pleomorphic rods, with no specific pattern.

iv. ***Bacilli spp.***

On blood agar medium, these bacteria were large, flat, haemolytic, and gray to brown in colour. The brown colonies were in most cases wrinkled with greenish colouration to the medium. The gray colonies were in most cases not haemolytic and had no pigment. On staining they were gram positive, medium to large rods in singles, twos or formed short chains. Those with spores, the spores were centrally placed while large rods were non-sporing.

v. ***Corynebacterium spp.***

The *Corynebacterium spp.* which were the most frequent isolates encountered from the survey (Table 6a) were the only group which was identified to species level. These were identified by biochemical tests to species level as shown in Table 7 and Appendix

3 was used as an aid to further identification. According to colonial morphology the most frequent isolates had the following characteristics:

- small, cream, dull, and non-haemolytic colonies.
- very small, light yellow or cream dry and non-haemolytic colonies.
- small, grey, smooth with entire edge and non-haemolytic colonies.
- small, whitish-gray, flat, dry and serrated non-haemolytic colonies.

Of the above four identified *Corynebacterium spp* colonies, the small, cream, dull and non-haemolytic colony was the commonest which belonged to *Corynebacterium hofmanni* (Table 7). Some of the morphological features of this genus are shown in Figure 15 and 16. Table 8 shows the percentages of the *Corynebacterium spp.* from the diseased and non-diseased cattle. While their association with the disease as determined by the Chi-square test are shown in Appendix 4.

Table 7: *Corynebacterium spp.* isolated from samples collected from the survey.

Colonial morphology	Cell morphology	Acid from gl su sal mal lac xyl	u. h.	*Corynebacterium spp*
Small cream dull and non haemolytic	gram +ve small rods with club-shaped ends, some are coccoids.	-ve -ve -ve -ve -ve -ve	-ve	*C. hofmanii*
very small light yellow or cream dry and non haemolytic	gram +ve small club shaped at both ends, few in pallisade arrangement.	-ve -ve -ve -ve -ve -ve	+ve	*C. renale*
Small gray smooth with entire edge non haemolytic	gram +ve coccoids to small rods, the coccoids form short chains of three to five cells.	-ve -ve -ve -ve -ve -ve	-ve	*C. spp* (not speciated)
Small whitish gray thin and flat with serrated edges and non-haemolytic.	gram +ve medium rods, curved, form v's, some are clubbed, some are coccoids.	+ve +ve -ve +ve -ve -ve	-ve	*C. kutscheri*

gl = glucose
su = sucrose
sal = salicin
mal = maltose
lac = lactose
xyl = xylose
u.h = urea hydrolysis

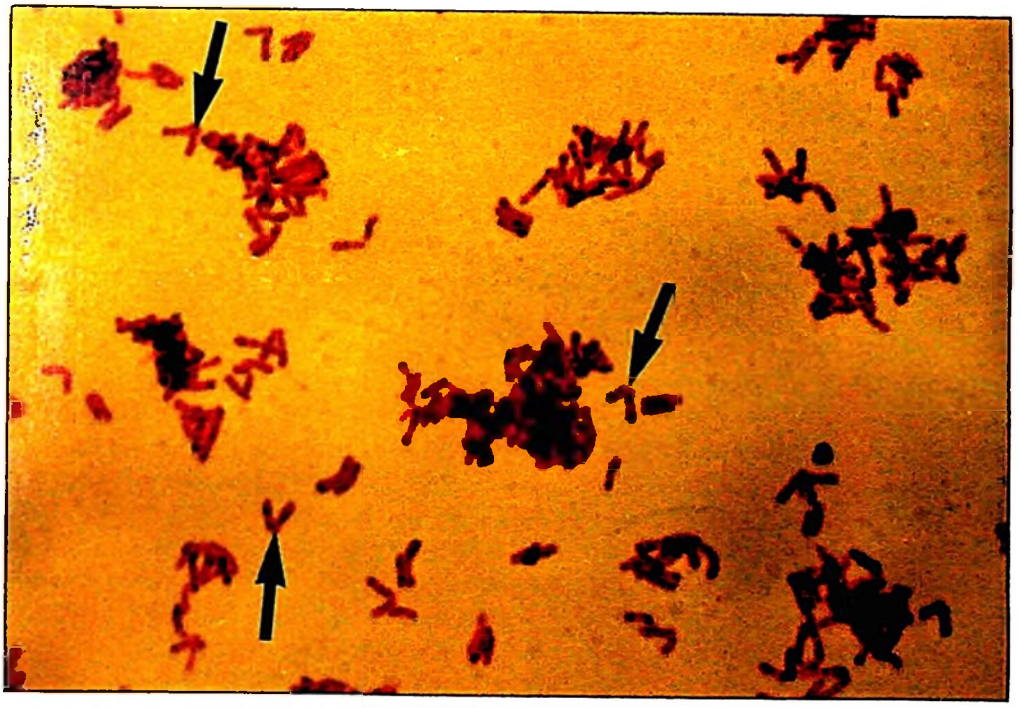

Figure 15: *Corynebacterium spp.* under Gram's staining. Arrows show the organisms in the characteristic V-shapes (Chinese letters).

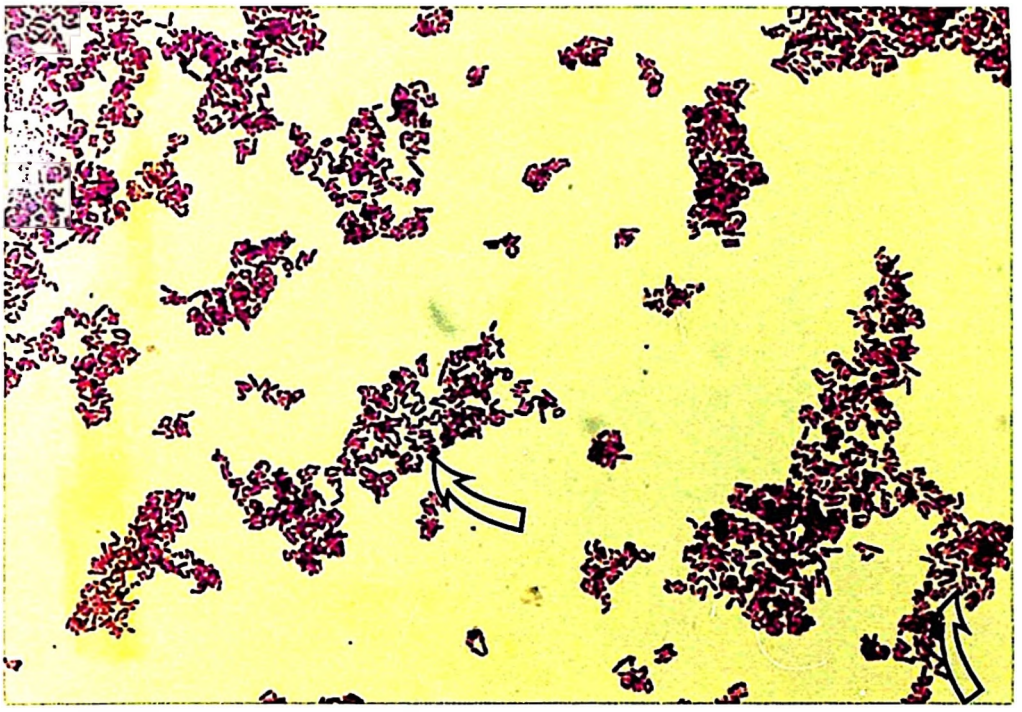

Figure 16: *Corynebacterium spp.* under Grams' staining: arrows show palisade arrangement of the cells (arrows), another feature of the genus.

Table 8. Occurrence of *Corynebacterium spp* from diseased and non-diseased ears of cattle.

Origin	Total	*Corynebacterium spp*	
		Non-diseased	Diseased
Mkata NARCO	41	-	41
Dakawa NARCO	5	-	5
Ruvu NARCO	53	8	45
Mkwaja ranch	22	8	14
Kongwa ranch	20	9	11
Ruvu DAFCO	21	9	12
Ngerengere DAFCO	17	6	11
Kibaha HMU	35	2	33
Kibaha EC	12	7	5
Kingolwira prison farm	35	4	31
Ubena prison farm	20	3	17
Sokoine farms	81	47	34
Lugoba traditional farms	30	2	28
Taji Mohamed	18	8	10
Gikas	27	27	-
Salum	16	8	8
Christopher	6	-	6
Wacha	8	-	8
Kapuratu	22	6	16
Total	**489**	**154**	**335**

4.1.3 The prevalence of *P. aeruginosa* and *A. pyogenes*

Out of the 1 062 samples examined, 652 were from diseased ears of cattle, and 410 from non-diseased. Sixteen (2.5%) samples from the 652 diseased ears and 35 (8.0%) out of 410 non-diseased ears revealed the presence of *P. aeruginosa*. Thirty three (5.1%) samples out of 652 from diseased ears and seven (1.5%) of the 410 from non-diseased ears revealed *A. pyogenes*. Twenty six samples (2.4%) had no growth on the medium. Fifty three isolates were not identified.

The associations of *P. aeruginosa* and *A pyogenes* with the bovine parasitic otitis using 2x2 contingency tables are shown in Appendices 5 and 6.

4.2 The role of *Rhabditis bovis, Pseudomonas aeruginosa* and *Actinomyces pyogenes* in the pathogenesis of bovine parasitic otitis.

4.2.1 Introduction

In order to determine the roles of *Rhabditis bovis, Pseudomonas aeruginosa* and *Actinomyces pyogenes*, a total of 49 weaner calves were infected with seven different combinations of these agents. While four calves were treated with phosphate buffered saline as controls.

The groups were inoculated weekly for three consecutive weeks at an interval of three weeks between groups.

4.2.2 Clinical manifestations of experimental calves

The type of inoculum, the number of calves that developed clinical manifestations in each group and the severity of the responses are shown in Table 9.

Table 9: Clinical responses of calves experimentally inoculated with combinations of *Rhabditis bovis* and bacteria *Pseudomonas aeruginosa* and *Actinomyces pyogenes*.

Gr.	Inoculum	Calves per group	Clinical response in calves		
			No.	%	Type of reaction
1	*Rh. bovis*	7	5	71.4	4 mild 1 moderate
2	*Rh. bovis* + *P. aeruginosa.*	7	6	85.7	5 mild 1 moderate
3	*Rh. bovis* + *A. pyogenes.*	7	6	85.7	3 mild 2 moderate 1 severe
4	*Rh. bovis* + *P. aeruginosa.* + *A. pyogenes.*	7	7	100	3 moderate 4 severe
5	*P. aeruginosa.*	7	0	0	none
6	*A. pyogenes.*	7	0	0	none
7	*P. aeruginosa* + *A.pyogenes.*	7	0	0	none
8	Control	4	0	0	none

A. =*Actinomyces*
P. =*Pseudomonas*
Rh. = *Rhabditis*
No. =number, Gr.=Group

Key to clinical responses

mild - scanty discharge with nematodes confined inside the ear canal
moderate - discharge with nematodes extending to the opening of the ear canal. the underlying tissue is hyperaemic, head shaking other signs not evident yet.
severe - plenty of discharge running out and soiling the pinna and area below the ear. The underlying tissue is hyperaemic and/or ulcerated, other signs such as dullness, swelling of the ear base and local lymph nodes and hypereaxic.

4.2.2.1 General clinical manifestations

The onset of clinical manifestations were characterized by an initial wetting of the ear canal which lasted from two days after the first inoculation to twenty one days after the third inoculation. The wet state of the ear canal was followed by the appearance of the nematodes that were visible only by the aid of an otoscope. With the appearance of the nematodes, the wetness changed to an obvious creamy discharge that increased in amount and became either thick or fluidy. The thick discharge was in most cases confined to the ear canal and a small portion of the convex part the pinna as shown by calves from groups one and two (Fig 17 and 18). The fluidy discharge oozed out of the ear canal and soiled the pinna and in few cases the area below the ear.

Other accompanying signs were, different degrees of hyperaemia of the tissue underlying the discharge, which bled easily when disturbed by scratching with the hooves, swabbing or rough handling. There was occasional shaking of the head and scratching of the ears with hooves. The affected calves showed reluctancy of being handled. In severe cases, the hyperaemic areas later became ulcerative and/or erosive, with the swelling of the ear base and the corresponding parotid lymph node. Such calves were dull, with hyperexia of up to 40.9°C.

The clinical signs were scored as mild, moderate, and severe. Mild clinical signs were characterized by scanty aural discharge with few nematodes which could only be seen by the aid of an otoscope. Such cases had no other accompanying signs. Moderate

clinical signs were characterized by an aural discharge extending to the outlet and involving the convex part of the pinna immediate after ear canal opening. The nematodes were visible in the discharge even without the use of an otoscope. The tissue underlying the discharge was hyperaemic. Severe clinical signs were characterized by an aural discharge which oozed out the ear canal and soiled the pinna and the area below the ear. The area underlying the discharge was ulcerative or erosive, with the swelling of the ear base and the corresponding parotid lymph node. The severe cases were dull, and pyrexic. Few cases (nos 24094 in group two and no. 96 in group three) manifested tilting of the head towards the affected ear and retention of the bolus in the cheek in calf no 96.

4.2.2.2 Group clinical manifestations

i. Group one

This group comprised of calves nos 85, 24091, 9084, 26048, 9081, 9084 and 97. Five calves out of seven which were infected with *Rhabditis bovis* alone, developed clinical signs. Four calves i.e calves nos 9084, 26048, 9081, and 24091 developed a mild response while calf no. 85 developed a moderate response. Calf no 85 was the first to show clinical manifestations just three days after the first inoculation. The discharge developed in both ears and was thick gray containing the nematodes whose movements could be seen by otoscope. There was a foetid smell which became stronger with time, and the amount of the discharge increased after the third inoculation but remained

inside the ear canal (Fig. 17). There after the discharge seemed to decrease from the 60th day i.e 57 days after the onset of the clinical signs. The decrease was characterized by the edges of lesions drying up and the contents becoming almost desiccated. This was the trend to the end of the experiment (eleven weeks). There was no change in demeanor throughout the experiment, and on swabbing after the experiment on the 84th day, there were plenty of nematodes from both ears.

Calf no 24091 developed mild clinical signs one day after the third inoculation i.e 22 days after first inoculation. The discharge was scanty through out the observation period. There was no change in the demeanor of the calf. The calf however slipped and broke the femur 48 days after the onset of experiment, the animal was destroyed as the fracture could not be repaired.

Calf no. 9084 developed mild clinical signs six days after the third inoculation and remained so to the end. Otherwise the demeanor was bright, and on swabbing few nematodes were recovered from both ears. Calf no. 26048 developed mild clinical signs 15 days after the third inoculation starting with the left ear while clinical manifestations of the right ear became apparent 43 days after the third inoculation. The discharge remained scanty throughout the observation period (25/5/1993- 13/8/1993), but towards the last two weeks (1/8/1993-13/8/1993), the discharge had dried off. On final swabbing (14/8/1993), there were few nematodes in both ears.

Calf no. 9081 was observed with scanty thick and grayish cream discharge with nematodes in the left ear 54 days after the third inoculation. The discharge was scanty throughout the observation period.

Calves nos 9086 and 97 did not develop any clinical signs at all even after the third infection and on swabbing there were no nematodes in their ears.

Although the overall clinical response indicated that five calves developed clinical signs, most were of mild nature.

Figure 17: Moderately thick aural discharge (arrow) from an experimental calf from group one inoculated with *Rhabditis bovis* alone.

ii. **Group two**

Group two consisted of calves no 24094, 26041, 9083, 9079, 9082, 86, and 24099 which were inoculated with *Rh. bovis* and *P. aeruginosa*. Six out of seven calves developed clinical manifestations. Calf no. 9083 developed moderate clinical manifestations six days after the first inoculation. The aural discharge was greyish in colour, very thick, and confined to the ear canal (Fig. 18). However the calf remained bright although very nervous during handling. Calves nos. 24099 and 26041 developed mild clinical manifestations seven days after the first inoculation. Nematodes were apparent in the discharge on the third day of the second inoculation in calf no 24099 and on the fourth day in calf no. 26041, the demeanor of these calves remained bright.

Calves nos. 24094 and 86 showed mild clinical response whereby there were aural discharges from both ears three days after the third inoculation. Calves nos. 9082 and 9079 did not develop any clinical response but on swabbing at the end of the experiment (3/9/1993), the right ear of calf no. 9082 had a few nematodes.

Calf no. 24099 that developed mild clinical manifestations was very nervous at handling and shook the head frequently and scratched the ears more vigorously than the rest of the affected calves in the group. Calf no. 24094 developed moderate clinical manifestations on the left ear that was seen drooping most times. The overall clinical response in this group was moderate. Ear swabs at the end of the experiment showed the presence of nematodes.

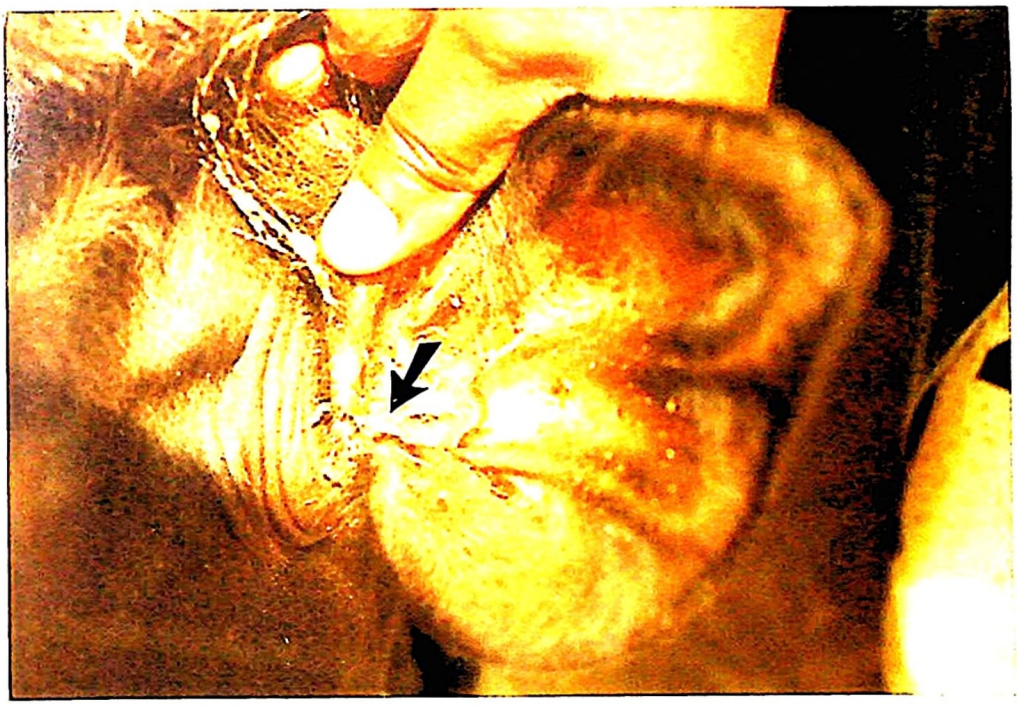

Figure 18: Calf no. 9083 from group two inoculated with *Rh. bovis* and *P. aeruginosa*. The photograph shows a thick creamy discharge confined to the ear canal outlet (arrow). This was one week after the third inoculation.

iii. **Group three**

Group three consisted calves nos 96, 24045, 91, 88, 90, 99 and 24096 which were inoculated with *Rh. bovis* and *A. pyogenes*. Six out of seven calves developed clinical manifestations, three calves nos 24045, 88, and 99 developed mild clinical manifestations while another three calves 96, 90 and 99 developed moderate clinical manifestations. Calf no. 24045 developed mild clinical signs three days after second inoculation on the right ear and five days for the left ear. The aural discharge was scanty, thick and creamy gray. Calf no. 90 developed clinical signs five days after the second inoculation. The right ear developed moderate clinical manifestations and the discharge was thick and creamy. Calf no. 91 developed clinical signs on the left ear six days after the second inoculation. However, nematodes were observed five days after the third inoculation. Six days after the third inoculation the aural discharge was oozing down the ear canal (Fig. 19). This condition continued for three weeks thus resulting into slight soiling of the hair below the ear. Later the discharge decreased in amount and was confined into the ear canal.

