Vaccine Trails Against Bovine Farcy

A thesis submitted in partial fulfillment for the requirement of the Degree of Master of Veterinary Science (M. V. Sc)

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Preface

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DEDICATION

To my Mother, Father, Sisters, Brother,
Relatives and Friends.
To my dear Fiance.
With deep love

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ABSTRACT

The present study was designed to develop and evaluate a live vaccine against bovine farcy. *Mycobacterium farcinogenes* (*M. farcinogenes*) strain A20, was passaged in a set of guinea pigs. The attenuated strain produced no lesions neither at the site of inoculation nor in any other organ of the tested guinea pigs. The organism, which was reisolated from the guinea pigs, was labelled as *M. farcinogenes* A24.

Another experiment was carried out in guinea pigs to determine the degree of protection following the vaccination with *M. farcinogenes* A24. Guinea pigs were divided into two groups. Vaccinated and non-vaccinated groups. At the end of the 9^{th} week, the challenge infection was performed using a freshly isolated *M. farcinogenes* SD117, which was isolated from a cow with bovine farcy. Ninety two percent (92%) of guinea pigs from vaccinated group showed no nodular lesions, whereas all the non-vaccinated guinea pigs showed visible nodular lesions.

Eleven calves were used for vaccine trials in bovine. The calves were divided into three groups: vaccinated group (n=8), non-vaccinated group (n=2) and control group (n=1). Seventy five percent (75%) of the vaccinated calves showed no lesions following the challenge infection while all of non-vaccinated calves showed the nodular lesions at site of inoculation.

ELISA readings among the vaccinated and non-vaccinated guinea pigs showed that the mean optical densities at 450 nm were 0.15 and 0.05, respectively.

In calves, antibodies titer reached its maximum level at the 5th week in vaccinated with O.D 2.722 and in non-vaccinated with O.D 0.229 which is highly significant. Then titer started to decline in vaccinated (O.D 0.248) and in non-vaccinated (O.D 0.175) which indicated no significant difference between two groups. The results were statistically verified.

We concluded, that the attenuated *M. farcinogenes* A24 that produced via passage in guinea pigs, could be used to initiate a protective humoral and cellular immunity against the casual agent of bovine farcy. Further studies are needed to adopt field trials vaccination to validate the current result and to determine the duration of immunity and the vaccine shelf life.

Introduction

Bovine farcy is a chronic infectious disease affecting Zebu cattle manly in the tropical countries. The disease is characterized by tumification and suppurative granulomatous inflamation of the lymphoid and subcutaneous tissues. All organs could be affected but the disease has more affinity to the lymphoid tissues. The disease develops very slowly and painlessly and can be noticed in the chronic form only (Mostafa, 1967, Hamid *et al*., 1991).

Pulmonary and cutaneous forms of the disease were described by Awad and Karib (1958). Awad (1960) reported nocardiosis of bovine udder and testis caused by *M. farcingenes* and *M. senegalense* (Chamoiseau 1979). In the past *N. farcinica* was believed to be the cause (Mostafa, 1966).

Bovine farcy is widely distributed and reported in many countries: France, Guadloupe, South America, India, Ceylon, Sumatra, Eritrea, Somali land, Kenya, Ghana, Nigeria, Chad and Sudan (Mostafa, 1966). Its history in France and Guadlloupe dates back to the first half of the nineteenth century. The disease then known as "arboulets" in the Anjou province and appeared to have been fairly prevalent in France. It was first observed in Charente.

Many workers in West Africa (Memery, *et al.,* 1958, Chamoiseau, 1974) and in Chad (Chamoiseau, 1979) have tackled the problem from microbiological point of view to identify the causal agent(s) of the disease. It has been suggested that the disease is endemic in Somalia (Abdulle, 1983).

In the Sudan the disease was reported in 1929 in Nuba Mountains, Southern Kordofan Region (Annual report of the Sudan Veterinary Services, 1929). The disease affected local cattle, but owners paid a little attention

because the losses from the disease were rare. However, the disease became known as an economical problem and the cattle owners in Western Sudan were seriously worried of the disease. The disease received little attention from the officials, hence no vaccination or effective treatment were attempted.

Awad and Karib (1958) showed the relationship between bovine farcy and bovine tuberculosis. There is cross-reaction between tuberculin test and bovine farcy. These cross-reactions may confuse the interpretation of tuberculin testing in countries where both diseases exist (Awad, 1958, Mostafa, 1967).

The condemnations of the carcasses due to bovine farcy were found to be 14.6% in Malkal Abattoir (Awad and Karib 1958). El-Nasri (1961) noticed the spread of the disease by 15% among the Arab herds in the Nuba Mountains region and this incidence was declined in the Southern region of the country.

Many investigations and research were conducted in Sudan, where the disease represents significant economic losses. These losses are particularly noticeable among the Bagara nomadic tribes of Western Sudan (Hamid, 1988, El-Hissien, 2001). Salih, *et al.* (1977) reported the incidence of 10.86% in Nuba Mountains and 3.12% in Nyala area.

Many aspect of bovine farcy have been studied during the last three decades. These included bacteriological, taxonomical, pathological and serological studies. The major break through has been in the bacteriology and the taxonomy of the causal agent, but there was no work done on the immunity and /or the production of protective vaccine. Although *M*

.*farcinogenes* and *M. senegalense* are sensitive to some antibiotics, yet no treatment in vivo could be of value. This will be especially true in case of the nomadic cattle where the nature of the granulomatous mycobacteriosis requires several months for complete recovery (Chamoiseau, 1979, El-Sanousi *et al*., 1979, Hamid and Goodfellow 1997).

Objectives

The objectives of the present study were:

1- Serially pass a laboratory-adapted strain of *M. farcinogenes* A20 in guinea pigs in order to hypothetically attenuate and render it non-virulent

2-Test the non-virulent *M. farcinogenes* strain in Zebu calves

3-Production of attenuated bovine farcy vaccine.

4- Monitor the immune response using ELISA technique

LITERATURE REVIEW

Chapter I

Literature Review

1.1 BOVINE FARCY

1.1 .1 **Definition**

Bovine farcy is a chronic infectious disease of cattle, in some tropical countries. It is considered as one of the most important mycobacterial infections (Timony *et al*., 1988). The disease was believed to be caused by *Nocardia farcinica* (*N. farcinica*), but now *Mycobacterium farcinogenes* (*M. farcinogenes*) and *Mycobacterium senegalense (M. senegalense*) were found to be the main causing agents (Chamoiseau, 1979). The disease is characterized by tumefaction and suppurrative granulomatous inflammation of superficial lymph nodes, vessels and subcutis. The prescapular, precrural and head lymph nodes are usually infected and undergone abscess formation. The disease was also found in internal organ and lymph nodules such as lung, liver and spleen (Awad and karib, 1958, Sulieman and Hamid, 2003).