Calf no. 96 developed scanty thick aural discharge in the right ear two days after the third inoculation. Five days later nematodes were apparent in the discharge. One week later the discharge from the right ear was oozing to the extent of the discharge actually dripped to the ground (Fig. 20). Along with this observation 26 days after third inoculation, the calf was observed to tilt its head towards the right ear (Fig. 21). Fifty days after the third inoculation, a small bolus of grass of about 4cm in diameter was

detected into the right side of the cheek. The bolus lasted for 3 days and disappeared. The demeanor of the calf appeared dull and the calf was observed to loose weight slightly. The left ear was observed with scanty discharge six days after third inoculation.

Calf no. 88 reacted mildly by developing slight aural discharge from the right ear five days after the third inoculation.

Calf no. 99 reacted mildly nine days after third inoculation, by developing scanty thick grayish discharge on the right ear. The condition continued to be mild in the right ear but on final swabbing even the left ear which did not respond had nematodes. The overall group response was moderate.

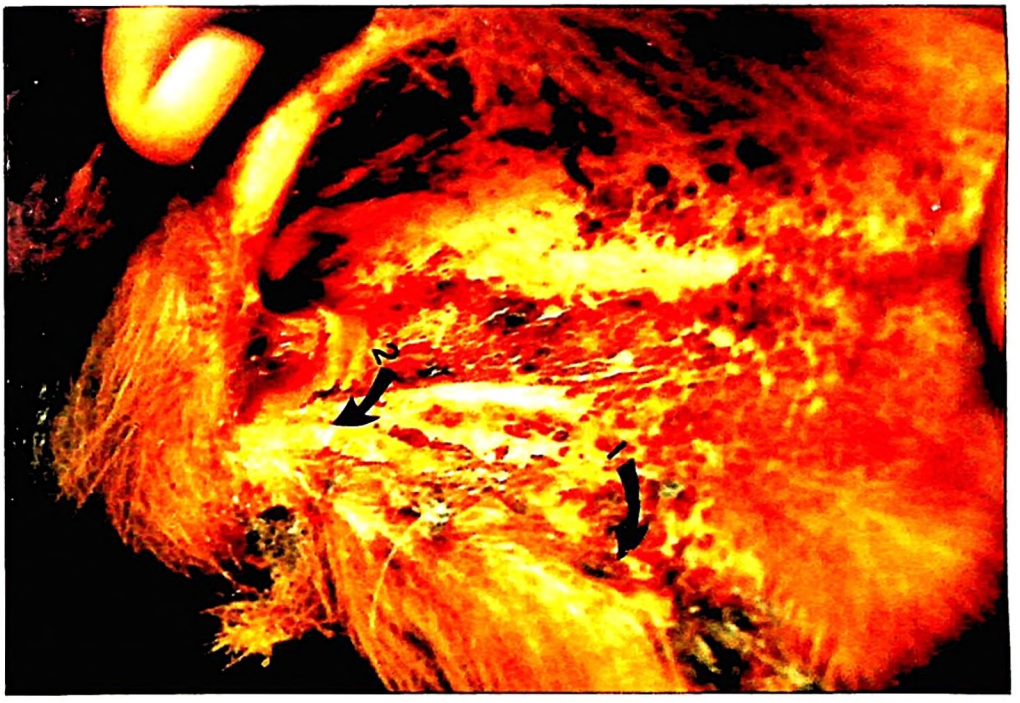

Figure 19: Calf no. 91 experimentally infected with *Rh. bovis* and *A. pyogenes* showing a fluid discharge which had soiled the pinna. Some of the discharge had dried up (arrow 1) and some was still running (arrow 2). This was 2 weeks post third inoculation.

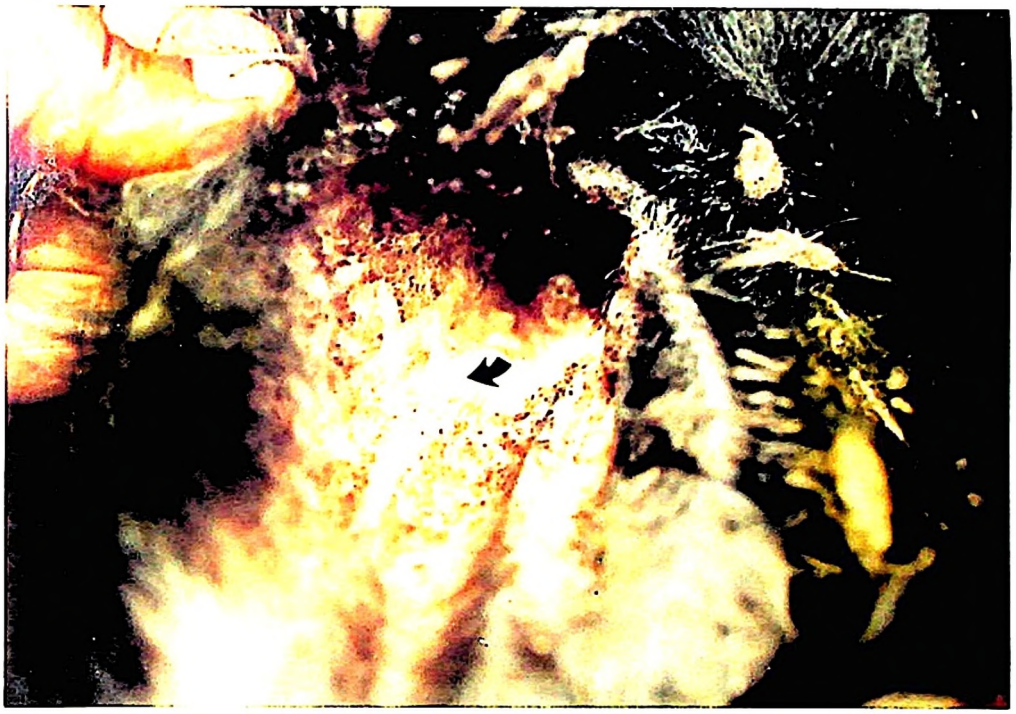

Figure 20: Calf no. 96, a closer view of the right ear with fluidly discharge which had soiled the pinna (arrow). The calf was inoculated with *Rh. bovis* and *A. pyogenes*.

Figure 21: Calf no. 96 inoculated with *Rh. bovis* and *A. pyogenes*. The right ear shows matting of the hairs of ear (arrow) and the head tilted to the most affected ear (right). This was four weeks after the third inoculation.

v. Group four

Group four consisted of calves nos 101, 103, 105, 269, 270, 374, and 375 which were inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*. All seven calves developed clinical manifestations whereby four had severe and three had moderate clinical manifestations. The clinical signs developed at different times. The first calves to respond were nos 105 and 270 which developed clinical signs five days after the third inoculation. However by the tenth day all calves had shown clinical signs. Initially the signs were mild characterized by scanty discharge only, then became moderate after one week whereby the discharge increased in amount and the aural lesions became more hyperaemic. By 24 days after the third inoculation, the clinical signs became severe in four calves, nos 103, 105, 269 and 270. At this stage, the clinical signs were characterized by swelling of the ear base, and the local lymph nodes especially the parotid lymph nodes were also swollen (Fig. 22). The aural lesions which extended to the pinna were characterized by erosion of the area underlying the discharge (Fig. 23).

These calves were dull, showed resistance when handled, and on occasions showed a considerable temperature reaction especially on the seventh week of the experiment. The pyrexia lasted for one week while the aural lesions however lasted for four weeks and started subsiding.

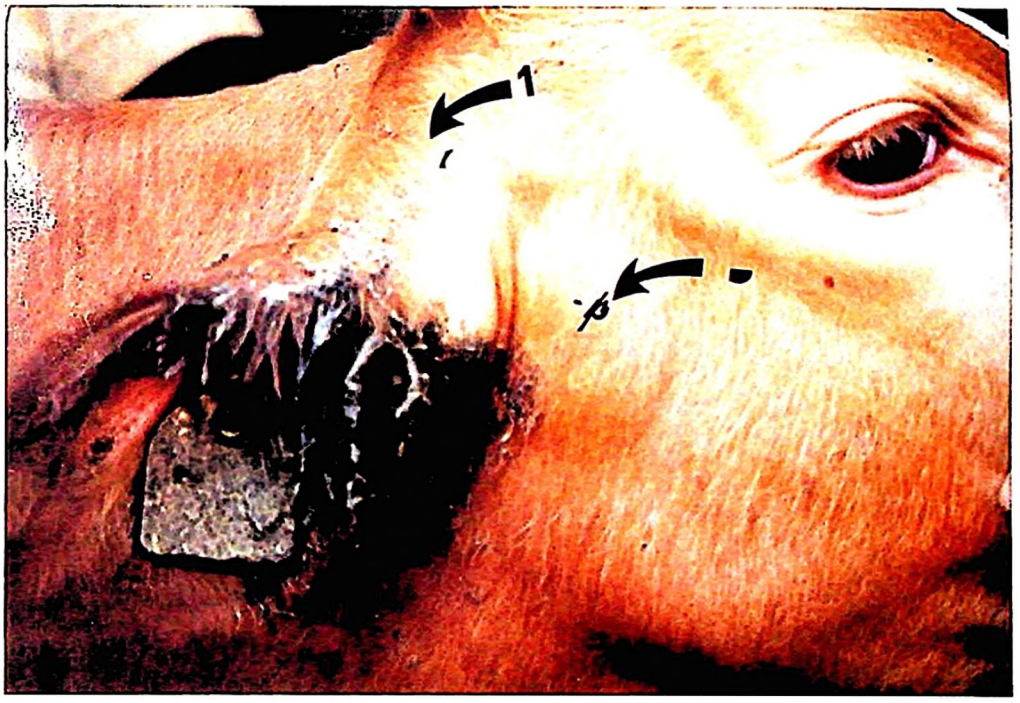

Figure 22: Calf no. 103 from group four inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*. This calf developed severe clinical manifestations characterized by swelling of the ear base (arrow 1) and the corresponding parotid lymph node (arrow *p*). At the 29th day post inoculation

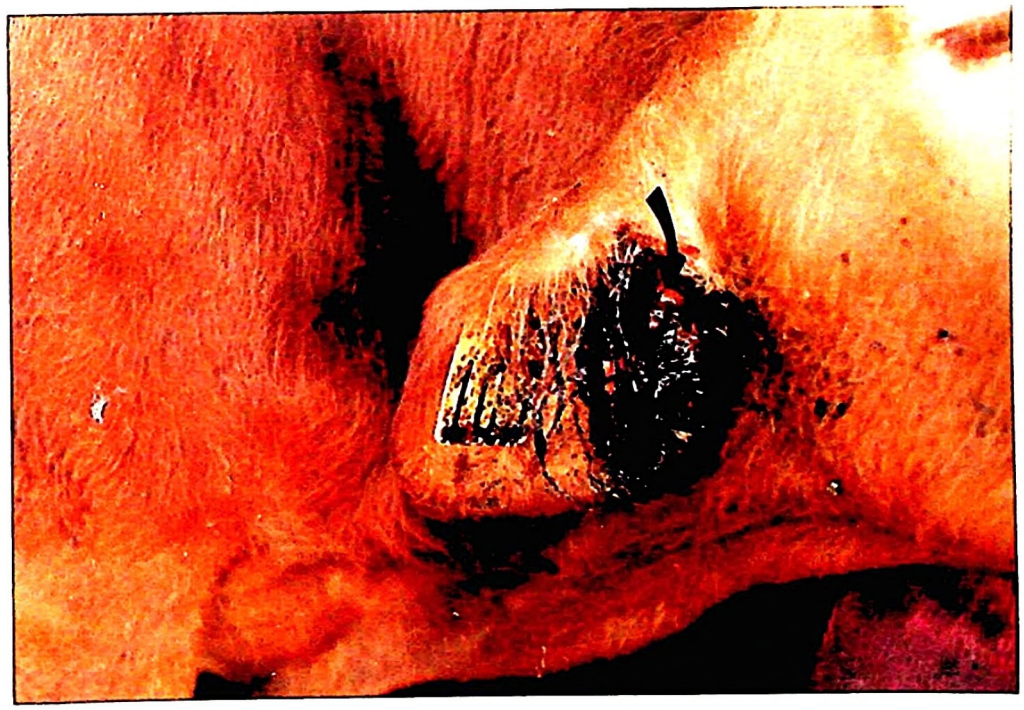

Figure 23: A closer view of the ear of calf no. 103. Note the eroded area shown by the arrow.

The recovery of the aural lesions were more apparent in calves nos 103 and 105 in which the discharge decreased in amount and the lesions dried by forming crusts. The crusts later disappeared on their own and the aural lesions were healed. Figures 24-27 shows calf no 105 with the lesions progressively decreasing in severity and finally healing up. The decrease in severity was accompanied by the decrease in the discharge and disappearance of the nematodes and in fact the left ear of calf no. 103 was completely healed at the end of the experiment and there were no nematodes recovered. The disappearance of the nematodes was confirmed by swabs taken on a fortnightly basis in this group after such a trend having been observed in other groups on swabbing at the end of the observation period of each group. The relationship of the severity of the lesions and the presence of the nematodes are shown in Appendix 7. The clinical manifestations in calves nos 101 and 270 subsided slightly and remained mild to the end of the observation period. The overall clinical manifestations were severe as all calves responded.

v. Group five

This group consisted of calves nos 360, 24022, 101, 26058, 9076, 24072, and 26045 which were infected with *P. aeruginosa*. All calves did not develop any clinical manifestations.

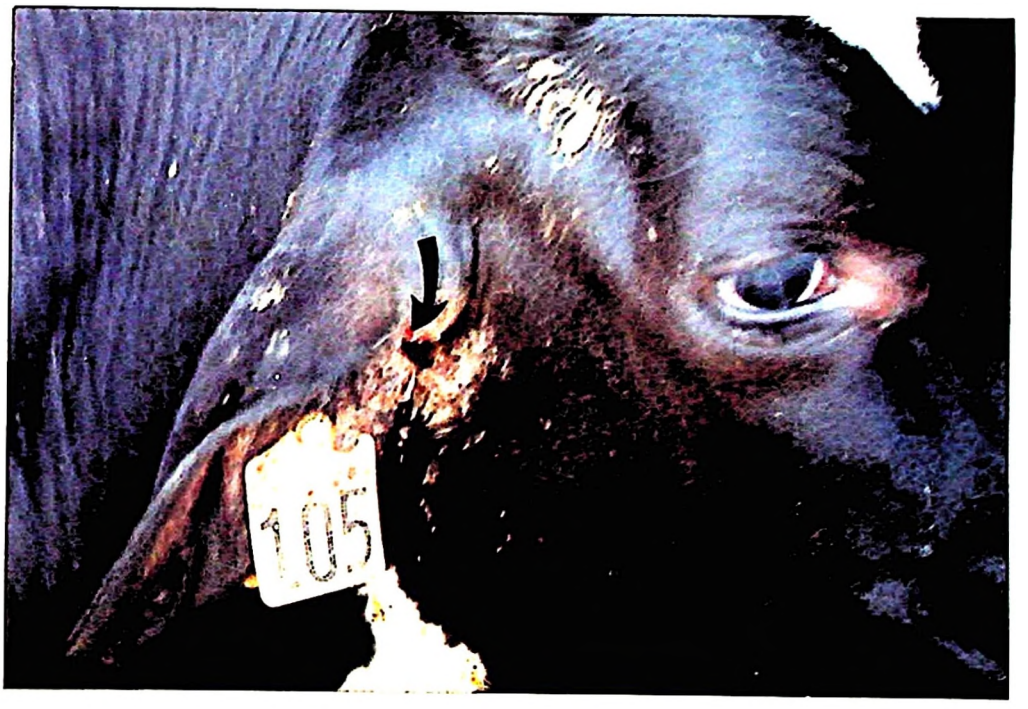

Figure 24: A calf no. 105 inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*, 35 days post first inoculation showing severe clinical manifestations. There was ulceration up to the ear canal outlet (arrow), swelling of the ear base and the parotid lymph node.

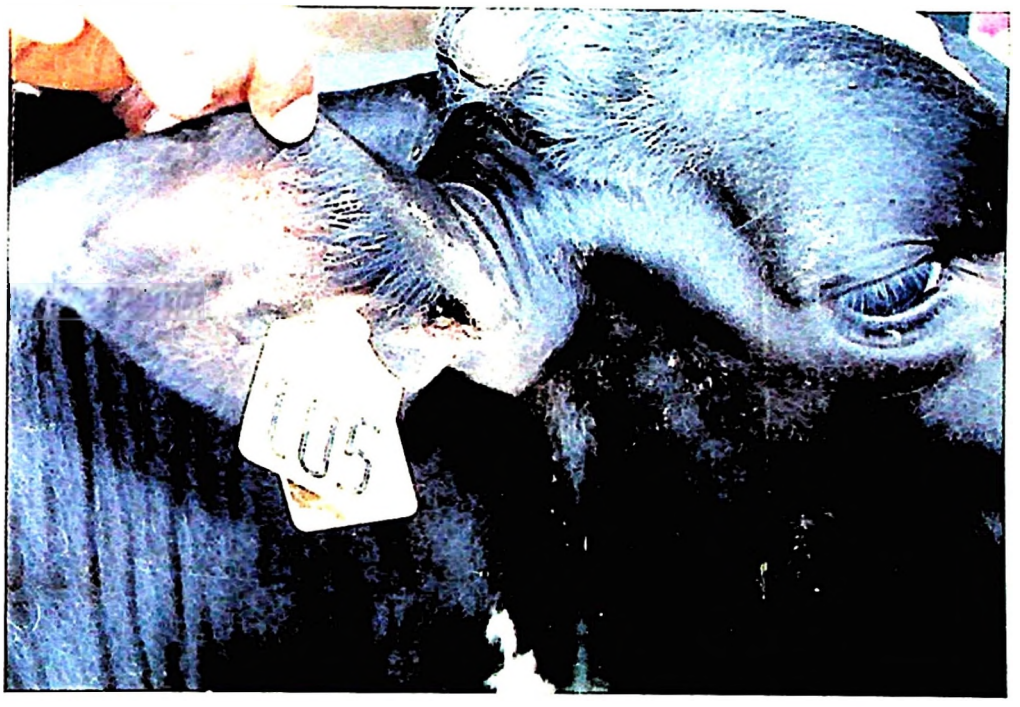

Figure 25: The same calf no. 105 as in fig. 24, 49 days post first inoculation, note the subsiding aural lesions (arrow) and swelling of the ear base and parotid lymph node.

Figure 26: The same calf no. 105 as in fig. 25, 63 days post first inoculation, the aural lesions had almost subsided (arrow).

Figure 27: The same calf no. 105 as in fig. 26, 77 days post first inoculation. There was much improvement on the lesion than the previous two 2 weeks (fig. 26) (arrow).

vi. **Group six**

This group consisted of calves nos 102, 123, 104, 125, 106, 107, and 108 which were infected with *A. pyogenes*. All calves did not develop any clinical manifestations.

vii. **Group seven**

This group comprised of calves nos 109, 110, 111, 112, 113, 114, and 252 which were infected with *P. aeruginosa* and *A. pyogenes*. All calves did not develop any clinical manifestations.

viii. **Group eight**

This group comprised of calves nos 259, 260, 356, and 354 which were inoculated with phosphate buffered saline as controls. All calves did not develop any clinical manifestations.

4.2.2 Analysis of data

The mean scores of the clinical manifestations which developed in experimental calves in groups 1-8 as described under 3.2.2 are shown in Appendix 8. The comparison of the means between groups was carried out by the General Linear Model procedure (Appendix 9) because of the uneven number of calves in each group. The responses between groups which were significantly different are shown by sign *** (Appendix 9).

4.2.3 Bacterial isolation from ears of experimental calves prior, during and post treatment.

The aural bacterial flora of the experimental calves had to be determined before the experiment to establish the type of bacteria present. Then the aural flora had to be determined at the end of the experiment to ascertain whether the same bacteria could be recovered. Bacteriological examination was also necessary to determine if the test organisms survived to the end of the experiment so as to relate them with the clinical disease.

Bacteria isolated from each group before and after the experiment are shown in Appendices 10-17. The tables also show that the most frequently occurring bacteria at both incidences were *Corynebacterium spp.* and *Staphylococci spp.* The test bacteria inoculated into the ears of calves were rarely recoverable at the end of each observation period. In group two, whereby calves were inoculated with *Rh. bovis* and *P.*

aeruginosa; no bacteria were recovered from the calves at the end of the observation period. The same for group three which was inoculated with *Rh. bovis* and *A. pyogenes*. In group four which was inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*, the bacteria were not recovered at the end of the observation period either. In group five *P. aeruginosa* was isolated from two ears out of 14 inoculated (14.29%) while from group six which was inoculated with *A. pyogenes*, the bacterium was not recovered. In group seven which was inoculated with *P. aeruginosa* and *A. pyogenes*; *P aeruginosa* was recovered from four ears out of 14 (28.58%) while *A. pyogenes* was recovered from only one ear out of 14 (7.15%). These results also show that the test organisms were recovered more from groups infected with bacteria alone (group 5, 6,and 7) than those infected with the nematode and the bacteria (groups 1-4).

Changes in aural bacterial flora of experimental calves were noted during the bacteriological examinations done before the experiments begun. Then a follow up of bacterial isolation during the experimental period was carried out in groups two and four to investigate these changes in aural bacterial flora. These changes are indicated in Appendices 18 and 19 showing sequential bacterial isolation in groups two and four. There was consistency in isolation of *Corynebacteria spp.* in the majority of calves and *Proteus spp* was dominant in few calves. The bacteria which were not constantly isolated were the *Actinomycetes* and *Bacilli spp.* Group two showed the same trend as group four with the *Corynebacteria spp.* dominating the type of isolates.

4.2.4 Histopathology

4.2.4.1 Normal histology of the external ear canal.

The normal histology of the bovine ear has not been well studied compared to that of small animals and human beings and thus has been thought to resemble that of other animal species(Dellmann and Brown, 1976).

The external meatus extends from the auricle to the tympanic memrane and is not perpendicular to the sagittal plane of the head, it is inclined at an angle upward. The approximate length of the canal to the temporal bone ranges from 3.5-4.5 cm. The outer two thirds are supported by the cartilage whereas the inner one third is supported by bone. At the beginning of the canal the epithelium is stratified squamous and slightly thicker with few dermal ridges. The sebaceous and ceruminous glands are numerous and conspicuous while the hair follicles are sparse(Fig. 28). At the outer two thirds of the canal, the epithelium lining changes gradually to a non keratinized stratified squamous epithelium. Both sebaceous and ceruminous glands decline in size and abundance, hair follicles and sebaceous glands disappear at the supporting cartilage. The ceruminous glands disappear further into the canal. Throughout the canal, small blood vessels are prominent in the dermal ridges close to the epithelial surface.

4.2.3.2 Histopathology of the experimentally infected ear with bovine parasitic otitis

Histopathological changes per group are shown in Table 10. The histopathological changes observed were the varying degrees of inflammatory cellular infiltration of mostly neutrophils and lymphocytes in the subepithelium immediately below the basement membrane. There was thickening of the epithelium without any cellular infiltration (Fig. 29), or accompanied by increased papillary interdigitation and slight cellular infiltration (Fig. 30). The thickening was in most cases characterized by development of extensive papillary interdigitation (rete ridges) (Fig. 30) and a magnified section showing the type of cells (Fig. 31). The infiltration varied from mild to severe, some of the severe infiltration formed aggregates of cells in the subepithelium (Fig. 32 and 33). Some sections showed discharge in the lumen of the ear canal in which sections of nematodes or whole nematodes could be observed (Fig. 36).

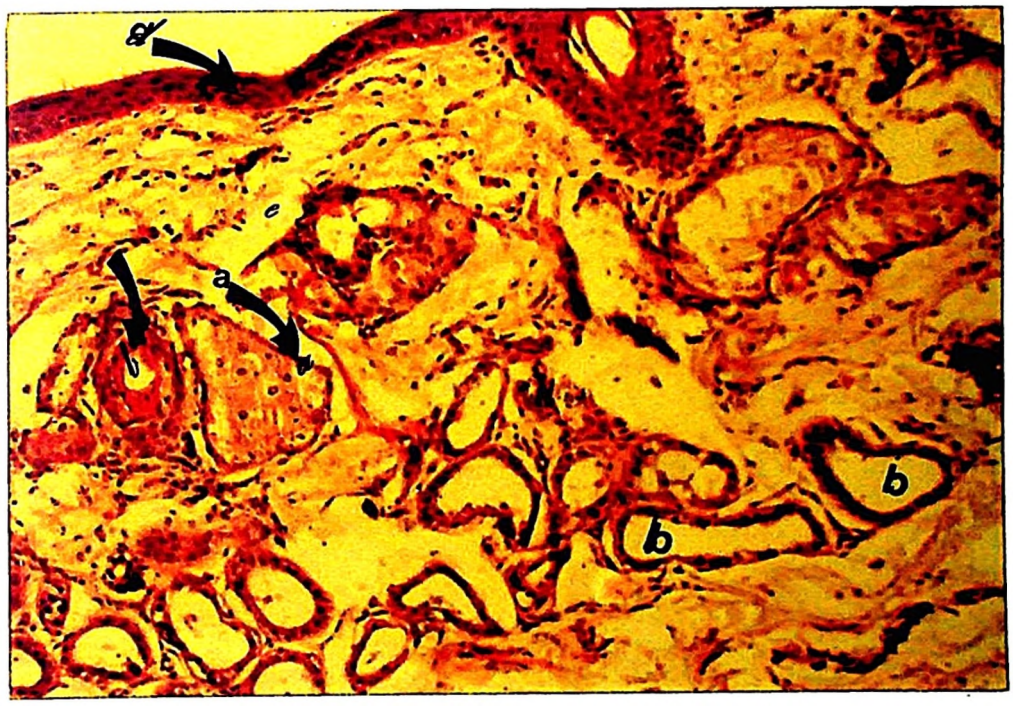

Figure 28: Histological section of the ear of a calf from the control group inoculated with phosphate buffered saline (H & E stain) where (a) sebaceous gland, (b) ceruminous glands, (c) subepithelium and (d) stratified squamous epithelium.

Table 10: Histopathological changes of calves experimentally infected with various combinations of *Rhabditis bovis*, *Pseudomonas aeruginosa* and *Actinomyces pyogenes*.

Group	Type of inoculum	Clinical response	Histopathological changes
1	*Rh. bovis*	mild	slight thickening of the epithelium with minimal cellular infiltration.
2	*Rh. bovis* and *P. aeruginosa*	mild	slight thickening of epithelium with cellular infiltration mostly with lymphocytes and slight increased dermal pappilae interdigitation.
3	*Rh. bovis* and *A. pyogenes*.	moderate	More thickening of epithelium with more cellular infiltration mostly by lymphocytes.
4	*Rh. bovis*, *P. aeruginosa* and *A. pyogenes*	severe	More thickening of epithelium with dermal papillae interdigitation with severe cellular infiltration and slight, desquamation of epithelium.
5	*P. aeruginosa*	none	no inflammatory change
6	*A. pyogenes*	none	no inflammatory change
7	*P. aeruginosa* and *A. pyogenes*.	none	no inflammatory change
8	Phosphate buffered saline	none	no inflammatory change

Rh.=Rhabditis
P = Pseudomonas
A.= Actinomyces

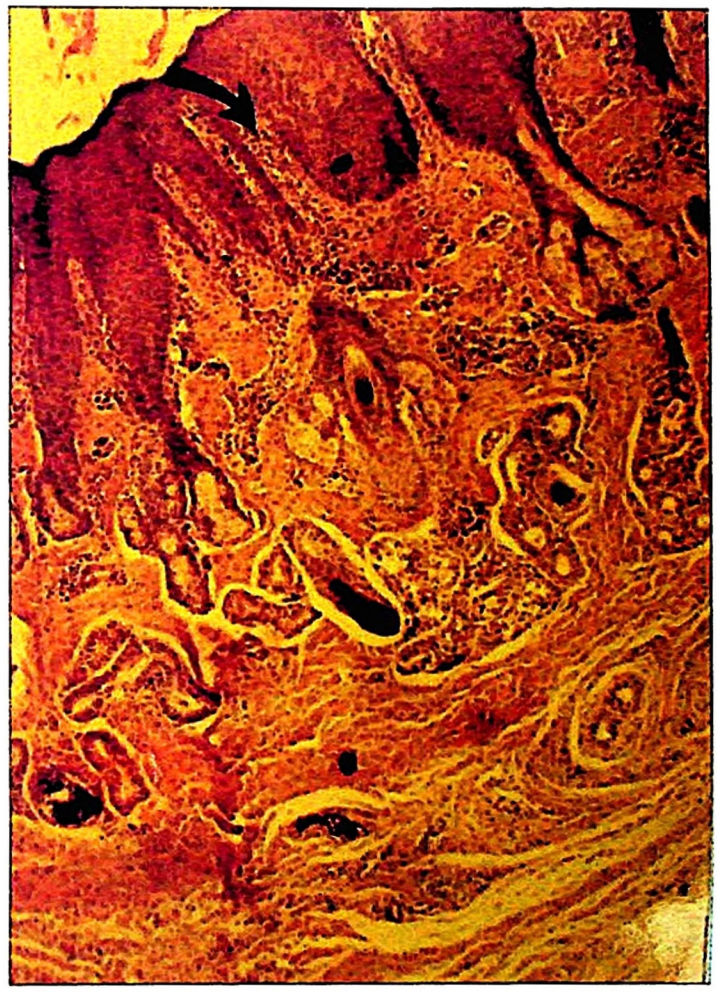

Figure 29: Histopathological section of the ear from a calf of group one with mild clinical manifestation. The epithelium is slightly thickened (arrow) and there is very minimal cellular infiltration x100 (H & E stain).

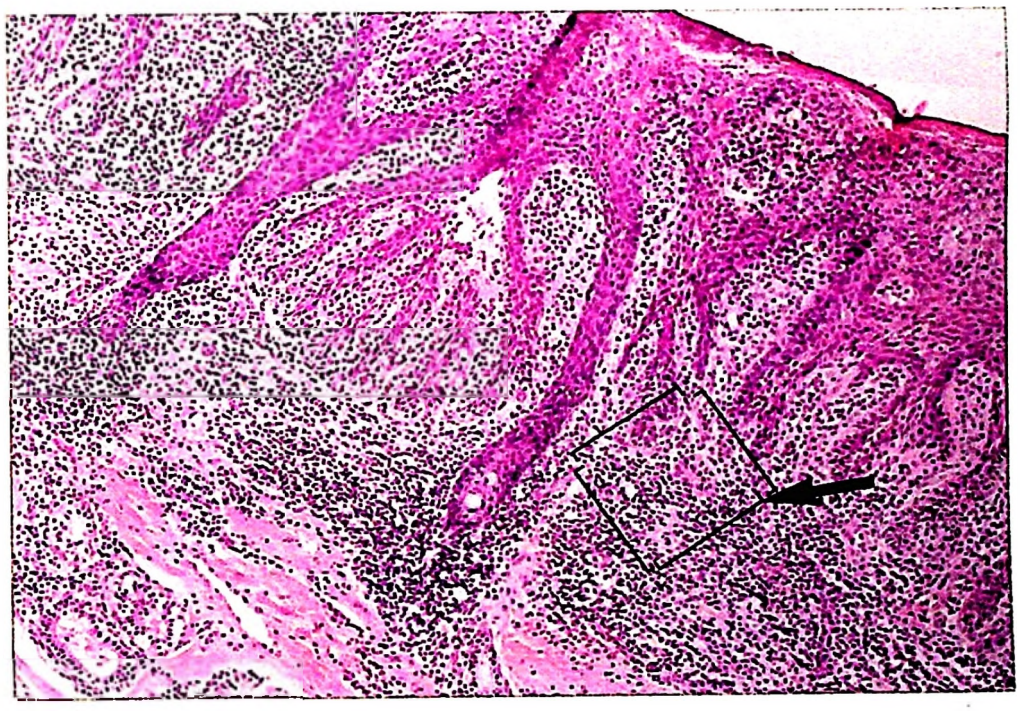

Figure 30: Histopathological section of the ear from a calf in group two, there is inflammatory cellular infiltration (arrow). x100. (H&E stain)

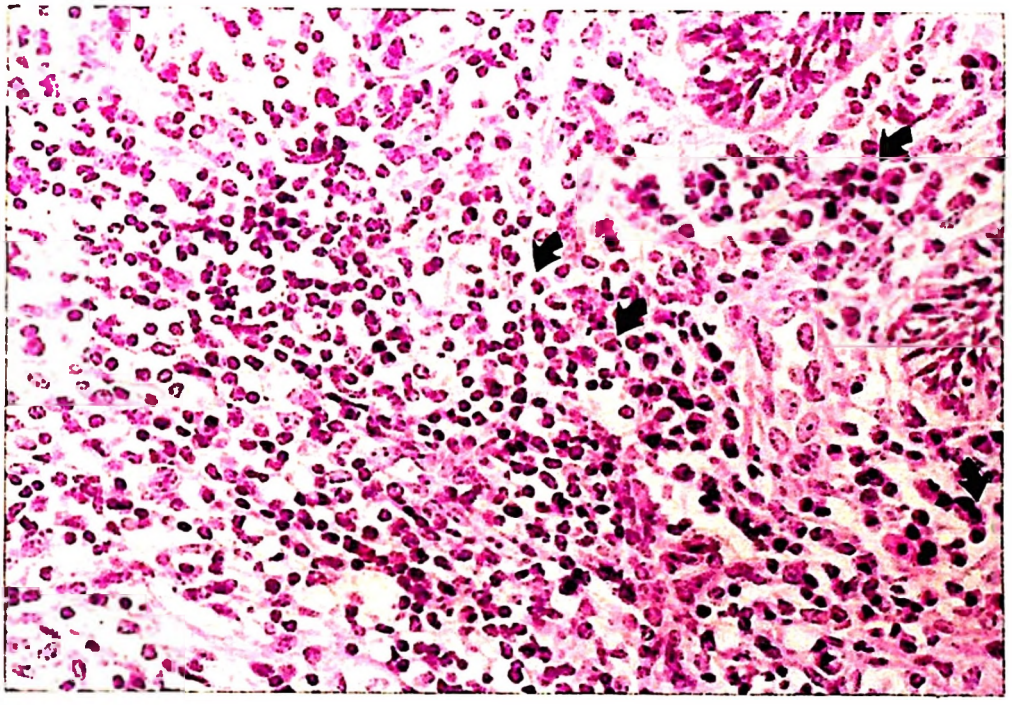

Figure 31: A magnified section from the area in squares of Fig. 30 showing lymphocyte infiltration (arrows). x400 (H&E stain).

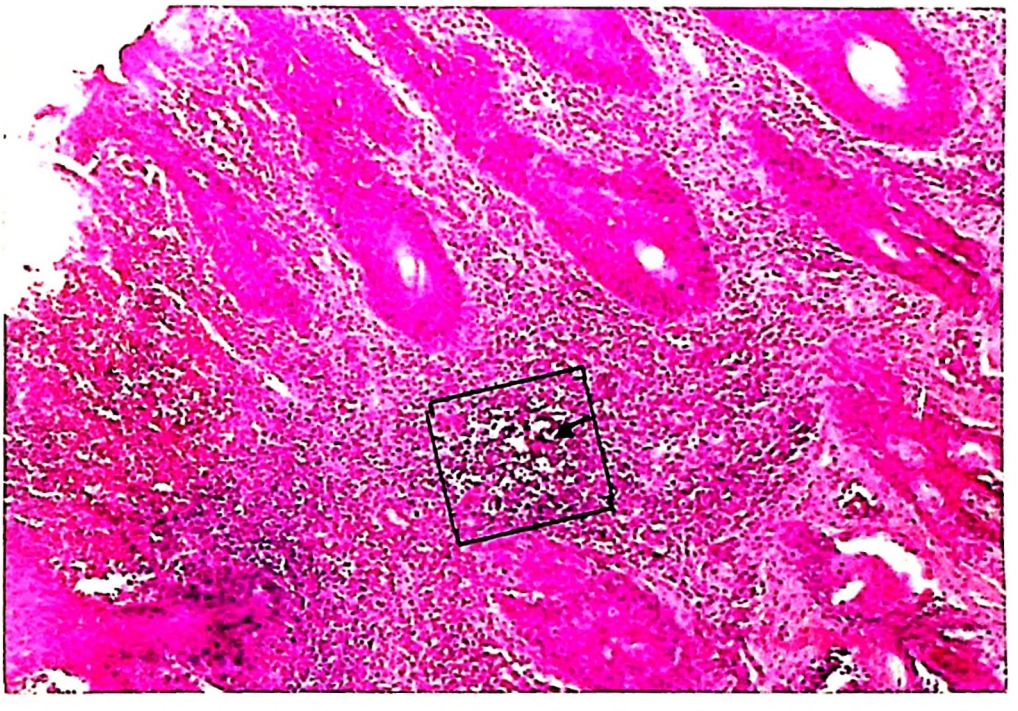

Figure 32: A histopathological section of the ear of a calf with severe cellular infiltration inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*. The square indicate an aggregate of cells. x200 (H & E stain).