1.1.2 Historical background

Mostafa (1966) mentioned the disease to have occurred in Anjou province in France with chronic circumscribed swelling to the limbs. He noticed that lesions persisted for a year or more and infected animals were difficult to fatten and owners disposed them when they become emaciated. He found that the chronic bovine farcy is characterized by suppurrative inflammation of superficial lymph nodes and lymphatic vessels; death was rare and signs like respiratory tuberculosis occurred.

The first isolate from a cow in Guadeloupe (In Caribbean) was described by Nocard (1888), latter named as *N. farcinica* by Trevisan (1889). Then the disease appeared in many countries (Mostafa, 1966). In the Sudan, the first official report was in 1929 (Annual report of Veterinary Service, 1929).

1.2 Aetological agents

1.2.1 Taxonomy

Bovine farcy organism was first discovered by Nocard (1888), who examined specimens collected from Guadeloupe in West Indies. He was able to isolate actinomycetes- like organisms, which appeared as tangled mass of branching threads, when he used Ziehl-Neelsen staining method. A year later, the organism was named *Nocardia farcinica* after Nocard (Trevisan, 1889).

The actinomycete responsible for bovine farcy in Africa was believed to be *N. farcinica* on the account of its morphological similarity to the causal agent of bovine farcy in West Indies (Mostafa, 1966).

Trevisan, 1889 described the disease and its causative agent(s) (*N. farcinica*), several workers in different countries followed his method of identifications which based on Acid fastness and branching filamentous with aerobically growth.

It is becoming increasingly evident that the actinomycete responsible for bovine farcy in Africa is *Mycobacterium* and not *Nocardia* (Asselineau *et al*., 1969, Chamoiseau, 1969, Lechevalier, *et al*., 1971, Chamoiseau, 1973; Chamoiseau, 1979; El-Sanousi, *et al.,* 1977; Ridell, *et al.,* 1982; El-Sanousi, *et al.,* 1986).

It was apparent that the organisms isolated from bovine sources in Chad was different in certain respects from those isolated in Senegal (Chamoiseau, 1973), as follows: (i) cultural characteristic: Chad strains grow slowly and Senegal strains grow rapidly (Chamoiseau, 1969). (ii) Biochemically: Senegal strains exhibit broader and more intense amidase glucolytic activities than do Chad strains (Chamoiseau, 1969, 1972). (iii) Lipid composition: Senegal strains contain the rarely encountered mycoside Ć (Laneelle, G., *et al*., 1971). (iv) Pathogenicity for experimental animals: Senegal strains produced a massive, generalized peritonitis in guinea pigs but Chad strains caused peritonitis with large but limited individual abscesses (Chamoiseau, 1973). Furthermore, it was known that the Chad strains also differ from the Senegal strains because of the low degree of homology between their deoxyribonucleic acid bases (Chamoiseau, 1973).

The above-mentioned differences led to the separation of the Chad and Senegal strains into varieties (subspecies) of the same species, both responsible for identical clinical syndrome. Chamoiseau, (1973) proposed the names *Mycobacterium farcinogenes* subsp. *tchadense* and *M. farcinogenes* subsp. *senegalense* for these organisms. It has subsequently been determined that the name of the type subspecies, *M. farcinogenes* subsp. *tchadense,* should have been *M. farcinogenes* subsp. *farcinogenes* (Lapage, *et al.,* 1975). the subspecies were raised to species status by Chamoiseau, (1979).

1.4.2 Morphology and cultural characteristics

The organisms of bovine farcy, from direct film of specimen, appear as long or short filaments, branched, come together, which do not fragment into

bacillary forms. When stained by Ziehl-Neelsen it is acid alcohol fast. The morphology of the organisms in smear from culture and from specimens is the same. When the organism cultured in Löwenstein-Jensen medium, the growth takes about 7-20 days for *M. farcinogenes* and 24-48 hours for *M. senegalense* at 37°C. The colonies are rough, convoluted and fairmly attached to the medium. It appears as wheaty color in case of *M. farcinogenes* and light green in case of *M. senegalense*. *M. farcinogenes* is difficult to emulsify in distilled water while *M. senegalense* is relatively easy to be emulsify in distilled water (Chamoiseau, 1979).

1.3 Pathology

1.3.1 Pathogenesis

The disease progresses very slowly and painlessly, and was more prevalent among adult cattle than calves in the Sudan (Mostafa, 1967). It is characterized by tumefaction and suppurrative granulonatous inflammation of superficial lymph nodes, vessels and subcutaneous tissue. The abscesses progress slowly, especially in the prescapular, precrural, prefemoral, retropharyngeal, submaxillary, inguinal, mediastinal lymph nodes, in the udder; under the tail and in the front of the shoulder joint. Some animals may develop the pulmonary form of the disease. Farcy nodules also occur along the lymphatics in the fore and hind limbs, around the head at the base of the ears, on the cheeks in parotid region and on the mandible along both angles. A varying number of pea-sized small cutaneous nodules may be seen under the skin of the legs, shoulder, flanks and abdomen (Mostafa, 1967). Lameness may be due to the presence of the lesions on the forelimbs. The swelling

which starts as small circumscribed nodule, develops slowly and may persist for along time without altering its form; after several months it may become as large as an orange or ostrichs egg. At times, several nodules form a rosary of beads along the lymphatics of the limbs, which may be enlarged and thickened. Different numbers, sizes of abscesses spread along the animal of the same herd. On palpation the abscesses appear either as hard swelling, which can be rolled under fingers or as an abscesses closely adherence to the skin (Mostafa, 1967).

In many cases the abscess become soft, uptake the skin and open discharging an odorless exudate. The exudate is thick grayish-white or yellowish -creamy material with soft cheesy content, which frequently contains small granules. Old lesions may discharge as well and have a dirty and foul odor due to secondary bacterial contamination. The wounds heal in a few days and new contents are formed within small-encysted abscesses, which continue to enlarge and develop. The lesions are usually surrounded with thick fibrous capsules (Mostafa, 1967).

1.3.2 Gross lesions

The internal lesions may be accompanied with an external lesion or may not when observed in slaughterhouses during meat inspection. The lesions may be local or generalized. The lungs, bronchial, mediastinal and mesenteric lymph nodes are mainly affected. The nodules in the lung are unevenly distributed, the pleura may be involved and lesions being either diffuse or localized nodular form, which look like tuberculosis pleurisy. Peritonitis may occur following infection of mesenteric lymph nodes and

viscera (Mostafa, 1967; Salih *et al*.,1978; Hamid *et al*.; Sulieman and Hamid, 2003).

In the chronic pulmonary form of bovine farcy, lesions are confined to the chest cavity. The lesions may take either the chronic suppurative granuloma or a chronic bronchopneumonia. A lung lobule may show encapsulated caseopurulent nodules with partially calcified centers. In the later stage of the disease, the organisms spread around by the lymph and blood stream, with ultimate secondary lesion in the lungs, liver, spleen, kidneys, testicles, udder, etc (Mostafa, 1967).