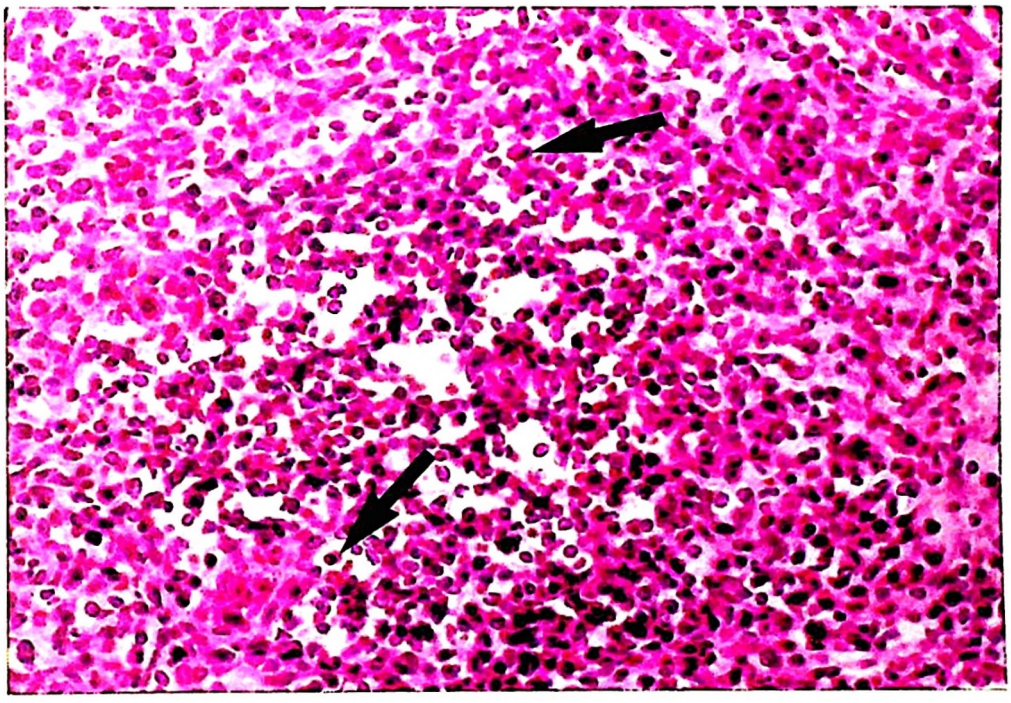

Figure 33: A magnified section of Fig. 32 marked with squares showing lymphocytes at x400 (arrows) (H & E stain).

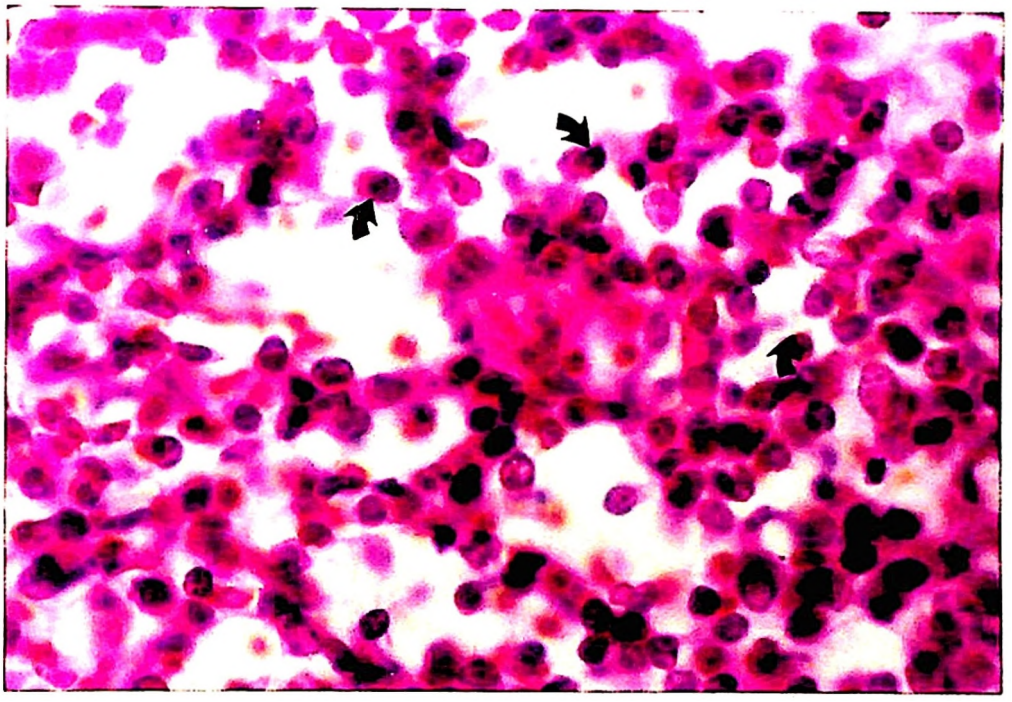

Figure 34: A magnification of x1000 of a section of Fig. 33 showing lymphocytes infiltration more clearly (arrows). (H&E stain).

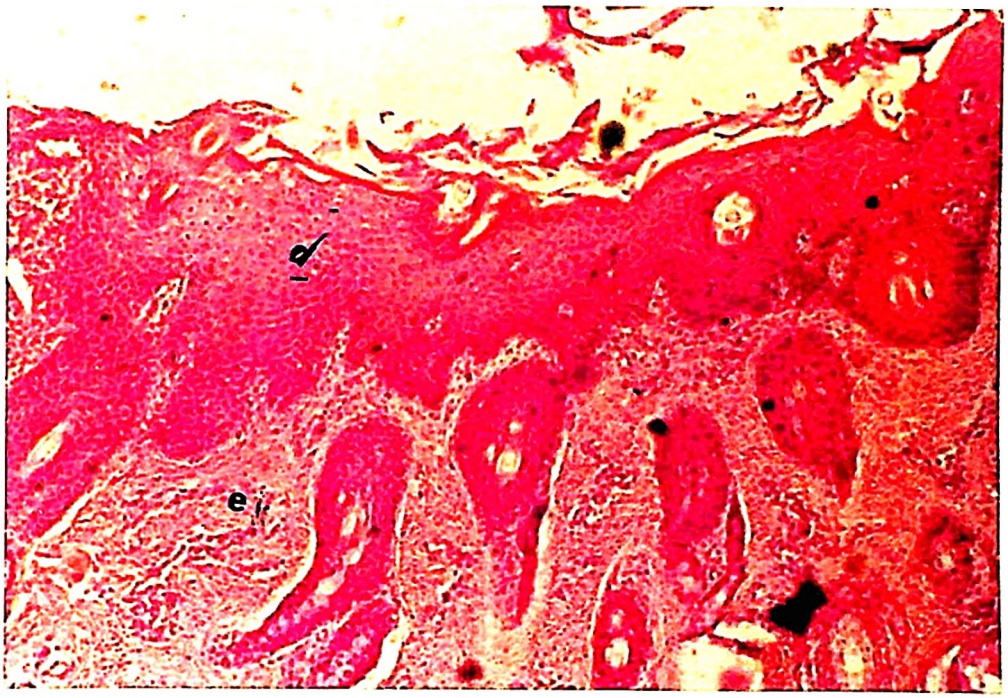

Figure 35: A histopathological section of the ear of a calf from group three inoculated with *Rh. bovis* and *A. pyogenes* showing thickening of the epithelium (d), there is mild cellular infiltration in the subepithelium(e).x200. (H&E stain).

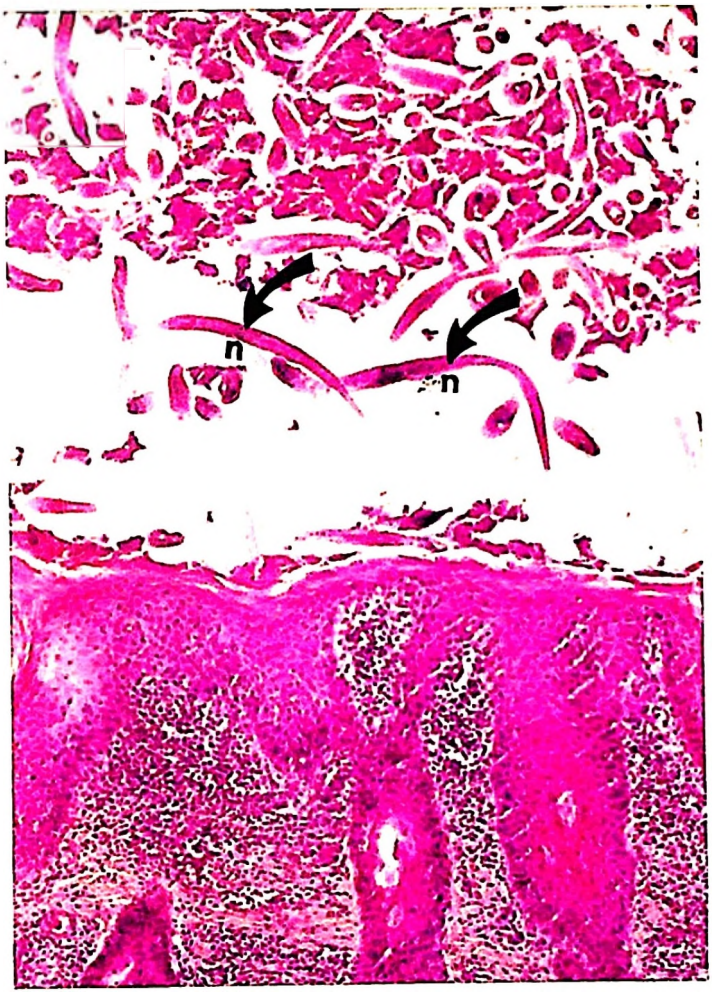

Figure 36: A histopathological section of the ear of a calf from group four showing the presence of nematodes in the lumen of the ear canal (arrows). There is also severe cellular infiltration and thickening of epithelium x400 (H & E stain).

4.3 The role of dips, sprayraces and soil/manure in the transmission of nematode *Rhabditis bovis*

4.3.1 Exposure of cattle to dipping, spraying and pour-on applications

Sixty cattle roughly aged two years were exposed to different tick control methods namely dipping, spraying and pour-on preparation for a period of five months. The objective was to determine the role of both dip tanks and spray races in the epidemiology of the disease. Pour-on preparation was used for the control group. Ear swabs from all groups were taken every two weeks to determine the presence of nematodes in the ears. Throughout the experiment, no nematodes were recovered from ear swabs indicating that no animal had contracted the disease from all the three exposure groups for the entire experimental period.

4.3.2 Isolation of *Rhabditis bovis* and *Rhabditis blumi* from the dipwash, soil and manure

To determine the presence of nematodes from dip wash, soil and manure, samples were collected from dip tanks and spray races. Samples were collected from Mkata NARCO ranch and examined for the presence of nematodes on the same day of dipping or spraying of the animals. An average of five dead nematodes per litre were recovered from the diptank at Mkata NARCO ranch while at Taji Mohamed diptank, an average of ten dead and six live nematodes were isolated per litre of dip wash. Three species of

Rhabditis namely *Rh. bovis*, *Rh. blumi* and an unidentified *Rhabditis spp.* were isolated from manure and soil from Taji Mohamed and Mianji farms. The various species of *Rhabditis* isolated are shown in Fig. 37, 38, 39, 40, 41, 42, and 43.

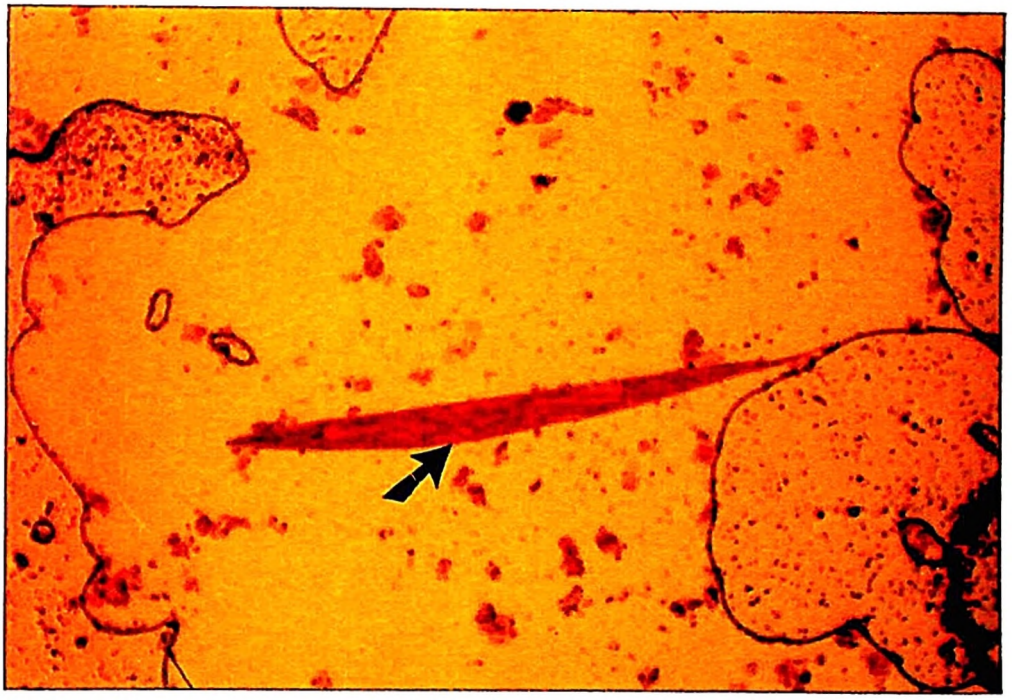

Figure 37: A wet preparation Lugol's iodine showing a female *Rh. bovis* (arrow) with the characteristic morphology of tapering at the anterior and posterior ends and widening at midbody. x100.

Figure 38: A wet normal saline preparation showing a male *Rh. blumi* (x100)(arrow). (At this magnification the morphology is similar to male of *Rh. bovis*).

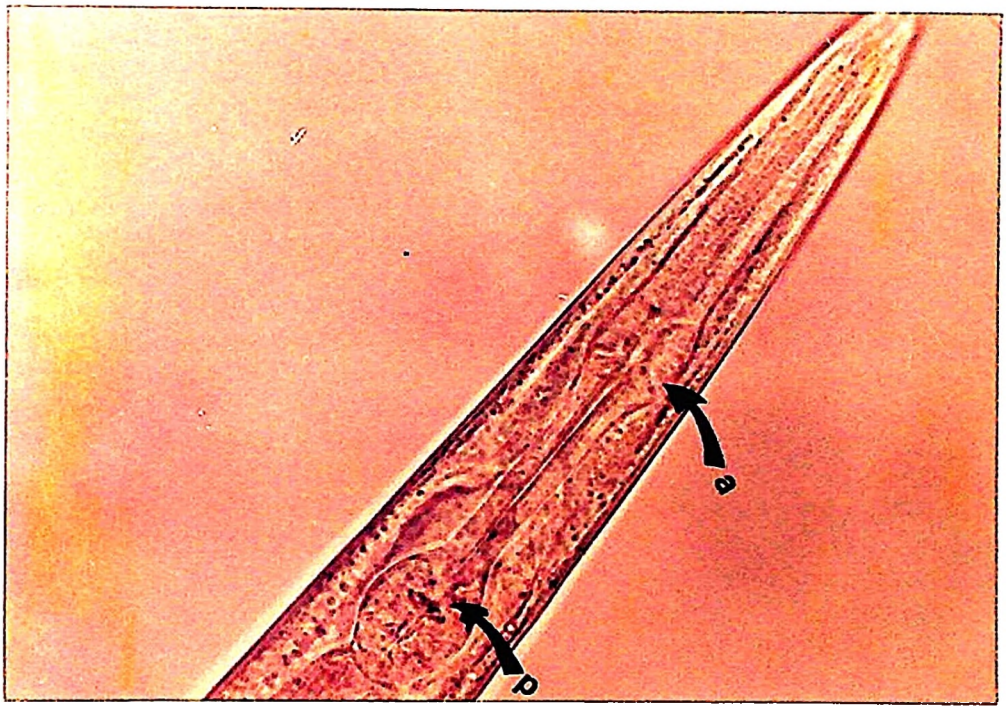

Figure 39: Anterior end of *Rh. bovis* showing the posterior (p) and the more developed anterior bulb (a), this feature is used to distinguish it from *Rh. blumi*. Isolated from Taji Mohamed farm.

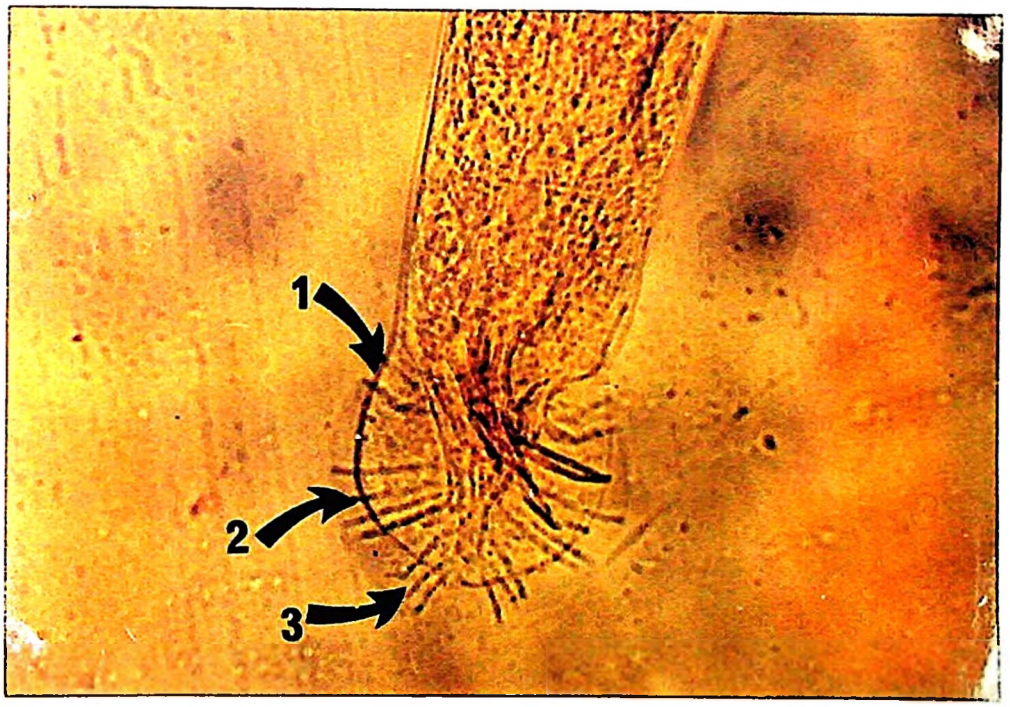

Figure 40: Posterior end of *Rh. bovis* male showing the bursal papillae arrangment in a bursal formula 2-()4-3 (arrows) 1,2 and 3 respectively. The paranthesis indicate the position of the cloacal opening. The nematode was isolated from Taji Mohamed farm.