1.3.3 Histopathology

The characteristic histopathological changes are well seen in the udder. They resemble those in infected lungs and lymph nodes. There is initially an abnormal cellular infiltration in the intravenous spaces showing increasing number of plasma cells (Mostafa, 1967). Macrophages appear in the infected foci; the glandular epithelial cells are shrunken and dissolved, and become detached from their nuclei (Mostafa, 1967).Severe granulomatous reactions appear, with a central zone of caseation and necrosis surrounded by a marked zone of inflammatory cells, which comprise neutrophils, lymphocytes, macrophages, epithelioid and giant cells. There were some dilatation of the blood vessels and in some sections there were severe heamorrhages (Awad and Karib 1958, Mostafa 1967, Perpezat *et al*., 1967, Hamid *et al*., 1991).

1.4 Epdimiology

1.4.1 Occurrence

Bovine farcy was reported in France, Guadeloupe South America, India, Ceylon, Sumatra, Eritrea, French West Africa and Sudan (Mostafa, 1966).

A Similar disease, called bovine lymphangitis, was reported in the United States (Traum, 1919). The clinical signs were typical to bovine tuberculosis. The causative agent was an acid-fast bacillus, which is positive to the bovine tuberculin test, but it is not typical to *M. tuberculosis* or one of the complexes, which had been isolated. In Sudan, the disease was reported in 1929 (Sudan Annual Report, 1929). El-Nasiri (1961) noticed the spread of bovine farcy (15%) among the nomadic Arabs cattle in Nuba Mountains area, Western Sudan. Salih *et al.* (1978), El-Sanousi *et al*. (1979) and Hamid *et al.* (1991), reported the incidence of the disease in Nyala (Western Sudan) in ratio of 10.86%, 4.19% and 31% respectively. In Omdurman area (Central Sudan), Mostafa (1967), Awad El-Kareem and Mostafa (1974), Salih *et al*. (1978), El-Sanousi *et al*. (1979) and Hamid *et al*. (1991) reported the incidence of the disease in a ratio of 36.6%, 7%, 3.4, 5.19% and 1%.

In Southern Sudan, Awad and Karib (1958) found that 14.6% of the carcasses condemned at Malakal abattoir were due to bovine farcy.The disease affects all breeds of cattle and both sexes. *M. farcinogenes* and *M.sengalense* do not infect human, wild animals or other domestic animals (Mostafa, 1966, Chamoiseau, 1979).

1.4.2 Source of infection

The way of transmission of the disease is not well known. Ticks were claimed to be a vector, but no clear evidences were indicated (Neumman, 1989, Daubney, 1929). There is no report proving that, the ticks are one of the means of transmission. The organism has not been isolated from the environment. Hamid (1988) identified that many ticks namely *Amblyomma variegatum* and *Amblyomma lipdium* were found feeding on infected farcy cattle, but no trials have been done to isolate the organism from these ticks. Al-Janabi *et al*. (1975) transmitted *M. farcinogenes (N. farcinica* at that time) from experimentally infected rabbit to a control one by using ticks. In Sudan the cattle owners think that ticks are responsible for the transmission of the disease from infected to healthy cattle.

1.4.3 Importance

The disease is responsible for significant economical losses due to death, culling, and condemnation of infected carcasses. It causes confusion when animal tested with tuberculin for eradication of bovine tuberculosis programs (Traum, 1919, Awad, 1958). The great economic loss is due to the condemnation of carcasses during meat inspection at slaughterhouses. The infected animals are mainly of no value because of emaciation, so the butchers refrain from purchasing them. The skins from infected animals are either sorted out or rejected (Awad El-Kareem and Mostafa, 1974).

1.5. Diseases other than bovine farcy caused by Mycobacteria

Diseases caused by mycobacteria in man and animals and their growth rate, are shown in Table (1.1)

Table 1 major division of Mycobacteria and diseases they cause *

Group	Species	Host	Disease and lesions
Rapid Growers	*M. fortuitum*	Human	Post - trauma abscesses
	M. chelonei	"	Skin lesions
	M. smegmatis	"	Rarely pathogenic
	M. phlei	"	"
	M. vaccae	"	"
	M. diernhoferi	"	"
	M. rhodesiae	"	"
	M. flaescens	"	"
	M. gilvum	"	"
	M. duvalli	"	"
	M. chitae	"	"
	M. thermoresistibile	"	"
	M. nonchromgenicum	"	"
Slow growers	*M. kansaii*	Human	Pulmonary diseases
	M. marinum	Fish	Swimming pool granuloma or fish tank granuloma
	M.scrofulaceam	Human	Cervical lymphadenits pulmonary diseases
	M. szugai		Rare, tuberculosis like infection, cervical adenitisan olecranon bursitis
	M .avium some strain	Cattle, sheep and goat	Para tuberculosis &pulmonary disease
	M. tuberculosis	Cattle and goat	Bovine tuberculosis
	M. avium lepraemurium	Rats	Rat leprosy

*** Runyun *et al*., (1974); William *et al.*, (2001)**

1.5.1 Bovine Tuberculosis

Bovine tuberculosis is a chronic disease of cattle, characterized by the formation of small masses of inflammatory tissue, or tubercles, in the organs where the bacilli lodged and multiply. The disease is cause by *M. bovis* (Hagan, 1973). Both the human and avian types of tubercle bacilli could affect the cattle; the disease can be localized or generalized. The later appear when the bacilli entered the blood stream (Hagan, 1973).

In adult cattle the lesions are found in the lungs and lymph nodes of the head and thorax, less often in the liver and the spleen and very rare in the udder. The disease affects all ages of animals; calves are infected when they feed on milk containing tubercle bacilli (Hagan, 1973).

Horses may be affected with bovine tuberculosis when they live in the same association with infected cattle; the lesions occur in pharyngeal region and mesentery lymph nodes. There are also some lesions in the liver, lungs and spleen. Dogs can be affected with both human and bovine types, but they are especially sensitive to human type. The lesions involve the thoracic organs. Cats are very resistant to human infection, but susceptible to bovine type of infection. The lesions are often found in the abdominal organs and lungs (Hagan, 1973).

The disease is rare in sheep and goats; the infection is usually contracted by direct contact with the infected animals. Chickens are susceptible to be infected by avian type, and not by mammalian tubercle bacilli. The infection is caused by the ingestion of bacilli from contaminated soil and water (Hagan, 1973).

The first report of tuberculosis in cattle in the Sudan was in 1915 (Annual Report, 1915), which stated that the disease was reported in Egypt, among cattle exported from Sudan.

1.5.2 Johne's Disease (Paratuberculosis)

The disease is a chronic, slowly developing, mild to severe gastroenteritis affecting younger cattle more severely than others and ending sometimes fatally (Abbas, 1981).