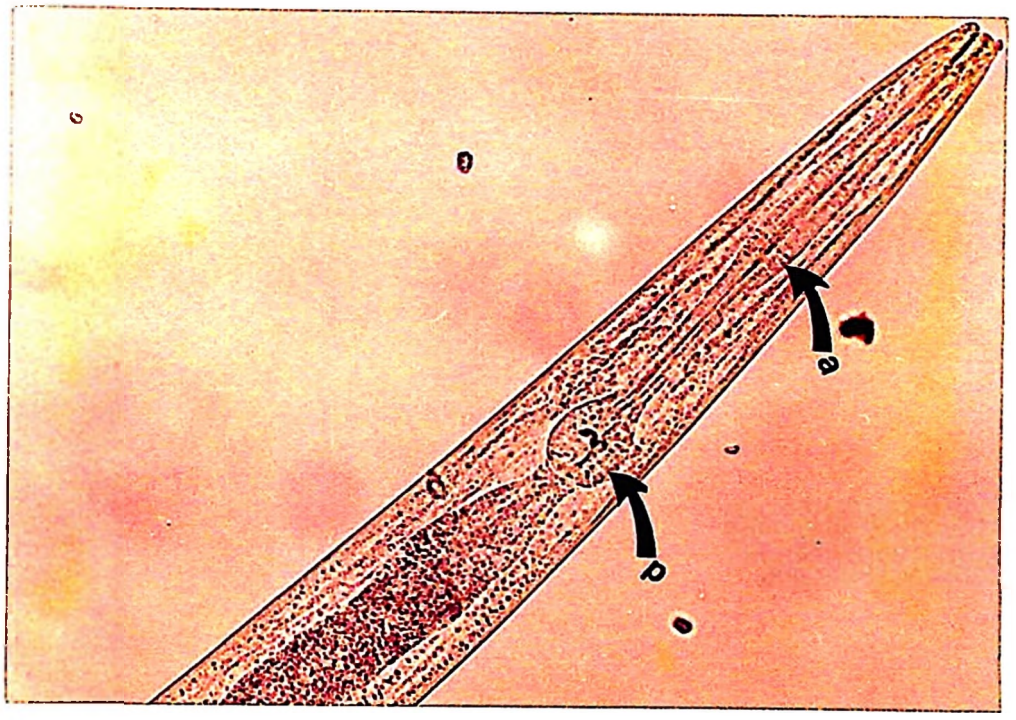

Figure 41: Anterior end of *Rh. blumi*, note the non-existance of the anterior bulb (a) and the presence of the posterior bulb (p). The nematode was isolated from Taji Mohamed farm.

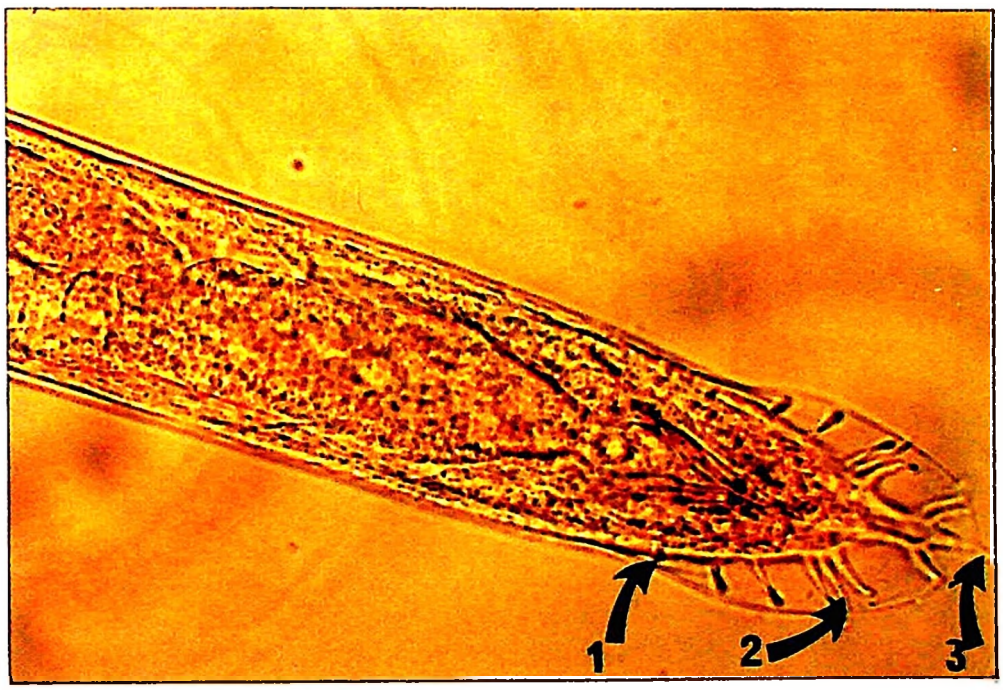

Figure 42: Posterior end of the male *Rh. blumi* showing the arrangement of bursal papillae in the bursal formula 1-2-()3-2. The papillae and the tail are pointed with arrows 1,2 and 3 respectively. This nematode was isolated from Taji Mohamed Farm.

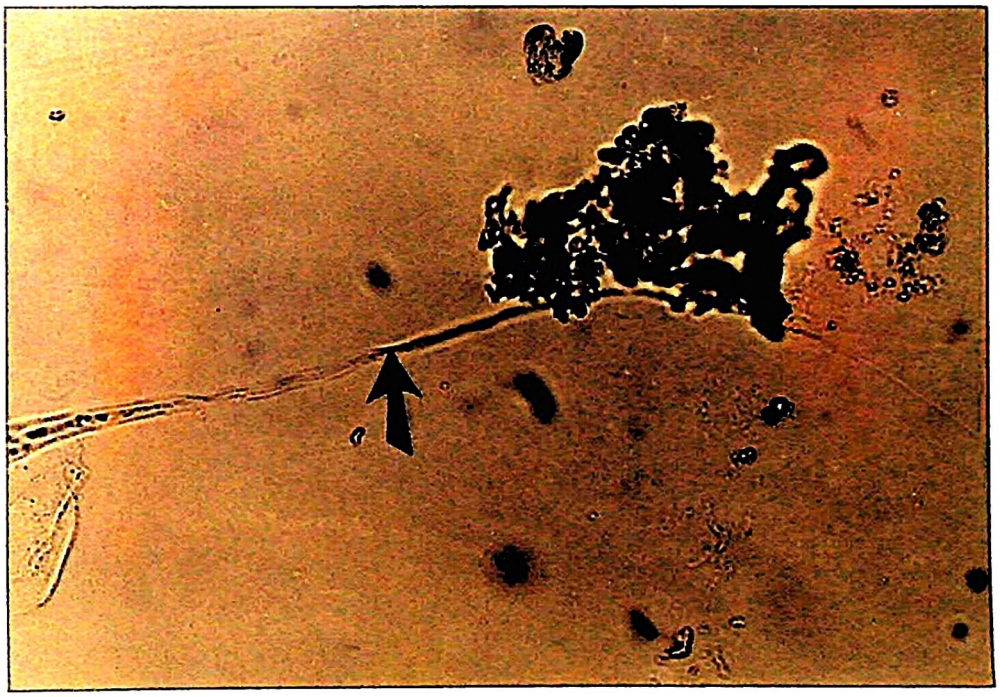

Figure 43: The posterior end of the female of an unidentified *Rhabditis spp.* isolated along with the other two species.

4.3.3 The viability of the nematode *Rhabditis bovis* in two fold serial dilution of Steladone®

Nematodes obtained from ears of naturally infected cattle, the majority being *Rhabditis bovis* were subjected to two fold dilution of Steladone®. Steladone was used because it is among the organophosphorus acaricides being used in the country for dipping and spraying cattle against tick borne diseases used as 0.17% emulsion.

A two fold serial dilution of Steladone® starting with a 0.17%, dilution for dipping cattle was made (Table 11). This strength of 0.17% was obtained by mixing one millilitre of the concentrate of Steladone® to six hundred millilitres of tap water. Viability of the nematodes in different dilutions of the acaricide was determined by exposing the nematodes in the acaricide and time taken for the nematodes to die was the basis for determining their viability in the acaricide. The time taken for the nematodes to die at each concentration is shown in Table 12. The total time taken for the all nematodes to die, ranged from 58 minutes in the highest concentration i.e 1:600 (the concentration used for dipping) and 31 hours in the lowest concentration i.e. 1:037 200. After the addition of the acaricides, the death of the gravid females was characterized by bursting thus releasing the larvae while other females, males and larvae died *in situ*. The females which did not release their mature larvae, the larvae remained alive inside the females uteri and could be seen wriggling while the mature female nematodes were dead. This implied that the larvae were protected from the exposure of the acaricide by the mature female nematodes. Most of these larvae were

released later at different intervals, and therefore different acaricide exposure time, consequently different survival times.

Although it is mentioned above that the nematodes died after 58 minutes, there were a few nematodes that died almost immediately after the addition of the acaricide. Those persisting for longer periods than 18 hours were sluggish and fewer. The larvae survived up to 48 hours in the lowest concentration of the acaricide i.e 1:307 200 (Table 11).

Table 12 shows the time taken for the nematodes to die in various concentrations at two fold serial dilutions.

Table 11: Two fold serial dilutions of a 0.17% solution of Steladone®

Serial number	Concentration
1.	1:600
2.	1:1 200
3.	1:2 400
4.	1:4 800
5.	1:9 600
6.	1:19 200
7.	1:38 400
8.	1:76 800
9.	1:153 600
10.	1:307 200

Table 12: Survival of *Rhabditis bovis* in two fold serial dilutions of Steladone®

Dilutions	Time exposed	Date	Time all adult died	Date	Total time	Date	Time all larvae died	Date	Total time
1:600	10.17	20/3/96	11.15	20/3/96	58min	20/3/96	12.30	20/3/96	2h 03min
1:1,200	10.18	"	12.30	"	2h 12min	"	06.50	21/3/96	20h 32min
1:2,400	10.19	"	14.30	"	4h 11min	"	06.55	"	20h 38min
1:4,800	10.20	"	18.32	"	8h 12min	"	07.00	"	21h 40min
1:9,600	10.21	"	08.30	21/3/96	21h 55min	21/3/96	08.15	22/3/96	46h 00min
1:19,200	10.22	"	10.30	"	24h 03min	"	08.20	"	46h 03min
1:38,400	10.23	"	12.20	"	25h 57min	"	08.28	"	46h 06min
1:76,800	10.24	"	13.15	"	26h 51min	"	10.25	23/3/96	72h 06min
1:153,600	10.25	"	15.10	"	28h 45min	"	12.30	"	74h 05min
1:307,200	10.27	"	17.30	"	31h 03min	"	18.00	"	79h 43min
tape water	10.29	"	07.12	"	20h 55min	"	08.25	22/3/96	45h 56min
distilled water	10.30	"	18. 30	20/3/96	8h 00min	20/3/96	10.35	21/3/96	23h 55min

CHAPTER FIVE

5.0 DISCUSSION

Pseudomonas aeruginosa and *Actinomyces pyogenes* are found as normal bacterial flora on the skin and mucous membranes of domestic animals (Merchant and Packer, 1967; Carter, 1973; Woolcock, 1991). These bacteria are also common inhabitants of water and soil and thus are in constant contact with animals in their environment and do contaminate water used by the animals for various purposes (McDonald and Pinheiro, 1967; Curtis, 1969). They thus cause disease when the body defenses are breached (Doxey, 1983; Carter and Chengappa, 1991). Among the numerous disease conditions caused by *P. aeruginosa* (Appendix 1), the organism has been reported to be a common cause of otitis in small animal species primarily the dog, cat and rabbit (Farrag and Mahmoud, 1953; Fraser, 1965, Bush, 1984; Bedford, 1989; Rai *et al.*, 1986). However, otitis due to *P. aeruginosa* in large animals, has only been reported in cattle (Azizuddin and Chandrasekharan, 1954) and in sheep (MacLeod *et al.*, 1972; Davies and Done, 1993). On the other hand, association of *A. pyogenes* with otitis in any animal species has not been reported.

Epidemiological studies that have been carried out on clinical cases of bovine parasitic otitis have revealed the presence of *P. aeruginosa* and *A. pyogenes* (Lweno *et al.*, 1983a, 1983b; Msolla *et al.*, 1987; 1989). Consequently, one of the objectives of this study, was to determine the prevalence of *P. aeruginosa* and *A. pyogenes* in clinically

normal ears and those affected with parasitic otitis in cattle.that had not been established. The results from the study demonstrated that *P. aeruginosa* occurrence in clinically normal ears was significantly higher ($P<0.05$) than in ears affected with the disease. This finding is in contrast with studies done in small animals and human beings where the organism is frequently associated with otitis (Frost, 1960; Fraser *et al.*, 1961; 1965; Grono, 1969; Shanon *et al.*, 1972; Gedek *et al.*, 1979; Bush, 1984). This could probably be due to species difference in the composition of microbial normal flora of different parts of the body as observed by Hoadley and McCoy, (1967) as well as the dynamic composition of normal aural bacteria flora as occurs in nature (Woolcock, 1991).

On the contrary *A. pyogenes* was isolated at a significantly higher rate ($P<0.05$) in the diseased ears than in non-diseased ears, this indicates probably it is rarely a normal flora in cattle as compared to *P. aeruginosa*. This finding also shows its high affinity to suppurative processes (Prescott and AnneMuckle, 1986). Although *A. pyogenes* has not been reported in otitis in any animal species, this study has shown that there is an association between the prevalence of *A. pyogenes* and bovine parasitic otitis ($P<0.05$). The role played by this bacteria was demonstrated later by the experimental infection of calves in which the combinations of *Rh. bovis*, *A. pyogenes*, and *P. aeruginosa*, displayed severe clinical manifestations followed by the group inoculated with *Rh. bovis* and *A. pyogenes*. In the group inoculated with *Rh. bovis* and *P aeruginosa* most of the calves developed only mild clinical manifestations.

The isolation of these bacteria from the ears of cattle in this field study is in agreement with the previous findings by Lweno *et al.*, (1983b); Msolla *et al.*, (1987; 1989). However, these workers did not indicate the prevalence isolation rates of these bacteria while this study was able to come up with some figures.

Apart from the selected bacteria of the study, a lot more bacteria were isolated from both diseased and non-diseased ears of cattle. The commonly isolated bacteria were *Corynebacterium spp.*, (41.9%) *Staphylococci spp* (22.9%) and *Proteus spp.* (13.9%). *Corynebacterium spp* were more prevalent in diseased ears than in non-diseased ears, while *Staphylococci spp* and *Proteus spp* were more prevalent in non-diseased ears than in diseased ones. This observation is in agreement with that of small animals(Griffin, 1990). The *Corynebacterium spp.* identified were *C. hofmanii*, *C. renale*, and *C. kutscheri*. The study has shown that *Corynebacterium spp.*, particularly *C. renale* was the most common bacterium of the genera. Nevertheless, its role in otitis has not been elucidated. *Proteus spp.* and *Staphylococci spp.* have been reported in otitis in other animal species especially the dog (Bedford, 1989; Griffin, 1990). However since most of the bacterial isolates are related to pyogenic lesions, they possibly exacerbate the clinical picture of bovine parasitic otitis.

To accomplish the second objective on the role of the nematode *Rh. bovis* and the bacteria, *P. aeruginosa* and *A. pyogenes*, the aural bacterial flora of experimental calves was determined before they were inoculated with various combinations of the nematode and the bacteria. The common bacterial flora isolated from experimental

calves was in agreement with the bacteria isolated from the both diseased and non-diseased cattle in the prevalence study. The ears of experimental calves could not be sterile, due to the fear that the nematodes which feed on bacteria could fail to establish.

The results from this experiment also indicated that *Corynebacterium spp.*, Staphylococci spp, and Proteus spp. dominated the bacterial flora before and after the experiment. This finding was similar to that of the prevalence study. The persistence of isolation of these bacteria indicated that they are probably the resident aural bacterial flora of cattle as in other animals. For example, *Staphylococcus aureus* is a normal inhabitant in the ears of dogs (Griffin, 1990), while *Corynebacterium spp.* are the normal inhabitant in the urogenital tracts of cattle, sheep and goats (Woolcock, 1991). The other bacteria not isolated before and after the experiment could be transient bacteria that changed with the environment that included *Bacilli spp., Actinomycetes* and *Escherichia coli.*

There was inconsistency of isolation of aural bacteria at the end of the experiment, both of the test organisms and other bacteria which could be due to the aural microbiology which keep on changing according to the environment (Carter, 1973, Woolcock 1991). Most of the test bacteria were not recovered at the end of the experiment. The failure to recover the test organisms from the experimental calves at the end of the experiment is an experience which has also been documented by other workers on experimental reproduction of otitis using *P. aeruginosa* in other animals species (Salvin and Lewis, 1946; Grono, 1969). These workers thought that this was due to lack of suitable

chronic inflammatory changes required for *P. aeruginosa* to establish. This is true as a defect in the integument of the skin is the most important predisposing factor in establishing an infection (Gyles, 1986).

The failure of isolation of bacteria could also be caused by the fact that there exists an association between bacteria and the nematodes of the genus *Rhabditis* (Nicholas, 1959; Thorne, 1961). This association is on the feeding habits of *Rhabditis spp.* that feed on live bacteria and thus could influence the type of aural flora. There are some preferences to this feeding so that the nematodes grow better in the presence of certain bacteria than others do (Nicholas, 1959). It is possible that some bacteria were preferred as source of food for the nematodes than others, hence failure to establish and subsequently failure to be recovered at the end of the experiment.