In cattle the infection involves the intestinal canal and the lymph nodes of the mesentery, the lower part of the small intestine, the caecum, and the beginning of the colon. The intestinal wall begins to undergo very obvious thickening (Hagan, 1973). The clinical signs of the disease are edema of intermaxillary space, the coat become dry, a diarrhea begins and follows with dehydration and general emaciation. The eyes sink into their sockets. Death may occur within a week after these signs. The animal may recover and the signs disappear or it may become permanent (Hagan, 1973).

Bovine John's disease is emerging as one of the most important diseases affecting the cattle industry in intensive dairy practices at the present time (Abbas, 1981), also infected sheep, goats and some wild animals. In the past *M. paratuberculosis* was the causal agent but know reclassified as *M. avium* subspecies paratuberculosis (Blood *et al.*, 1983).

1.5.3 Rat leprosy

The disease is caused by *M. avium lepraemurium*. The disease was reported for the first time in Odessa (Stephansky 1901). 5% of the animals are infected with a disease, which look like the human leprosy. The disease

affects the lymph nodes of axillary and inguinal regions. In other cases in the skin, subcutaneous tissue and underlying muscles. The hair is lost from the area and ulcers may be formed from the thick discharge exudates. Lesions are rare in internal organs except some nephritis. The rat leprosy is transmitted to many of the domestic animals and has no relation to human leprosy (Hagan, 1973).

1.5.4. *Mycobacterium avium*

M. avium infections are a common problem in large swine in the United States and cause substantial financial losses at slaughter inspection due to carcass condemnation. Once the infection is established in a swine herd, it is difficult to effectively prevent or eliminate the disease. Swine mycobacteriosis is not a nation or worldwide problem, however, the incidence in regions with large swine populations is high (Hines *et al* 1998).

Normal young children (1-4 years of age) and immune compromised individuals such as AIDS patient, bone marrow transplant recipients and cancer patient often become infected with opportunistic members of the *M. avium* intracellular complex (MAIC) (Gallo *et al*., 1983; Kurzrock *et al*., 1984; Young *et al*., 1986), therefore, improperly handled MAIC infected meats may be potential sources of human infection and cause of food safety concerns. It has been estimated over 50% of AIDS patients have co-existing MAIC infections that may significantly contribute to the high mortality rate observed in individuals with HIV-1 infections (Helbert *et al*., 1990). Due to recent increase in mycobacterial infections of both animals and humans, development of effective methods for prevention and control is highly appreciated.

1.6 Mycobacterial Vaccines

1.6.1 *M. tuberculosis*

A vaccine against *M. tuberculosis* had been available since early in the twentienth century. It was produced from Bacille Calmette-Guerin (BCG), by attenuating a strain of *M. bovis*. When injected intradermillay, it produces tuberculin hypersensitivity, and enhanced ability to activate macrophages that kill the pathogen. The World Health Organization (WHO) uses this vaccine in mass immunization campaigns for children in several European countries (William *et al.*, 2001). However, recommendation by public health officials in United States suggested that vaccination could be considered only for tuberculin-negative individuals under sustained heavy risk of infection, such as special groups of health care workers, or those at high risk in areas where multiple drug-resistance TB is common. The reasons for its restricted use is that the vaccine causes a positive tuberculin reaction for several years after it is given, and this greatly limits the usefulness of diagnostic tuberculin test. BCG should not be given to AIDS or other immuno-suppressed individuals, because in such cases it has occasionally cause clinical disease. The use of genetically engineered BCG tailored for improve efficacy against tuberculosis is currently under investigations (William *et al.*, 2001). Because BCG activates macrophages, it has also been used experimentally to stimulate cellular immune functions in the treatment of some malignancies, for example, cancer of bladder (William *et al.*, 2001).

1.6. 2. *M. avium*

M. avium infections in swine are of great economic importance to many swine producers in USA (Pritchard *et al*., 1963; Cole *et al*., 1975; Thoen, 1992). There have been no reports of vaccination for prevention of swine mycobacteriosis. Initial studies suggested that Macrophage Inhibitory Factor –A3 (MIF-A3) is a virulence factor of *M. avium* and a potential antigen for vaccine development. MIF-A3 has been shown to inhibit the ability of murine and bovine macrophages to kill a target organism e.g. *Candida albicans* (Hines II *et al*., 1995). MIF-A3 has also been shown to exhibit a greater inhibitory effect on macrophages from inbred mice (c57BL/6) that do not possess the BCG resistance gene (Hines *et al*., 1996). In their study, they evaluated the efficacy of killed (whole cell) *M. avium* serovar 2 bacterin and MIF-A3 subunit vaccine in preventing infection and disease in swines challenged with virulent *M. avium* serovar 2.

After many trials Hines *et al.,* (1998) found that the killed bacterin (whole cell) did not prevent infection, but did reduced the severity of gross and microscopic lesions by 47% as compared to infected control and MIF-A3 subunit vaccinated pigs. The conjugated MIF-A3 subunit vaccine failed to prevent infection and lesion scores, were generally similar to the (sham) vaccinated infected control group. Correlation of tests in the diagnosis of swine mycobacteriosis was quite good. Approximately 91 (21/23) of challenge animals were positive for mycobacterial disease by at least two tests on one or more specimens. A combination of histology (lesion score > 2

lesions) and culture demonstrated, that 82.6% (19/23) of challenged animals had evidence of mycobacterial infection, while the combination of histology and polymerase chain reaction (PCR) demonstrated an infection rate at 65.2% (15/23).

1.6.3 *M. paratuberculosis* (Johnes disease)

The control of paratuberculosis in domestic cattle, sheep and goats by conventional methods has proved to be very difficult. Isolation and slaughtering of clinically affected and serologically positive animals together with hygienic precautions and suitable husbandry have been unsuccessful. For this reason vaccination has in several countries turned out to be the final resource in controlling the disease (Blood *et al*. 1983, Riemann and Abba*s*, 1983).

In 1926 Vallée initiated vaccination in France followed by Rinjard vaccinated cattle with live a virulent strain of *M. paratuberculosis* mixed with liquid paraffin, olive oil and pumice powder (Vallée 1934). The principle of this method was to establish state of sensitivity, which protected the animals as long as the bacteria persisted in the fibrocaseous nodule formed at the site of vaccination. The safety of this method was emphasised by Vallée *et al.* (1934) and confirmed experimentally by Doyle (1945).

The value of vaccination of cattle was emphasized in France by Rinjard (Saxegaard 1985).

In Iceland, Sigurdsson, (Saxegaard, 1985) successfully vaccinated 450000 sheep with a heat- killed strain of *M. paratuberculosis* suspended in mineral oil. An attempt to control the disease in goats in Norway by vaccination was described by Saxegaard (1985) who used two British bovine

reference strains of *M. paratuberculosis,* for vaccine production. These strains, which originated from the Central Veterinary Laboratory, Weybridge, had been adopted to grow in liquid synthetic media. These strains were closely related to Norwegian goat strains with regard to antigenic structure, thus indicating that their immunizing properties were similar. The efficacy of the vaccine was judged by postmortem examination of vaccinated and non-vaccinated goats performed as described by Fodstad and Gunnarsson (1979).

Postmortem examination of material from slaughtered goats shows that the adjuvanated vaccine with live *M. tuberculosis* bacteria offers a high degree of protection against paratuberculosis in goats. The gradually decreasing infection rate after vaccination was initiated. Farmers have also reported that clinical signs of the disease have gradually disappeared and milk production has increased (Fodstad and Gunnarsson, 1979).

Results also indicated that it might be possible to eliminate the disease from a herd, since vaccinated animals are kept in an environment where infection was prevalent show good resistance. Regarding the age at which the animal should be vaccinated there are discrepancies in the literature. According to Hole (1958) cattle of all ages were vaccinated in France, while only calves were vaccinated in Great Britain. However, according to Thoen *et al*. (1979) and Blood *et al*. (1983) animals can contract infection very early in life, usually before they are three months old, but may not develop signs until two years later. Moreover, the finding of Taylor (1953) indicates that there is an age bar of about six months to experimental infection in cattle.

There is a general agreement that revaccination of cattle against paratuberculosis is not recommended. On the other hand, revaccination of

goats has not been performed in countries like Norway (Gilmour 1976, Blood *et al*.1983, Riemann *et al*., 1983).

It is well known that vaccination against paratuberculosis sensitize animals both to mammalian and avian tuberculin (Hole 1958, Blood *et al*., 1983). Vaccination as a control measure is therefore only practiced in countries with no tuberculosis eradication programs or in selected herds known to be free from tuberculosis (where there is little risk of it being introduced). As bovine tuberculosis was eradicated in Norway since 1963 (Saxegaard, 1985), the interference of vaccination against paratuberculosis with tuberculin testing represents no problem. Vaccination alone does not control paratuberculosis in cattle, sheep or goats. As emphasised by Riemann *et al*. (1983), other procedure such as the removal of clinically affected and serologically positive animals, improved by hygiene and appropriate husbandry should be practiced together with vaccination.

MATERIALS AND METHODS

Chapter II

Materials and Methods

2.1 Mycobacterial strains, culture and staining methods

2.1.1 Strains

M. farcinogenes Strain M62 was previously isolated from a case of bovine farcy at El-Obeid (Hamid, 1988). This strain was then subjected to serial passages in modified synthetic media (Hamid, unpublished data). The strain was received and labeled *M. farcinogenes* A20, which was then used for guinea pigs inoculation.

M. farcinogenes strain SD117, was isolated from a case of bovine farcy which was purchased from Elshiek Abuzaid market, Omdurman. This strain was used for challenge test. Previously identified *M. farcinogenes* strains of (M39, M217 and M16) were included as control.

2.1.2 Isolation of *M. farcinogenes*

The method of isolation and identification was done according to Hamid *et al*. (1993a). Infected lymph nodes which had been collected from cases of bovine farcy were decontaminated by burning the outer surface with spirit, then a straight loop was aseptically inserted inside the lymph node and its contents (purulent materials), were cultured on slant of Löwenstein Jensen (L.J) medium as described by Board and Feltham (1993). (Appendix A). The cultures were done in universal vials, incubated at 37˚C for up to six weeks, and examined daily for the presence of any growth. *M. farcinogenes* was identified by the growth of acid-fast branching filamentous organisms. These branching filaments were differentiated from *M. senegalense* and *Nocardia*

farcinica by the analysis of the mycolic acid and presence of stable unfragment acid-fast filaments in *M. farcinogenes* and *M. senegalense* but fragmenting partial acid fast filaments in *N. farcinica* (Wayne and Kubica, 1986).

2.1.3 Smears and staining methods

Smears were prepared from purulent materials of infected farcy cattle as follows:

A loopfull of the purulent materials was spread on a clean slide and left to dry. The smear was fixed by passing over a flame. Fixed smears were stained with Ziehl–Neelsen according to Board and Feltham (1993). (Appendix B).

2.1.4 Purification of *M. farcinogenes* culture

Purified well-separated colonies of *M. farcinogenes* were subcultured onto glucose yeast- extract agar (GYEA) (Gordon and Mihm, media, 1962) and incubated as above (appendix A). Purification procedure was done wherever there is contamination or in presence of doubtful colonies. *M. farcinogenes* colonies are recognized according to the followings characters: Slow growth, rough convoluted appearance, no aerial hyphae and wheat-colored (Chamoiseau, 1979).

2.1.5 Preservation of culture

All test strains were preserved at room temperature in GYEA media and as suspension at -20˚C in glycerol (20%, v/v) according to Willngton and Williams (1978).

The glycerol suspension were prepared by scraping colonies from a heavily inoculated plate of GYEA medium and incubated at 37˚C for 14 days. The heavy suspension was transferred as 3 ml volumes of glycerol in Bijou vials. Frozen glycerol suspensions were used for long-term preservation of the strains and also to provide a source of inoculums for different purposes. Inocula were obtained by thawing suspensions at room temperature for about 15min. and were then used for culture, or restored.

2.2 Identification of *M. farcinogenes*

2.2.1 Cultural and morphological characteristics

Growth of *M. farcinogenes* was identified by the presence of acid –fast branching filamentous organisms. The branching filaments were differentiated from *M. sengalense* and *Nocardia farcinica* by the analysis of glycolipids and mycolic acids. Glycolipids and mycolic acids analysis were used to confirm and identify the *M. farcinogenes* A24 vaccine strain, *M. farcinogenes* SD117 challenge strain in comparison to reference strains. They were analyzed by thin layer chromatography for diagnostic glycolipids and mycolic acid patterns to confirm their identity.

2.2.2 Glycolipids analysis

The glycolipids were extracted according to Hamid *et al*. 1993b as follows:

Test strains were grown in GYEA media at 37˚C until good growth was obtained for the lipid analysis. Five mg from the test strains and reference strains were shaken in 4 ml chloroform / methanol (2:1, v/v) in sterile bijou vials, each separately. The contents were mixed with shaker for an hour,

centrifuged and supernatant was transferred in another tube. The extraction was repeated with 2 ml chloroform/methanol (2:1,v/v) with continuous shacking. The supernatant was added to the previous one and evaporated to obtain small volume (10µl). Analytical one–dimensional thin layer chromatography (TLC) of the least polar class in mycobacterium polar lipid fractions was performed with 6x13cm pieces (6x13cm) of (TLC aluminum sheets silica gel 60 F254. MERCK, KgaA, 64271 Darmstadt, Germany) as described by Hamid *et al.* (1993b). Five µl of the previously prepared 10µl volumes were spotted onto (TLC) aluminum sheet. TLC plate was developed in chloroform/ methanol/ H_2O (100:14:0.8,v/v) as described by Dobson *et al*. (1985).