Actinomyces pyogenes is the major opportunistic pathogen of cattle but many aspects of how it expresses its virulence has not been well elucidated as compared to *P. aeruginosa* (Lovell, 1937; 1941). The bacterium produces a haemolytic exotoxin which causes necrosis in laboratory animals when given intravenously (Lovell, 1944). The conditions required for the production of the toxic products remains to be investigated. However, *C. pyogenes* has been frequently isolated in mixed bacterial infections particularly with *Fusobacterium necrophorus* and *Peptostreptococcus indolicus* because of the production of stimulatory growth factors for these species (Prescott and AnneMuckle, 1986).

The other commonly isolated bacteria in the experimental calves were *Corynebacterium spp.*, *Staphylococci spp.* and *Proteus spp.*. The *Corynebacterium spp* identified were *C. hofmanii*, *C. kurtscheri (murium)* and *C. renale*. *Corynebacterium kurtscheri* is pathogenic to mice and rats and its pathogenicity to other animals is low (Morrison *et al.*, 1982) whereas *C. hofmanii (pseudodiphtheriticum)* is reported non-pathogenic (Morrison *et al.* , 1982; Carter and Chengappa, 1991). However, *C. renale* is pathogenic although it has been isolated from healthy animals and exists in three immunological types, I, II and III which stand for *C. renale, C. pilosum* and *C. cystitidis,* respectively. The bacterium causes pyelonephritis, ureteritis, and cystitis in cattle and kidney abscesses in pigs (Carter and Chengappa, 1991). The bacterium causes disease as a result of inflammatory reaction caused by the production of urease (Carter and Chengappa, 1991). However, *Corynebacterium spp.* have also been isolated from the respiratory tract, skin of clinically healthy cattle, sheep, goats and in the urogenital tract of chickens (Woolcock, 1991). In view of this ubiquity, their involvement in bovine parasitic otitis can not be over-ruled. This is especially so when the urine from infected cattle is voided in the premises where animals spend most of their time. The contaminated manure could be a source of infection due to the habit of cattle scratching their ears with hooves carrying the contaminated manure. However, no attempts were done to isolate this bacterium from manure.

Other ways bacteria do express their virulence is the presence of certain predisposing factors such as osmolality, iron concentration of the microclimate, genetic and phenotypic expressions such as presence of flagella and pili (Totten *et al.*, 1990). For

example, with *P. aeruginosa*, it has been shown that flagella and pili are important factors for attachment on mucosal and other epithelial surfaces (Drake and Montie, 1988; Mekalanos, 1992). There are also some strains of bacteria that do not possess these structures and thus are non-pathogenic (Totten *et al.*, 1990). In this study, the pathogenicity of the strain of *P. aeruginosa* used was not determined experimentally. However, the use of a pigment producing strain in this experiment was enough indication of the pathogenicity of the strain.

The regime of inoculation of calves adopted in this study was based on the frequency of dipping recommended in the country, which is once or twice per week. This was also used as the sole criterion of infecting because dipping has been observed to be the main method of transmission (Jibbo, 1966; Msolla *et al.*, 1986b). Other source of infection were completely absent as the calves were placed in completely disease free pens. Thus the calves were inoculated once per week using nematodes obtained from naturally infected calves. This interval was convenient as it provided enough time for the nematodes to multiply inside the ears from one swabbing to the next.

A high dose of nematodes was required for infection because some nematodes were lost when the animals shook their heads during inoculation procedure. Other nematodes too died in an effort of acclimatizing to the new environment in the ear canal. The nematodes that died were confirmed when ear swabs were taken during the experiment for confirming the establishment. Even with the relatively high dose for each calf, the incubation period varied over a wide range of three to 53 days. In the

field the incubation period could be even longer because the dips which are thought to be the main source of infection are usually not that heavily infested.

The results from the experimental infection of calves demonstrated the real field situation, where the disease is manifested as mild, moderate and severe. The group mean responses showed significant differences ($P<0.05$) between all groups but the differences differed in magnitude as the difference was more marked between group. Taking into account that group one was mildly affected, it leaves group four as the most affected. Calves in this group were inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*. From the results, it is apparent that *Rh. bovis* was shown to be the primary causative agent of the disease and bacteria played a secondary role. The study has therefore been successful in experimentally reproducing the disease with the nematode and the bacteria *P. aeruginosa* and *A. pyogenes* in contrast to a similar study using *Staphylococci spp.* and *Bacillus cereus* which produced only mild response (Lweno *et al.*, 1983b). This difference indicates that the bacteria used in this study are more important role in the pathogenesis of bovine parasitic otitis. The clinical signs in all experimental calves were characterized by an initial scanty aural discharge that contained the nematodes. The discharge was confined in the ear canal thus could only be detected by use of the otoscope, an observation similar to the field cases whereby early cases pass unnoticed unless a physical examination are carried out (Jibbo, 1966). With time however, the discharge increased in amount and became thick, yellowish brown and the smell of the discharge became apparent while the underlying tissues became hyperemic. Later, the discharge became thicker as evidenced in-groups one

and two or more fluid as seen in-groups three and four. The consistency of the discharge probably depended on the bacteria involved, as observed in this study. For example, the groups inoculated with *Rh. bovis* and *A. pyogenes* had more fluid aural discharge compared to those inoculated with *Rh. bovis* alone or with *Rh. bovis* and *P. aeruginosa*. It was also observed that there was a relationship of the nature of the lesions and the type of inoculum. Mild lesions were mainly observed in calves of group one that were inoculated with *Rh. bovis* alone. Moderate lesions were seen in calves of group two that were inoculated with *Rh. bovis* and *P. aeruginosa*. Severe lesions were found in calves of group three which were inoculated with *Rh. bovis* and *A. pyogenes* and group four which was inoculated with *Rh. bovis*, *P. aeruginosa* and *A. pyogenes*. The groups inoculated with bacteria alone did not develop any clinical manifestations. These results show that *Rh. bovis* does play a primary role in producing aural lesions. However, it would seem that the presence of *A. pyogenes* exacerbated the clinical disease more so than the presence of *P. aeruginosa* as the later only caused most mild lesions. However the combination of the two bacteria demonstrated the most severe lesions indicating the presence of a synergestic effect.

The mild and moderate lesions were characterized by the presence of scanty or thick discharge and a few nematodes usually remained so throughout the observation period. These two types of clinical manifestations were manifested by the majority of experimental calves and equally represent the chronic form of the disease that affects the majority of the animals in the field(Msolla *et al.* 1985).

Tilting of the head towards the affected ear as observed in few of the experimental calves was in agreement with cases observed in the field. In the field it was assumed the tilting was due to the affection of the CNS(Jibbo, 1966; Msolla *et al.* 1993). In this study post-mortem findings of one of the most affected calf showed no lesions in the brain. This indicated that tilting of the head to the affected ear was not necessarily caused by central nervous aberrations but could have been caused by other factors such as pain, irritation and affection of the organs of balance. Further histopathological studies are required on long standing field cases.

Swelling of the lymph nodes was only present in severe cases and such calves had a pyrexia of up to 40.9°C. Affected calves were dull and resisted being handled as a sign of pain. Temperature reaction was higher compared to the rest of the groups which probably was caused by the severe inflammatory reaction. The swelling of lymph nodes and the pyrexia however subsided as the lesions got less severe. The above findings are in keeping with the field observation where a severe disease is seen in immatures (Msolla *et al.*, (1993), but the presence of pyrexia or its absence has not been reported as a feature of such cases.

As observed in this study, there was head shaking and scratching of the ears with the hooves probably due to irritation caused by the nematodes and the pain due to inflammatory reaction. This observation in experimental animals is in keeping with what is observed in the field (Jibbo, 1966). However, an interesting observation was that head shaking and scratching of ears with hooves were more marked in boran

crosses than exotic breeds (Friesian and Aryshire crosses). The boran calves were also noted to be more nervous especially during handling and thus could easily traumatize the delicate and hyperemic lesions. The frequent scratching probably enhances lesions becoming repeatedly traumatized leading to long standing and complicated aural lesions as seen in the field cases. It was of interest also to note that although boran crosses were nervous the majority developed only mild to moderate clinical manifestations while the majority of the exotic breeds developed the severe clinical manifestations. This finding demonstrates that behaviour/breed is an important predisposing factor in bovine parasitic otitis. Jibbo, 1966 was of the opinion that it was the aural anatomical difference between zebu and exotic cattle that was the main contributing factor. This factor has not substantiated by experiments but in small animals especially the dog, anatomy of the aural canal has been documented as an important factor in otitis(Bedford, 1989). The observation that the disease affects zebu cattle than exotic was most probably due to the fact that the studies carried out were on herds of boran cattle than exotic cattle (Round, 1962; Jibbo, 1966; Lweno *et al.*, 1983a). Round in 1962 when he first observed the disease, it was in a boran herd where the morbidity rate was a 100%. Lweno *et al.* (1983b) working with mainly zebu cattle observed the same. In herds of mixed breeds of cattle, the breed prevalence of the disease has not been established.

Although the experimental calves demonstrated most of the features characteristic of BPO, there were some clinical manifestations that were not apparent. Manifestations such as central nervous system aberrations, gradual loss of weight, obliteration of ear

canals, drooping of ears and myiasis as observed in the field were not detected in the experimental calves. These symptoms were not observed because they are characteristic of long standing infections which was not the case in this study. The observation period for the experiment was only eleven weeks and therefore not sufficient to elicit such changes. In the field, clinical cases may last for long periods of up to several years (Msolla *et al.*, 1993).

There are some factors that possibly do enhance the severity of the aural lesions in the field to an extent of affected animals loosing ears and horns as well as invasion by maggots. Moisture does facilitate the development of the lesions by favouring the survival of the nematodes since the nematodes are very susceptible to drying (Nicholas, 1959; Thorne, 1961). In this study in the absence of additional moisture as occurs in dipping, resulted in the discharge decreasing in amount and the aural lesions dried up and the eroded areas healing spontaneously. Other cases maintained a mild or moderate form throughout the experiment. In this study, the drying up of the aural discharges and lesions, which led to recovery, was in agreement with the field cases which also do recover spontaneously (Msolla *et al.*, 1989). The possible explanation to this spontaneous recovery is a point of interest for further studies.

The nematodes obtained for inoculating the experimental calves were from naturally infected calves and a mixture of *Rh. bovis* and *Rh. blumi*. However, *Rh. blumi* occurs in very small numbers in the ears of infected animals as opposed to *Rh. bovis* (Msolla *et al.*, 1987; 1989). This trend is supported by the present findings whereby

suspensions of aural swabs when examined under the stereo microscope showed a ratio for *Rh. blumi* and *Rh. bovis* of approximately 1:10.

The nematodes used to infect calves were washed twice in sterile water by sedimentation to reduce the bacterial contamination. This was carried out because axenic cultures (without bacteria) of *Rh. bovis* have not successfully been established as was the case in this study. Axenic cultures have been difficult to establish even with other species of *Rhabditis* (Glaser, 1940; Doughtery, 1953a; 1953b). The fact that mixed cultures of *Rh. bovis* and *Rh. blumi* end up by being dominated by *Rh. blumi* as demonstrated by Msolla *et al.*, (1986b), necessitated the use of freshly obtained nematodes. The use of antibiotics to disinfect the nematodes as suggested by Nicholas (1959), was dismissed because in the preliminary study it was demonstrated that antibiotics did interfere with reproduction of the nematodes by killing the bacteria which are a source of food to the nematodes (Nicholas, 1959; Thorne, 1961).

On the other hand, the histopathological studies of the ears of calves that developed aural lesions revealed that there was no direct involvement of the nematode in the initiation of the aural lesions. Immune response due to parasitic infestation is demonstrated by the presence of eosinophils that were not demonstrated in this study. The explanation to these observations could be that the damage to aural epithelium was probably due to some excretory products by the nematodes. Damage to the aural canal epithelium could also be due to soaking of tissues by the discharge formed in the process of the inflammatory reaction and that of dipping animals. Soaking or

maceration of the aural epithelium has been shown to be among the predisposing factors in the pathogenesis of otitis in most animal species (Grono, 1969; Siegmund and Fraser, 1975). The soaked epithelium that is soaked is probably easily removed by the nematodes in the process of feeding.

Another factor that was noted in the experimental calves, which possibly exacerbated the severity of the lesions, was the self-mutilation caused by frequent scratching of irritated ears by hooves. As mentioned earlier, scratching resulted the top layer of the aural epithelium being easily removed leaving raw and even bleeding areas. Besides using their hooves for scratching their ears, affected cattle in the field were observed to scratch/rub their ears on hard objects such as tree stumps and walls (personal observation). In this way they erode the tender tissues of the affected ears, which bleed and make good environment for the multiplication of bacteria and attract flies.

A further observation from group four of the experimental calves was the disappearance of nematodes from two ears of the severely affected calves in the group. This was noted during the last three weeks of the experiment or eighth week post inoculation of the nematodes. The disappearance of the nematodes could be explained by the fact that these lesions were drying up and healing spontaneously thus denied the nematodes of a suitable medium.

The results from this study indicate that the inflammatory processes were characterized by infiltration of inflammatory cells predominantly neutrophils and lymphocytes

mainly in the subepithelium. The extent of the infiltration varied from one area to another in the same ear canal. There were apparently no eosinophils in the subepithelium which are normally characteristic of parasitic infections. This could have resulted from the fact that the nematodes did not penetrate the epithelium into the subepithelial mucosa where most of the infiltrated cells were found. It would seem that the nematodes occupy only the lumen of the canal and cause superficial erosion of the epithelium by possibly excreting substances that could be responsible for the observed changes. The fact that some of the ear canals of recovered animals in the field become occluded, this was not experienced in this study. The occlusion probably occurs in long standing cases of the disease.

An interesting observation was the presence of nematodes in ears with no inflammatory response and therefore could not have been visualized macroscopically without the assistance of an otoscope. This was a feature in calves in group one and two. This implies that there are many subclinical cases in the field which are missed. This finding is also in keeping with the absence of eosinophils indicating that there was no severe inflammatory response either. Bovine parasitic otitis is a chronic disease condition whose duration in the field can go up to several years being on and off and thereby allowing such changes to occur. Apparently this was the information collected from many places where sampling was carried out. The chronic form of the disease was observed in older animals and owners would confirm that such animals were infected when they were calves.

Dipping has been incriminated as the main method of transmission. However, there are cases where the disease has been maintained in undipped cattle for a period of several years (Semuguruka personal communication, 1996). This is also in agreement with the information obtained from Lugoba pastoralists in the coastal humid areas where, despite the fact that dipping had stopped since the early nineties, to date there are still cases of otitis especially in older animals which had the disease at that time.

The role of dips, spray races and soil or manure in the transmission of *Rh. bovis* and *Rh. blumi* have previously been reported (Msolla *et al.* 1987 and 1989). However, further work was deemed necessary in view of the ever-changing use of acaricides in the field. When previous studies on the role of dips were being carried out (Msolla *et al.*, 1986a), the acaricide in use in the country was Toxaphene®, an orgonochlorine. However, during the present study, Steladone® an organophosphorus acaricide was in use in most parts of Tanzania. In the previous study, it was shown that up to twenty live nematodes per litre of dip wash were isolated. The studies went on to show that the nematodes could survive in used acaricide of toxaphene for up to 28 days and up to 15 days in freshly prepared 0.25% toxaphene (Msolla *et al.*, 1986a).

The results from this study have demonstrated that fewer (up to ten) live nematodes of *Rh. bovis*, were isolated from the dip wash and soil but none from the spray races. These results were in agreement with the previous findings in nematode isolation although in this study, the nematodes isolated were fewer and some were dead possibly due to the use of a different acaricide. Although both nematodes were isolated from

manure mixed with soil in sleeping pens; *Rh. blumi* was isolated more abundantly than *Rh. bovis*, a feature which was in agreement with the finding by Msolla *et al.*, (1989).

In this study no nematodes were isolated from the spray race possibly be due to several factors. The small tank used to mix the acaricide of the spray race, was cleaned after spraying thus even if the nematodes were present they would easily be washed away. The other factor would be the way by which the animals go through the spray race. During spraying, the animals pass through the spray race holding their heads up with ears directed posteriorly and thus very little wash gets into the ears. Where as during dipping, animals plunge into the dip wash with such force and the nematodes present in the ears get washed into the dip tank. Another factor could be the force by which the nozzles of the spray race spray the animals. The jet effect of the spray nozzles may have a mechanical effect on the nematodes if present. It is also possible that the freshly prepared acaricide for the spray races allowed no accumulation of nematodes. When all these factors work together, very few, if any nematodes could survive in the spray race wash.

The fact that fewer nematodes were isolated from the dip wash in contrast to the previous studies was possibly due to the acaricidal effect of (Steladone®) which in the *in-vitro* experiment demonstrated that the nematodes survived for about 24 hours at most, compared to 15 days in Toxaphene®.(Msolla *et al*; 1985) Thus, with Steladone® in use very few nematodes could possibly survive from one dipping to another. The other supporting evidence was the reduced prevalence of the disease in these farms

with the new acaricide (Steladone®) had been introduced has declined from 70% to around 20% (Mtae personal communication, 1995).

Rhabditis blumi was also isolated from farms that had no history of having otitis indicating that the nematode is a saprophyte and is found in the ears of cattle as an accidental parasite. Another *Rhabditis* species, which was not identified, was isolated from the same locations where *Rh. blumi* was isolated. This species was similar to *Rh. bovis* and *Rh. blumi* morphologically under low magnification (x2) of the stereo microscope, and if the nematodes were not examined at an appropriate magnification (x40), they could easily be mistaken for *Rh. bovis*. The lack of clinical manifestations of BPO after the exposure of the animals to the three types of acaricides application could have been due to the following factors.

The small number of live nematodes isolated from the dip wash, the time required for an animal to acquire the disease, and the critical number of nematodes required to initiate a clinical disease. It could possibly be due to the acaricidal effect to the nematodes rendering them less pathogenic.

CHAPTER SIX

6.0. CONCLUSION AND RECOMMENDATIONS

These studies have been able to demonstrate the roles played by the nematode *Rhabditis bovis,* the bacteria *Pseudomonas aeruginosa* and *Actinomyces pyogenes* in the development of BPO. The studies have demonstrated that *Rh. bovis* is the primary agent in bovine parasitic otitis whereas the bacteria are secondary. Thus, severe clinical manifestations of BPO do occur when primary *Rh. bovis* infestations are superimposed with *A. pyogenes*, *P. aeruginosa* or a combination of *Rh. bovis* and the two bacteria. However, the mechanisms by which both the nematodes and bacteria cause the damages to the auricular canal as observed in this study is of interest and warrants further study.

Of major interest would be the determination of the nature of excretory products produced possibly by *Rh. bovis* that probably are responsible for the primary lesions. Further work on the role of other bacteria isolated from the ears of cattle in the pathogenesis of the disease would be of interest if effective treatment and control measures are to be put in place. These studies have also confirmed the spontaneous recovery that has been reported in field cases. This aspect if researched extensively, more so on immunological aspects, could give way to developing a vaccine or reliable treatment against BPO.