The developed sheets were stained by spraying with 5% ethanolic molybdophosphoric acid, followed by heating at 180˚C for 15 min.
Glycolipids appear as golden pink streaks on a light- brown background

2.2.3 Mycolic acid analysis

Mycolic acid was extracted by acid methanolysis following the method of Minnikin (1988).

Five mg of dried biomass of the *M. farcinogenes* A24 vaccine strain, *M. farciogenes* SD117 the challenge strain, *M. farcinogenes* M39 and the reference strain were placed in a separate corning tube. One ml of methanol / toluene / concentrated sulphuric acid (30: 15: 1, v/v) was added and the mixture heated overnight at 75˚C. After cooling, the preparation was shaken with 2ml petroleum ether (b.p.60-80 ˚C), and the upper layer, which contained the fatty acid esters, was discarded and the lower layer evaporated to dryness at 37˚C.

Analytical one-dimensional TLC was performed using pieces of aluminum sheets (6x10 cm). The dried lipid extract were each dissolved in 0.1ml petrolium ether, 5µl of it applied to TLC aluminum sheets Minnikin (1988). The later were developed two times with petroleum ether/ acetone (95:5, v/v). Then sprayed with 5% ethanolic molybdophosphric acid followed by heating at 100˚C for 15 min.

2.3 Vaccination

2.3.1 Preparation of the vaccine

Locally isolated wild strain of *M. farcinogenes* M62 was previously subjected to serial passages (20 times) in synthetic modified Sauton's broth (Hamid, unpublished data). The resultant *M.farcinogenes* strain was labelled as *M.farcinogenes* strain A20. This was used to inoculate guinea pigs.

2.3.1.1 Attenuation of *M. farcinogenes* A20 in guinea pigs

The procedure for attenuation of *M. farcinogenes* was done according to El Sanousi *et al.* (1979). Colonies from grown culture of *M. farcinogenes* A20 was harvested and homogenized in normal saline. The turbidity of the cell suspension was adjusted to that of McFarland tube number 1 (150 million bacteria / ml). Six guinea pigs were inoculated subcutaneous with 0.1ml of this suspension, and classified as group 1. Lesions such as nodular abscesses were observed at the site of inoculation. The nodules were opened and their contents were cultured on slant of L.J.medium. The grown strain, which was lablled A21, was used to inoculate a new set of guinea pigs (group 2). The second set was monitored as before. The process was repeated for a third, a fourth and a fifth set of guinea pigs then classified and labelled A22, A23, and

A24 respectively. An attenuated avirulent strain (*M. farcinogenes* A24) was achieved after four passages in guinea pigs.

2.3.1.2 Preparation of the challenge strain *M. farcinogenes* (SD117)

A virulent strain of *M. farcinogenes* (SD117) was used in the challenge infection. It was freshly isolated from a case of bovine farcy purchased from El-Sheikh Abuzeid market; Omdurman (Figure 3.1). Isolation, purification and preservation of this strain followed the method described in section 2.1.

2.3.2 Vaccination of guinea pigs

For this purpose 35 guinea pigs were brought from Central National Research Laboratory. Their weights ranged between 250-300g and their ages between 4-6 weeks. These animals were divided into three groups. Group I and II had 13 animals each, group III had 9 animals. Group I (vaccinated group) was inoculated with 0.1ml of the vaccine (*M. farcinogenes* A20) subcutaneously at the right side of the abdomen. Group II (non vaccinated group) was inoculated with 0.1ml normal saline. Group III (control group) was inoculated with 0.1ml normal saline. The vaccinated and non-vaccinated guinea pigs (group I and II) received challenge infections with the virulent strain of *M. farcinogenes* (SD117) after five weeks post vaccination. The inoculation was given S/C as 0.1ml on the left side of the abdomen opposite to the vaccination side (right side).

2.3.3 Vaccination of calves

Eleven calves, (six month old) were used in the vaccination trial. They were purchased from El Sheik Abuzed market, Omdurman. Group I, which had 8 calves, was inoculated with 1ml of the prepared vaccine, at a

concentration of 1McFarland (150 million bacteria/ml). The inoculation was done subcutaneously at the left side of the abdomen. Group II, contained 2 calves, inoculated with 1ml normal saline, and used as negative control. Group III, contained one calf, inoculated with 1ml normal saline but received neither vaccine nor challenge infection (control group).

The vaccinated and non-vaccinated calves received challenge infections after five weeks post vaccination with 1 ml McFarland from a young culture of the virulent *M. farcinogenes* SD117. The inoculation was done using 1ml subcutaneously at the right side of the abdomen (opposite to the left side used for vaccination).

2.3.4 Monitoring and assessment of lesions

Four weeks after injection of calves with virulent strains there was some swellings which started as small-circumscribed nodules that developed slowly (several months) forming different larger size. These types of abbesses were usually multiple raises for two to three nodules on each animal. In comparison of vaccinated animals these nodules were smaller and less in number.

2.4 Indirect Enzyme – linked Immunesorbant Assay (ELISA)

ELISA was done according to Hamid *et al.*, (1998).

2.4.1 Preparation of antigens

Cells of *M. farcinogenes* SD117, were harvested from grown culture and kept in sterile Bijou vial in 0.5mg amount, these were then washed three times with sterile distilled water and finally suspended in 1.5ml sterile distilled water. One gram of glass beads (100µl Sigma), was added, then intergraded

with vortex (Vortex- gene) for 15 minute and centrifuged at 3000 rpm for 15 minute (Ground, 1970).

The supernatant was transferred into another sterile Bijou vial and stored at -20°C for further use.

2.4.2 Collection of sera

Blood samples were aseptically collected from calves and guinea pigs for sera preparation. Heart punctures were performed from guinea pigs using 5ml sterile disposable syringes, (6 weeks after the beginning of vaccination).

For calves, blood was collected from the jugular veins, according to the following protocol:

Before vaccination (zero time), 3week, 5week, 17week, 32weeks, and 52 weeks after vaccination.

Blood samples were allowed to stand at 37°C for one hour and at 4°C overnight to clot. Sera were separated using Pasteur pipettes and kept in eppendroff tubes as 1ml volume, labeled and stored at -20°C until they used.

2.4.3 Equipment

Microtitre plates, made of polystyrene flat bottom were used for ELISA (Corning, New York). The pipettes, used were single and multichanel with volume size 100 and 1000µl.

Washing bottles were used for the washing purposes.

A Multiscan MS, version 3.0 (Lab System, Finland), ELISA reader, (spectrophotometers machine) was used for reading the plates.

2.4.4 Reagents

(Appendix C)

2.4.5 ELISA Procedures

Coating with Antigen

The antigen was diluted with PBS in the ratio 1:2 (antigen: PBS) at pH 7.2 and used as coating buffer. 100 µl of antigen was added into separate wells of microtetire plate and incubated overnight in a humid chamber at 37°C (Hamid *et al*. 1998). After incubation, the contents of microplates were washed three times with PBS-Tween 20 (washing solution).

Test sera

100µl volumes of the test sera diluted as 1:100 in PBS were added in duplicate wells to the coated plates and incubated at 37°C for one hour. The plates were washed as previously described.

Conjugated

100µl volumes of the conjugate, freshly diluted 1:1000 in PBS were added to the, plates washed and incubated at 37°C for one hour. The plate was washed again as described before.

Substrate

Finally, 100 µl volumes of a freshly prepared p-NPP tablets in diethanolamin buffer (pH 9.2 at conc. 1mg/ml) were added into the wells of the plates and incubated at 37°C.

Reading the plates

Plates were incubated for one hour and then read at 450 nm..

2.5 Statistical analysis

T-test was used to compare the means of the optical density in the vaccinated cohort and the non-vaccinated cohort of calves across the six weeks of the experiment. The data were screened for consistency, independence and normality and it was found independent and approximately normally distributed. SPSS statistical software was used to carry out the analysis.

RESULTS

Chapter III

Results

3.1 Isolation and identification of *M. farcinogenes*

M. farcinogenes strain SD117 was isolated from lesions in a cow suspected to be infected with bovine farcy (Fig. 3.1). The smear made from the lesions showed acid-fast branching filamentous organisms (Fig. 3.2), the strain was further identified by mycolic acid and glycolipids analysis.

All *M. farcinogenes* strains (A20, SD117, M39) were cultured on glucose yeast-extract agar (GEYA) medium, the cultures showed rough wrinkled colonies very tedious in growth with elevated margins, loosely attached to the medium. The colonies were nodular, rough and wheaty in colour (Fig. 3.3).

Smears of strains from cultures showed extensive filamentous branching organism. (Fig. 3.4).

The TLC of glycolipids analysis appears as golden pink streaks on light brown background.

The TLC analysis of mycolic acid showed the presence of α and epoxy mycolate methyl esters from *M. farcinogenes*. Mycolate from the test strains have co-chromatographed with reference *M. farcinogenes* (Fig.3.5).

3.2 Attenuation of *M. farcinogenes* M20 in guinea pigs

The first set of the guinea pigs, which were inoculated with *M.*

farcinogenes A20, showed gross lesions at the site of inoculation after 10 days. The lesions appeared as small-circumscribed nodules ranged from 0.5 to 1cm in diameter.

The nodules increased in size and by the end of the 3rd week the abscesses were opened. Successful isolation of the *M. farcinogenes* A21 from the pus was achieved using L.J.medium. The second set of guinea pigs, which were inoculated with *M. farcinogenes* A21, showed similar lesion to those produced by *M. farcinogenes* A20. Successful isolation of *M. farcinogenes* A22 from the pus was obtained using L.J.medium. The third and fourth sets of g. pigs used for attenuation gave similar results to the first and second sets but with marked decrease in size of the abscesses. Isolation of the *M. farcinogenes* A23, and A24 was achieved from abscesses of the third and fourth sets, respectively. No isolation was obtained from the fifth set which showed no visible lesions. No visible lesions were seen neither at the site of inoculation nor elsewhere on carcasses following the post-mortem examination at the end of the 5th weeks.

3.3 Vaccination

3.3.1 Vaccination of guinea pigs

The results of the vaccination in guinea pigs using *M. farcinogenes* strain A24 and the challenge infection with *M. farcinogenes* strain SD117 were as follows:

Ninety two percent (92%) of the guinea pigs in group I (vaccinated group) produced no visible lesions neither at the site of inoculation nor any where in the carcasses following post mortem examination at the end of the ninth

week. One of the guinea pigs in this group showed a very small nodule, yet it was scored positive.

All guinea pigs in group 3, which were the negative control, showed nodules at the site of challenge infections. Abscesses were found to be containing *M. farcinogenes* when examined microscopically.

3.3.2 Vaccination in calves

The result of vaccination in calves using *M. farcinogenes* strain A24 and the challenge infection with *M. farcinogenes* strain SD117 were as follows:

Seventy five percent (75%) of the calves in-group I (vaccinated group) produce no lesions at the site of inoculation neither after 3 weeks nor after 52 weeks post inoculation (Fig. 3.6A). Twenty five percent (25%) of the calves showed small nodules at the site of inoculation, which were smaller than those, produced in the non-vaccinated group. All the calves in group 2, which contained the negative control, showed nodules at the site of inoculation (Fig. 3.6B). Among the latter a very big abscess (1-2cm) was found to contain *M. farcinogenes* when examined microscopically.

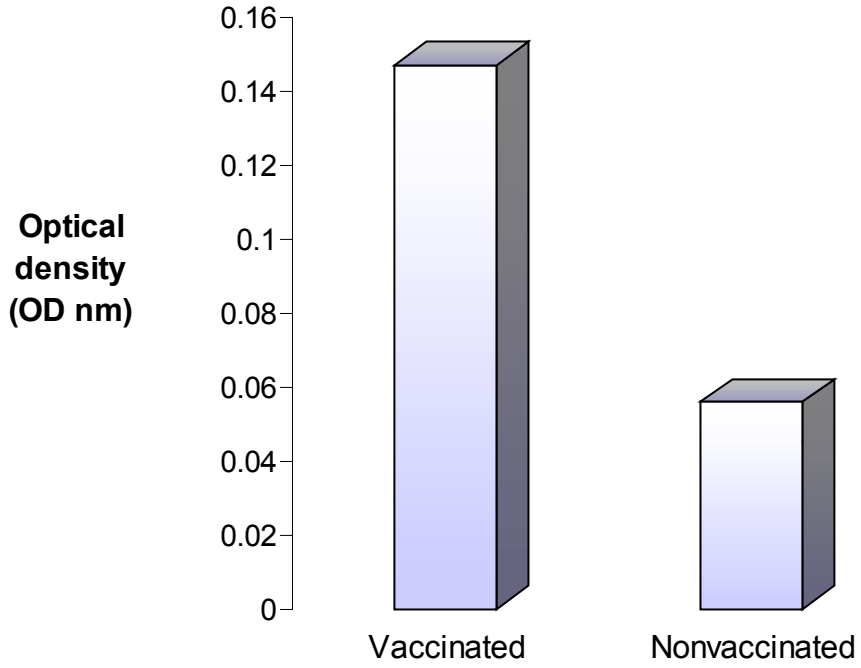
3.3 ELISA

3.4.1 ELISA test in guinea pigs

ELISA was carried out to detect humoral antibody. The result of ELISA for guinea pigs were as follows:

The mean O.D at 450nm was 0.15 and 0.05 in vaccinated and non-vaccinated group respectively (Fig.3.7).