On the prevalence study, it has been demonstrated that the bacteria *P. aeruginosa* and *A. pyogenes* occur in both clinically normal ears of cattle and those affected with BPO. Their involvement in the disease is due to their opportunistic presence in the aural canals as normal flora and when the aural epithelium has been damaged they exacerbate a clinical disease of BPO.

Recommendations.

From the present study it is recommended that:

(i). The mechanism by which the nematodes cause the primary aural lesions need further studies.

(ii). The role of flies in the transmission of the disease also need further study.

(iii). The role of the *Corynebacterium spp*, especially *C. renale* the most commonly isolated bacteria from the infected animals warrants further detailed research.

(iv). Methods for establishing axenic nematode cultures and of preserving the nematode for longer periods should be researched for experimental purposes.

(v). Field experiments on the role of dips, spray races, soil and manure in the transmission of the nematodes should be carried as a long term research project.

(vi). The use of calves (cattle) in such experiments is very expensive. Model experimental animals should be investigated which will be cheaper to use.

(vii). The *in vitro* experiment on the survival of the nematodes in various acaricides in use could be of further interest. This could be done in conjunction with the acaricide manufacturers with a view to coming up with an acaricide that could be more effective in both treatment and control of the disease.

(viii). Studies on the incorporation of nicotine at 2.08 ppm in commercially prepared acaricides for the treatment and control of bovine parasitic otitis. This is an important link between research, industry and extension services.

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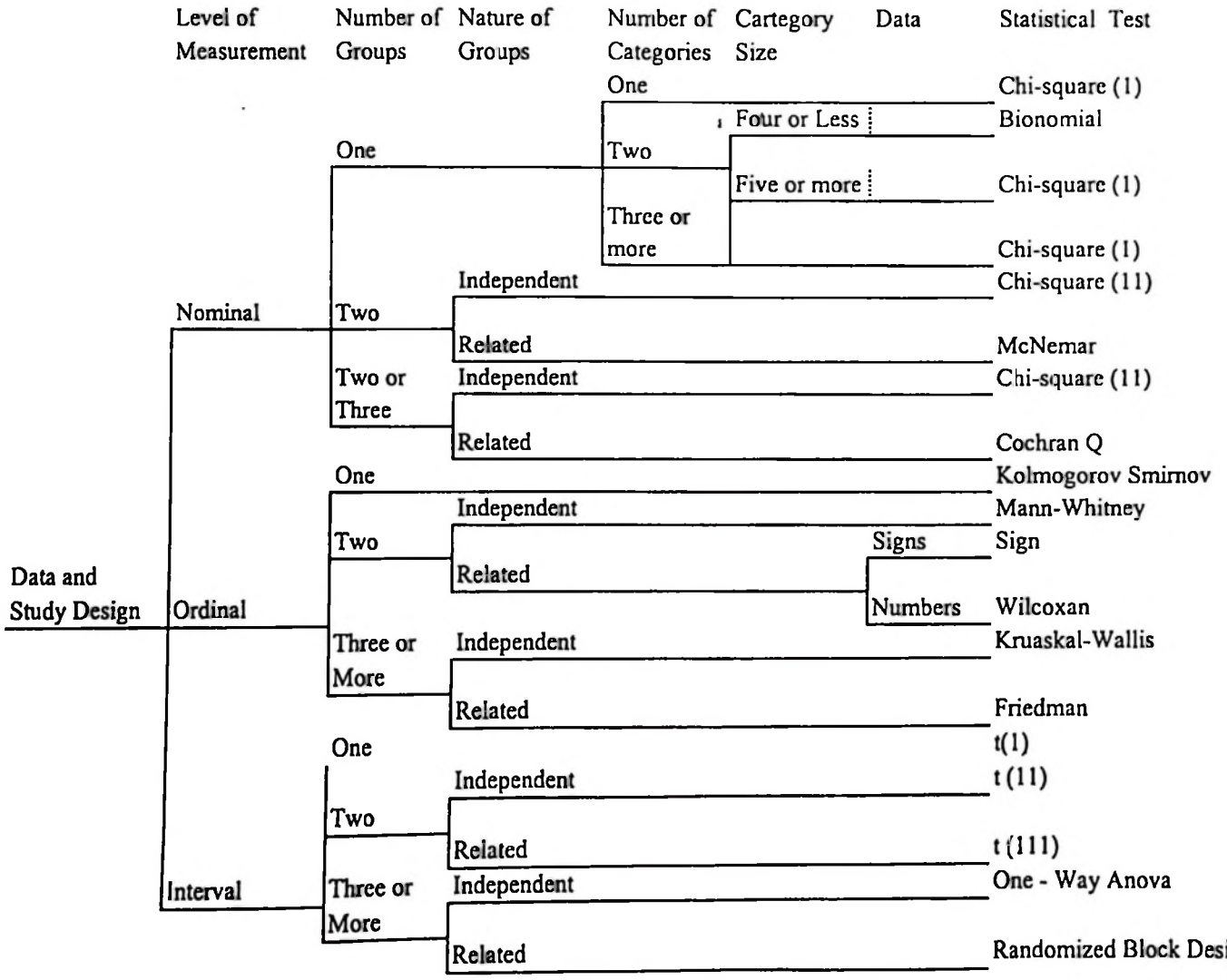
APPENDICES

Appendix 1: Diseases caused by *Pseudomonas aeruginosa* in animals (summarized from Lusis and Soltys (1971) and revised by the author).

Species	Diseases	Selected references
Cattle	Generalized	Gardiner and Craig (1961). Korzh *et al.*(1990)
	Mastitis	Cherrington and Gildow,(1931). Okello and Gibson,(1979) Kirk and Barlett (1984)
	Reproductive tract infections	Getty and Ellis (1967).
	Respiratory tract infections	Prasad *et al.*(1968).
	Enteritis	Matthews and Fitzsimmons (1964).
	Otorrhea,myocarditis, Synovitis,laryngitis.	Azizuddin and Chandrasekharan (1954).
	Arthritis	Buxton and Frazer (1967)
Buffalo	Lung and liver abscesses, otorrhea	Azizuddin and Chandrasekharan (1954)
	Mastitis	Pal and Verma (1989) Kapur *et al.*(1990)
Sheep	Mastitis	Azizuddin and Chandrasekharan (1954) Honhold and Carter (1987)
	Fleece rot	London and Griffith (1984). Chin and Watts 1991
	pneumonia, lung abscess	Azizuddin and Chandrasekharan (1954)
Goats	mastitis	Larrivee and Elvehjem (1954).
Horses	reproductive tract infection	Hughes *et al.* (1966a). Krishnappa *et al.* (1979). Atherton and Pitt (1982)
	lung abscess,enteritis	Hoadley and McCoy (1967)
	mastitis	Roberts (1986)
Pigs	Enteritis	Choudhary *et al.* (1983). Prasad and Chauchan (1984).
	respiratory system	Prasad and Chauchan (1984)
	otitis	Baker (1962)

	pelvic abscesses	Nador (1991)
Dogs	generalized	Bush (1984).
	infertility	Ticer (1965); James (1965).
	respiratory tract	Azizuddin and Chandrasekharan (1954)
	pneumonia	-----„----
	otorrhea	Farrag and Mahmoud (19753) Grono and Frost (1967) Ozaki *et al.* (1990)
	endocarditis	Carter and Chengappa (1991)
Cat	Conjunctivitis	Gerald and Ruby (1979).
	Urethroadenocystitis	Gentilini *et al.* (1990)
Mink	pneumonia and septicaemia	Beckenhauer and Miner, (1960). Farrell *et al.* (1958). Trautwein *et al.* (1962).
Chinchilla	generalized infections, including conjunctivitis,otitis,	Bowden, (1959).
	pneumonia,enteritis and reproductive tract infections	Larrivee and Elvehjem, (1954).
Laboratory animals	generalized infections	McDonald and Pinheiro (1967).
mice	enteritis	
rats	otitis	Rai *et al.*(1986)
rabbits		
guinea	generalized infections	
pigs	pneumonia	Bush, (1984)
Captive wild animals (primates, carnivores, rodents, artiodactyla, marsupials)	septicaemia, pneumonia, enteritis,hepatitis,nephritis	Fiennes (1961). Oettle *et al* (1990)
Fowls	Generalized	Essex *et al.* (1930).
	Enteritis	Chute, (1949)
	Edema	Chute, (1949).
	keratitis and keratoconjunctivitis	Essex *et al.* (1930).
	lowered egg production	Niilo (1959). Sidhu *et al.*(1989). Bapat *et al.* ,(1985)
Fish	generalized	Ahmed *et al.*(1990)
Insects	generalized	Gordon and Stephens.(1957)
Plants	generalized	Elrod and Braun (1946) Kabashnaya *et al.* (1988)

Appendix 2 Tree diagram for selection of an appropriate statistical test depending upon character istic of the the study design and data analyzed. Source Sharp, (1979) cited by Smith, (1995).



t (1) = t test with one group
t (11) =t test with paired sample
t(111)=t test with two related groups

Appendix 3.

Distinguishing features of species of the genus *Corynebacterium*. Source: Morrison *et al.*, (1982).

			Acid production									
Spp	Colonial morphology	Cellular morphology	glucose	lactose	sucrose	maltose	salicin	mannitol	trehalose	indole	urea
C. diphtheriae	varies 1-3mm 24hours show a narrow band of haemolysis	rods, frequently swollen at one or both ends, usually stain unevenly	+	_	_	+	D	_	_	_	_
C. pseudotuberculosis (ovis)	yellow white opaque convex col with matty surface about 1mm at 24hr often with a narrow band of haemolysis around the colony	small irregular rods staining irregularly with club forms & metachromatic granules	+	d	d	+	_	_	_	_	+
C. bovis	white to cream circular colonies	Irregular rods, usually barred and clubbed coccobacillary forms may occur.	+	_	_c	+	_	.	_	.	_c
C. renale	dry, granular, cream to pale yellow colonies not haemolytic	rather large staining bacillus often with pointed ends irregular staining	+	_	_	_	_	_	.	.	+
C. xerosis	small circular col (1mm or less in 24hours) on BA colonies are large may be pale yellow to tan colour non haemolytic rough or smooth	often barred rods occasional granules and club forms	+	_	+	+	_	_	_	_	_
C. kutscheri (murium)	small, thin, yellowish or grayish white serrate colonies	irregular staining slender rods often clubbed, sometimes pointed ends	+	_	+	+	+	_	+	.	d
C. pseudodiptheriticum	white to cream coloured, regular and smooth, butyrous consistancy	short regular rods which stain evenly except for transverse medial unstained septum. club forms pallisade arrangement	no acid from any carbohydrate								
C. paurometabolum	white to gray, entire circular, small	small rods occuring singly, in pairs	no acid from any carbohydrate								

	dry, granular on nutrient agar, slightly μ haemolysis.	and masses, metachromatic granules present.								
C. enzymicum	bacillary form produce very small colourless coccoid forms	pleomophic beaded and club shaped rods, sometimes coccoid forms	+	+	+	+	.	.	.	.	.
C. hoagii	small, pale pink, dull entire colonies on nutrient agar.	small rods occuring singly, short forms show polar staining, larger forms are barred & slightly club shaped.	+	.	+	–	.	.	.	.	.
C. striatum	On blood agar slight haemolysis around deep colonies	pleomophic rods often club shaped coccoid forms and long filaments found in old cultures.	+	+	d	+	.	.	.	.	.
C. phocae	Very small (0.1mm) circular smooth colonies on agar transparent and colourless at 24hr whitish & larger at 48hr on blood agar are slightly larger non haemolytic.	small rods occuring in chains of three to seven or more or in pairs occasinal singles	+	.	+	+	+	.	.	.	–
			no acid from any carbohydrate							

Appendix 4: A 2x2 contingency table showing the strength of association of the *Corynebacterium spp* and bovine parasitic otitis.

Corynebacterium spp	Diseased animals BPO (+)	Non-diseased animals (-)	Total
Positive (+)	335	154	489
Negative (-)	317	256	573
Total	**652**	**410**	**1062**

P =0.00001
Chi-square = 19.33
OR = 1.76
RR = 1.24

Where
P = Probability, OR = Odds Ratio, RR = Relative Risk

Appendix 5: A 2x2 contingency table showing the strength of association of *Pseudomonas aeruginosa* with bovine parasitic otitis

P. aeruginosa	Diseased animals BPO (+)	Non-diseased animals (-)	**Total**
positive (+)	16	35	**51**
negative (-)	636	375	**1011**
Total	**652**	**410**	**1062**

P = 0.00001
OR= 0.27
RR = 0.50
Chi-square = 20.35

Where
P = Probability
RR= Relative Risk
OR= Odds Ratio

Appendix 6: A 2x2 contingency table showing the strength of association of *Actinomyces pyogenes* with bovine parasitic otitis

A. pyogenes	Diseased animals (+)	Non-diseased animals (-)	**Total**
positive (+)	34	7	**41**
negative (-)	618	403	**1021**
Total	**652**	**410**	**1062**

P = 0.004
OR= 3.17
RR = 1.37
Chi-square = 8.34

Where
P=Probability
RR=Relative.Risk
OR=Odds.Ratio

Appendix 7. Nematode isolation in comparison to the severity of the lesions

Calf no	16\11\95		29\11\95		16\12\95		30\12\95		15\1\96	
	s	nem	s	nem	s	nem	s	nem	s	nem
105R	3+	3+	3+	-ve	2+	1+	2+	-ve	1+	-ve
105L	2+	1+	2+	2+	2+	3+	2+	3+	2+	2+
270R	3+	1+	2+	3+	2+	2+	2+	2+	2+	3+
270L	3+	1+	2+	4+	2+	2+	2+	3+	2+	2+
269R	2+	1+	3+	2+	2+	3+	2+	2+	2+	3+
269L	3+	-ve	2+	3+	2+	3+	2+	3+	1+	2+
103R	3+	1+	3+	-ve	3+	-ve	1+	-ve	0	-ve
103L	3+	3+	3+	4+	2+	3+	2+	3+	2+	2+
375R	2+	1+	2+	4+	3+	2+	3+	3+	2+	2+
375L	2+	2+	2+	3+	2+	3+	2+	3+	2+	2+
101R	3+	2+	2+	4+	2+	3+	2+	3+	2+	3+
101L	3+	2+	2+	3+	2+	3+	2+	3+	2+	3+
374R	3+	-ve	3+	-ve	2+	2+	2+	2+	2+	3+
374L	2+	-ve	2+	3+	2+	2+	2+	2+	2+	2+

s	score
nem	nematodes
R	Right
L	Left

For scoring system 3+= severe, 2+= moderate 1+= mild

Appendix 8: Group means scores per group used for computing the statistical differences between groups.

Group	No. of observations	Minimum score	Maximum score	Mean score
1.	520	0.0	2.0	0.588
2.	546	0.0	2.0	0.807
3.	546	0.0	3.0	1.007
4.	546	0.0	3.0	1.865
5.	546	0.0	0.0	0.000
6.	546	0.0	0.0	0.000
7.	546	0.0	0.0	0.000
8.	312	0.0	0.0	0.000

Appendix 9: General Linear Models Procedure for group comparison.

General Linear Models Procedure
Class Level Information

Class Levels Values

GROUP 8 1 2 3 4 5 6 7 8

Number of observations in data set = 4107

NOTE: Due to missing values, only 4054 observations can be used in this analysis.

General Linear Models Procedure

Dependent Variable: RESPONSE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	572.2308513	81.7472645	255.44	0.0
Error	4046	1294.8446297	0.3200308		
Corrected Total	4053	1867.0754810			

R-Square	C.V.	Root MSE	RESPONSE Mean
0.306485	157.5137	0.565713	0.35915146

General Linear Models Procedure

Dependent Variable: RESPONSE

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	7	572.2308513	81.7472645	255.44	0.0

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	7	572.2308513	81.7472645	255.44	0.0

General Linear Models Procedure

T tests (LSD) for variable: RESPONSE

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 Confidence= 0.95 df= 4046 MSE= 0.320031
Critical Value of T= 1.96055

Comparisons significant at the 0.05 level are indicated by '***'.

General Linear Models Procedure

GROUP Comparison	Lower Confidence Limit	Difference Between Means	Upper Confidence Limit	
1 - 3	-0.4730	-0.4045	-0.3361	***
1 - 2	-0.2648	-0.1963	-0.1279	***
1 - 4	0.1022	0.1706	0.2390	***
1 - 5	0.5066	0.5750	0.6435	***
1 - 6	0.5066	0.5750	0.6435	***
1 - 7	0.5066	0.5750	0.6435	***
1 - 8	0.4951	0.5750	0.6550	***
2 - 3	-0.2758	-0.2082	-0.1406	***
2 - 1	0.1279	0.1963	0.2648	***
2 - 4	0.2993	0.3669	0.4345	***
2 - 5	0.7038	0.7714	0.8390	***
2 - 6	0.7038	0.7714	0.8390	***
2 - 7	0.7038	0.7714	0.8390	***
2 - 8	0.6921	0.7714	0.8506	***
3 - 2	0.1406	0.2082	0.2758	***
3 - 1	0.3361	0.4045	0.4730	***
3 - 4	0.5076	0.5751	0.6427	***
3 - 5	0.9120	0.9796	1.0472	***
3 - 6	0.9120	0.9796	1.0472	***
3 - 7	0.9120	0.9796	1.0472	***
3 - 8	0.9004	0.9796	1.0588	***

4 - 3 -0.6427 -0.5751 -0.5076 ***
4 - 2 -0.4345 -0.3669 -0.2993 ***
4 - 1 -0.2390 -0.1706 -0.1022 ***
4 - 5 0.3369 0.4045 0.4720 ***
4 - 6 0.3369 0.4045 0.4720 ***
4 - 7 0.3369 0.4045 0.4720 ***
4 - 8 0.3252 0.4045 0.4837 ***

5 - 3 -1.0472 -0.9796 -0.9120 ***
5 - 2 -0.8390 -0.7714 -0.7038 ***
5 - 1 -0.6435 -0.5750 -0.5066 ***
5 - 4 -0.4720 -0.4045 -0.3369 ***
5 - 6 -0.0676 0.0000 0.0676
5 - 7 -0.0676 0.0000 0.0676
5 - 8 -0.0792 0.0000 0.0792

6 - 3 -1.0472 -0.9796 -0.9120 ***
6 - 2 -0.8390 -0.7714 -0.7038 ***
6 - 1 -0.6435 -0.5750 -0.5066 ***
6 - 4 -0.4720 -0.4045 -0.3369 ***
6 - 5 -0.0676 0.0000 0.0676
6 - 7 -0.0676 0.0000 0.0676
6 - 8 -0.0792 0.0000 0.0792

7 - 3 -1.0472 -0.9796 -0.9120 ***
7 - 2 -0.8390 -0.7714 -0.7038 ***
7 - 1 -0.6435 -0.5750 -0.5066 ***
7 - 4 -0.4720 -0.4045 -0.3369 ***
7 - 5 -0.0676 0.0000 0.0676
7 - 6 -0.0676 0.0000 0.0676
7 - 8 -0.0792 0.0000 0.0792

8 - 3 -1.0588 -0.9796 -0.9004 ***
8 - 2 -0.8506 -0.7714 -0.6921 ***
8 - 1 -0.6550 -0.5750 -0.4951 ***
8 - 4 -0.4837 -0.4045 -0.3252 ***
8 - 5 -0.0792 0.0000 0.0792
8 - 6 -0.0792 0.0000 0.0792
8 - 7 -0.0792 0.0000 0.0792

Appendix 10: Bacterial isolates before and after the experiment and the clinical responses of calves in group one.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
1	9084	L-*Staphylococci spp*	*Rhabditis bovis*	L-none	L-*Staphyloccoci spp.*
		R-*Corynebacterium spp*		R-mild	R-*C. spp & Bacilli spp*
	26048	L_*Proteus spp, C. spp & Actinomycetes*		L-mild	L-*Corynebacterium spp*
		R-*Proteus spp*		R-mild	R-*Proteus spp.*
	9081	L-*Proteus spp.*		L-mild	L-*Corynebacterium spp.*
		R-*E. coli & Corynebacterium spp*		R-none	R-*Corynebacterium spp.*
	9086	L-*Staphylococci spp.*		L-mild	L-*Staphylococci spp.*
		R-*Corynebacterium spp.*		R-none	R-*Corynebacterium spp.*
	97	L-*Corynebacterium spp.*		L-none	L-*E. coli & Corynebacterium spp*
		R-*Staphyloccoci spp*		R-none	R-*Corynebacterium spp.*
	85	L-*Actinomycetes spp*		L-moderate	L-*Corynebacterium spp.*
		R-*Actinomycetes & Corynebacterium spp.*		R-moderate	R-*Corynebacterium spp.*
	24091	L-*Corynebacterium spp.*		L-none	L) -
		R-*Corynebacterium spp.*		R-mild	R) destroyed on 15/7/1993

R = Right
L = Left
E = Escherichia
C = Corynebacterium

Appendix 11: Bacterial isolates before and after the experiment and the clinical responses of calves in group two.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
2	24094	L-*Corynebacterium spp.*	*Rhabditis bovis & P. aeruginosa*	L-moderate	L-*Actinomycetes & Corynebacterium spp.*
		R-*Staphyloccoci spp.& Corynebacterium spp.*		R-none	R-*Staphyloccoci spp.*
	26041	L-*Staphyloccoci spp.*		L-mild	L-not identified
		R-*Staphyloccoci spp.*		R-mild	R-*Escherichia coli & Corynebacterium spp.*
	9083	L-*Corynebacterium spp.*		L-moderate	L-*Escherichia coli*
		R-*Corynebacterium spp & Pseudomonas spp*		R-moderate	R-*Actinomycetes*
	9079	L-*Corynebacterium spp.*		L-none	L-*Staphyloccoci spp*
		R-*Corynebacterium spp*		R-none	R-*Corynebacterium spp.*
	9082	L-*Pseudomonas aeruginosa*		L-mild	L-*Corynebacterium spp.*
		R- *Staphylococci spp.*		R-mild	R-*Proteus spp.*
	86	L-*Staphyloccoci spp.*		L-mild	L-*Corynebacterium spp.*
		R-*Corynebacterium spp. & Staphylococci spp.*		R-moderate	R-contaminated
	24099	L-*Staphyloccoci spp.*		L-mild	L-*Corynebacterium spp. & Actinomycetes*
		R-*Pseudomonas spp.*		R-mild	R-*Actinomycetes & Staphyloccoci spp.*

R = Right
L = Left
P = Pseudomonas

Appendix 12: Bacterial isolates before and after the experiment and the clinical responses of calves in group three.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
3	96	L-*Corynebacterium spp.*& *Staphylococci spp.*	*Rhabditis bovis* & *A. pyogenes*	L-mild	L-*Proteus spp.*
		R-*Proteus spp.*		R-severe	R-*Corynebacteriumm spp.*
	24045	L-*Proteus spp.*		L-mild	L-*Actinomycetes* & *Corynebacterium spp.*
		R-*Proteus spp.*& *Corynebacterium spp.*		R-mild	R-*Corynebacterium spp.*
	91	L-*Corynebacterium spp.*		L-mild	L-*Corynebacterium spp.* & *Actinomycetes*
		R-*Corynebacterium spp.*		R-severe	R-*Proteus spp.*
	88	L-*Staphyloccoci spp.*		L-none	L-*Staphyloccoci spp.*
		R-*Corynebacterium spp.*		R-mild	R-*Corynebacterium spp.*
	90	L-*Corynebacterium spp.*		L-mild	L-*Corynebacterium spp.*
		R-*Bacilli spp., Actinomycetes & Corynebacterium spp.*		R-mild	R-*Proteus spp.*
	99	L-*Staphyloccoci spp.*		L-mild	L-*Corynebacterium spp.* & *Actinomycetes*
		R-*Corynebacterium spp.*		R-mild	R- *Corynebacterium spp.* & *Actinomycetes*
	24096	L-*Staphylococci spp.*		L-none	L-*Corynebacterium spp.*
		R-*Pseudomonas spp.*		R-none	R-*Proteus spp.*

R = Right
L = Left
A = Actinomyces

Appendix 13: Bacterial isolates before and after the experiment and the clinical responses of calves in group four.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
4	101	L-*Bacilli spp.& E. coli*	*Rhabditis bovis & P. aeruginosa & A. pyogenes*	L-moderate	L-*Corynebacterium spp.*
		R-*Proteus spp.*		R-moderate	R-*Corynebacterium spp.*
	375	L-*Proteus spp.*		L-moderate	L-*Corynebacterium spp &*
		R-*Proteus spp.*		R-moderate	R-*Staphylococci spp. & Corynbacterium spp.*
	105	L-*Staphyloccoci spp., Corynebacterium spp. & Bacilli spp.*		L-moderate	L-*Corynebacterium spp.*
		R-*Proteus spp.*		R-severe	R-*Proteus spp.*
	374	L-*Corynebacterium spp. & Staphylococci spp*		L-moderate	L-*Corynebacterium spp.*
		R-*Proteus spp.*		R-moderate	R-*Corynebacterium spp.*
	270	L-*Proteus spp.*		L-severe	L-*Corynebacterium spp.*
		R-*Corynebacterium spp.*		R-severe	R-*Corynebacterium spp.*
	103	L-*P. aeruginosa*		L-severe	L-*Corynebacterium spp.*
		R-*Proteus spp.*		R-severe	R-*Corynebacterium spp.*
	269	L-*Staphyloccoci spp. & Corynebacterium spp.*		L-severe	L-contaminated
		R-*P. aeruginosa & Corynebacterium spp.*		R-severe	R-*Corynebacterium spp & Actinomycetes*

R = Right
L = Left
P = Pseudomonas
A = Actinomyces
E = Escherichia

Appendix 14: Bacterial isolates before and after the experiment and the clinical responses of calves in group five.

Group	Calf no	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
5	360	L-*Corynebacterium spp.* R-*Corynebacterium spp.*	*P. aeruginosa*	L-none R-none	L-*P. aeruginosa* R-*Corynebacterium spp.*
	24022	L-*Corynebacterium spp.* R-*Proteus spp.*		L-none R-none	L-*Staphyloccoci spp.* R-*Staphyloccoci spp.*
	101	L-*Staphylococci spp.* R-*Corynebacterium spp.* & Yeast		L-none R-none	L-*Corynebacterium spp.* R-*P. aeruginosa.* & *Corynebacterium spp.*
	26058	L-*Proteus spp.* R-*Proteus spp.*		L-none R-none	L-*Corynebacterium spp.* R-*Staphyloccoci spp.*
	9076	L-*Staphylococci spp.* R-*P. aeruginosa*		L-none R-none	L-*Proteus spp.* R-*Proteus spp.*
	24072	L-*Proteus spp.* R-*Proteus spp.*		L-none R-none	L-*Staphyloccoci spp.* R-*Proteus spp.*
	26045	L-*Proteus spp.* R-*Proteus spp.*		L-none R-none	L-*Staphyloccoci spp.* R-*Staphyloccoci spp.*

R = Right
L = Left
P = Pseudomonas

Appendix 15: Bacterial isolates before and after the experiment and the clinical responses of calves in group six.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
6	102	L-*Staphylococci spp & Bacilli spp.*	*A. pyogenes*	L-none	L-*Staphyloccoci spp.& Corynebacterium spp.*
		R-*Bacilli spp. & Staphylococci spp.*		R-none	R-*Staphyloccoci spp. & Corynebacterium spp.*
	103	L-*Staphylococci spp.*		L-none	L-*Staphyloccoci spp*
		R-*Staphylococci spp.*		R-none	R-*Staphyloccoci spp.*
	104	L-NG		L-none	L-*Staphylococci spp. &* Yeast
		R-NG		R-none	R-*Actinomyces pyogenes & Staphyloccoci spp.*
	105	L-*Staphylococci spp.*		L-none	L-*Staphyloccoci spp.*
		R-*Corynebacterium spp.*		R-none	R-*Corynebacterium spp & Staphylococci spp.*
	106	L-*Staphylococci spp.*		L-none	L-*Staphyloccoci spp.*
		R-*Staphylococci spp.*		R-none	R-*Staphyloccoci spp.*
	107	L-*Corynebacterium spp.*		L-none	L-*Staphyloccoci spp.*
		R-*Staphylococci spp.*		R-none	R-*Staphyloccoci spp.*
	108	L-*Staphylocccoci spp.*		L-none	L-*Staphylococci spp. & Corynebacterium spp.*
		R-not identified		R-none	R-*Staphyloccoci spp.*

A = Actinomyces
R. = Right
L = Left
NG = No growth

Appendix 16: Bacterial isolates before and after the experiment and the clinical responses of calves in group seven.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacteria flora after the experiment
7	109	L-*Staphylococci spp.*	*P.aeruginosa & A.pyogenes*	L-none	L-*Staphylococci spp.,A. pyogenes* & fungus
		R-*Staphyloccoci spp.*		R-none	R-*P. aeruginosa*
	110	L-*Staphyloccoci spp.*		L-none	L-*Corynebacterium spp.*.
		R-*Staphyloccoci spp.*		R-none	R-*Bacilli spp.*
	111	L-*Staphyloccoci spp.*		L-none	L-*P. aeruginosa, Staphyloccoci spp. & Corybacterium spp.*
		R-not identified		R-none	R-*Proteus spp.*
	112	L-*E. coli*		L-none	L-*Staphyloccoci spp.*
		R-*Corynebacterium spp.*		R-none	R-*Proteus spp.*
	113	L-*Staphyloccoci spp.*		L-none	L-*Staphylococci spp. & Bacillus spp.*
		R-not identified		R-none	R-*Proteus spp.*
	114	L-*Bacilli spp.*		L-none	L-*Staphyloccoci spp.* & fungus
		R-Yeast		R-none	R-Yeast & *Staphyloccoci spp.*
	252	L-Yeast		L-none	L-*P. aeruginosa & Staphylococci spp.*
		R-Yeast		R-none	R-*P. aeruginosa*

L = Left
R = Right
P = *Pseudomonas*
A = *Actinomyces*

Appendix 17: Bacterial isolates before and after the experiment and the clinical responses of calves in group eight.

Group	Calf no.	Aural bacterial flora before the experiment	Type of inoculum	Response	Aural bacterial flora after the experiment
8	259.	L-*Staphylococci spp.*	PBS	L-none	L-*Staphylococci spp.*
		R-*Staphylococci spp.*		R-none	R-*Staphylococci spp.* & Yeast
	260	L-*Staphyloccoci spp.*& *Corynebacterium spp.*		L-none	L-*Staphylococci spp.*
		R-*Corynebacterium spp.* & *Bacilli spp.*		R-none	R-*Corynebacterium spp.* & *Staphyloccoci spp.*
	356	L-*Corynebacterium spp.* & *Staphylococci spp.*		L-none	L-*Staphyloccoci spp.*
		R-*Corynebacterium spp.*		R-none	R-*Staphylococci spp.* & *Bacilli spp.*
	354	L-*Staphyloccoci spp.* & *Corynebacterium spp.*		L-none	L-*Staphylococci spp.* & *Bacilli spp.*
		R-*Staphyloccoci spp.*		R-none	R-*Staphyloccoci spp.*

A.= *Actinomyces*
C.= *Corynebacterium*
E.= *Escherichia*
K.= *Klebsiella*
P.= *Pseudomonas*
L= Left
R= Right
PBS= phosphate buffered saline

Appendix 18: Sequential bacterial isolates during the course of the infection in calves from group four.

Calf no	Bacterial isolates						
	Dates						
	13/10/95	4/11/95	18/11/95	1/12/95	18/12/95	1/1/96	17/1/96
101 R	*Proteus spp*	*A. pyogenes & C. spp.*	*C. spp.*	*C. spp.*	*Actnomycetes& C. spp.*	*Actinomycetes & C spp.*	*C. spp.*
101 L	*Bacilli spp*	*A. pyogenes & C. spp.*	*Actnomycetes & C. spp.*	*A. pyogenes,C. spp., E. coli & Actinomycetes*	*P. aeruginosa & K. spp*	*Actinomycetes & C spp.*	*C. spp*
375 R	*Proteus spp*	*C. spp & E. coli.*	*Actinomycetes, C. spp & E. coli*	*A. pyogenes, C. spp. & Actinomycetes*	*C. spp*	*C. spp.*	*C. spp.*
375 L	*Proteus spp.*	*C. spp. & P. spp*	*Actinomycetes, C. spp, K. spp & P. spp.*	*C. spp.*	*E. coli*	*C. spp. & K. spp.*	*C. spp. & Staphylococci spp.*
105 R	*Proteus spp.*	*Actinomycetes E. coli & A. pyogenes*	*C. spp.*	*Proteus spp*	*Proteus spp*	*Proteus spp*	*Proteus spp.*
105 L	*Bacilli spp. & C. spp*	*Actinomycetes E. coli,K. spp & A. pyogenes*	*Actinomycetes & C. spp.*	*E. coli.*	*Actinomycetes & C. spp*	*C. spp.*	*P. spp & C. spp.*
374 R	*Proteus spp.*	*Proteus spp..*	*Actinomycetes C. spp. & E. coli*	*Staphylococci spp.*	*C. spp*	*C. spp.*	*C. spp.*
374 L	*Staphylococci spp, & C. spp.*	*C. spp.*	*Actinomycetes, C. spp & E. coli*	*Proteus spp.*	*Actinomycetes*	*C. spp.*	*C. spp.*

A.= Actinomyces
C.= Corynebacterium
E.= Escherichia
K.= Klebsiella
P.= Pseudomonas

Appendix 18 continued.

Calf no	Bacterial isolates						
	Dates						
	13/10/95	4/11/95	18/11/95	1/12/95	18/12/95	1/1/96	17/1/96
270 R	*C. spp.*	*C. spp. & K. spp.*	*A. pyogenes , C. spp & Staphylococci spp.*	*C. spp.*	*E. coli & C. spp.*	*Actinomycetes & C. spp.*	*C. spp.*
270 L	*Proteus spp.*	*C. spp & K. spp.*	*Bacilli spp.*	*C. spp.*	*C. spp.*	*C. spp., Staphylococci spp. & Actinomycetes*	*C. spp.*
103 R	*Proteus spp.*	*Actinomycetes & K. spp.*	*P. spp.*	*C. spp. & Staphylococci spp.*	*C. spp.*	*C. spp.*	*C. spp.*
103 L	*P. aeruginosa*	*Actinomycetes & C. spp.*	*C. spp. & A. pyogenes*	*Actinomycetes & K. spp.*	*Actinomycetes & C. spp.*	*C. spp.*	*C. spp.*
269 R	*C. spp. & P. aeruginosa*	*C. spp., Bacilli spp. & A. pyogenes*	*C. spp.*	*C. spp.*	*P. spp. & K. spp.*	*C. spp.*	*C. spp & Actinomyectes* contaminated
269 L	*C. spp. & Staphylococci spp.*	*C. spp & Staphylococci spp.*	*Proteus spp.*	*C. spp.*	*C. spp.*	*C. spp.*	

A.= Actinomyces
C.= Corynebacterium
E.= Escherichia
K.= Klebsiella
P.= Pseudomonas
L= Left
R= Right

Appendix 19: Sequential bacterial isolates during the course of infection in calves from group two.

Serial no.	Calf no.	Bacterial Isolates			
		dates			
		14/5/1993	4/6/1993	8/8/1993	3/9/1993
1	9082	*Pseudomonas spp.*	*P. aeruginosa*	*Proteus spp.*	*Corynebacterium spp.*
2	9082	*Bacilli spp. & Corynebacterium spp.*	*Staphylococci spp.*	*Proteus spp.*	*Proteus spp.*
3	9083	*Corynebacterium spp. & Staphylococci spp.*	*Corynebacterium spp.*	*Proteus spp.*	*Actinomycetes*
4	9083	*Corynebacterium spp. & Staphylococci spp.*	*Corynebacterium spp.*	*Actinomycetes & Corynebacterium spp.*	*Corynebacterium spp.*
5	86	*Corynebacterium spp.*	*Corynebacterium spp.*	*Corynebacterium spp. &* unidentified	contaminated
6	86	*Proeus spp.*	*Staphylococci spp.*	*Bacilli spp.& Corynebacterium spp.*	*Corynebacterium spp.*
7	26041	*Proteus spp.*	*Staphylococci spp.*	*Corynebacterium spp.*	*Staphylococci spp.*
8	26041	*Corynebacterium spp.*	*Staphylococci spp.*	*Staphylococci spp.*	*Corynebacterium spp.*
9	24099	*Corynebacterium spp.*	*Staphylococci spp.*	*Corynebacterium spp.*	*Actinomycetes & Corynebacterium spp*
10	24099	*Corynebacterium spp.*	*Pseudomonas spp.*	*Proteus spp.*	
11	24094	*Proteus spp.*	*Corynebacterium spp. & Staphylococci spp.*	*Bacilli spp. & Staphylococci spp.*	*Corynebacterium spp.*
12	24094	*Corynebacterium spp.*	*Corynebacterium spp.*	not identified	*Actinomycetes & Corynebacterium spp.*
13	9079	*Pseudomonas spp.*	*Pseudomonas spp.*	*Corynebacterium spp.*	*Staphylococci spp.*
14	9079	*P. aeruginosa*	*P. aeruginosa*	*Corynebacterium spp.*	*Staphylococci spp.& Corynebacterium spp.*

P.=Pseudomonas