Fig.3.7 Humoral antibodies responses in vaccinated and non-vaccinated guinea pigs

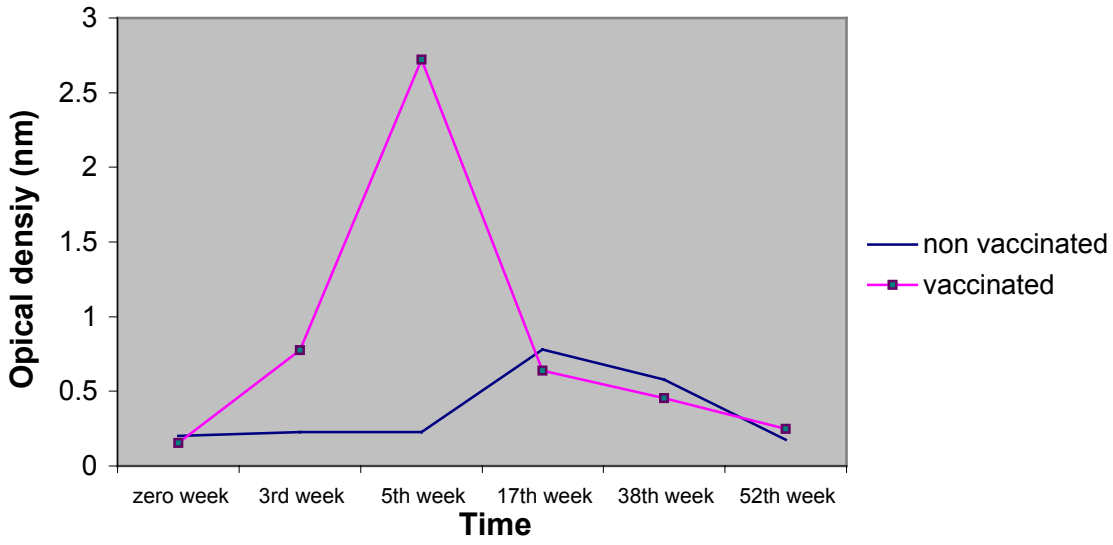


3.4.2 ELISA test in calves

ELISA was carried out to detect humoral antibody titers in vaccinated and non-vaccinated calves. The mean optical densities of vaccinated calves for zero week, 0.153 nm; 3rd week 0.777 nm; 5th week 2.72 nm; 17th week 0.64 nm; 38th week 0.45 nm and 52nd week 0.24 nm. Detailed account of the mean, median, minimum and maximum of the data obtained is shown in: -

Table 2. The mean optical density of non-vaccinated calves was found to be in zero week, 0.20; 3rd week, 0.229; 5th week, 0.229; 17th week, 0.78; 38th week, 0.577 and 52nd week, 0.175. The titer of antibodies rose in the 5th weeks and then declined till it disappeared at the 52Th weeks after vaccination. The optical densities of humoral antibodies shown graphically in Figure (3.7). Detailed account of the mean, median, minimum and maximum of the data obtained is shown in table2.

Fig 3.7 Humoral antibodies response in vaccinated and unvaccinated calves



3.5 Comparison between optical density of vaccinated and non-Vaccinated Calves

T-test was used to compare the mean of the optical density in the vaccinated and non-vaccinated calves in each week of the experiment. No significant difference ($P>0.05$) between the optical densities was detected in the zero weeks, 17^{th} week, 38^{th} and 52nd week. However high significant difference ($P<0.05$) was recorded between the two groups in the 3^{rd} and 5^{th} week. The calculated T-value and P-value is shown in Table (3).

3.5.1 Comparison between the OD of vaccinated calves in different weeks of the experiment.

One-way analysis of variance (ANOVA) was used to explore the significance of the difference between the optical densities of sera collected from vaccinated animals in the different weeks of the experiment. The difference was found to be highly significance ($P< 0.05$).The ANOVA table is shown in table **(**4).

The least significant difference of statistical test was implemented to determine the significance of the difference between the weeks. The difference between the OD of sera collected before vaccination was found to be statistically significant compared with the OD of sera collected in the subsequent weeks except the OD of sera collected in 52nd week. The OD of sera collected in the 5^{th} week was found to be the highest; a highly significant difference between the OD of sera collected in this week and the other weeks was established. Statistical difference between the OD of sera collected in 17^{th} and sera collected in other weeks was detected with exception of the 3^{rd} week. The average and level of significance using LSD is shown in table (5).

Table 3 Comparison between the optical densities of the t-test, P-value one tailed and P-value two tailed of vaccinated and non-vaccinated calves

Time	OD of non vaccinated	OD of vaccinated	t-test	P-value one tailed	P-value two tailed
Zero week	0,202667	0,153	No significant	0,316	0,632
3rd week	0,229	0,777	Highly significant	0,000613	0,001
5th week	0,229	2,722	Highly significant	2,06E-07	4,13E-07
17th week	0,78	0,640	No significant	0,277	0,553
38th week	0,576667	0,453	No significant	0,063	0,126
52nd week	0,175333	0,249	No significant	0,093	0,186

Table 4 ANOVA table for comparison between OD of vaccinated calves through the weeks of the experiments

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31,88962	5	6,377923	340,4099357	3,04E-29	2,477165
Within Groups	0,674496	36	0,018736			
Total	32,56411	41				

Table 5 least significant differences for comparison between OD in each week of the experiments

Multiple Comparisons. Dependent Variable: OD						
LSD		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) TIME	(J) TIME				Lower Bound	Upper Bound
Zero week	3rd week	-0.623857143*	0.077315	1.37E-09	-0.78066	-0.46705
	5th week	-2.568714286*	0.077315	0	-2.72552	-2.41191
	17th week	-0.565428571*	0.077315	1.28E-08	-0.72223	-0.40863
	38th week	-0.200285714*	0.077315	0.013751	-0.35709	-0.04348
	52th week	-0.123	0.077315	0.120379	-0.2798	0.033803
3rd week	Zero week	0.623857143*	0.077315	1.37E-09	0.467055	0.78066
	5th week	-1.944857143*	0.077315	0	-2.10166	-1.78805
	17th week	0.058428571	0.077315	0.454735	-0.09837	0.215231
	38th week	0.423571429*	0.077315	3.46E-06	0.266769	0.580374
	52th week	0.500857143*	0.077315	1.6E-07	0.344055	0.65766
5th week	Zero week	2.568714286*	0.077315	0	2.411912	2.725517
	3rd week	1.944857143*	0.077315	0	1.788055	2.10166
	17th week	2.003285714*	0.077315	0	1.846483	2.160088
	38th week	2.368428571*	0.077315	0	2.211626	2.525231
	52th week	2.445714286*	0.077315	0	2.288912	2.602517
17th week	Zero week	0.565428571*	0.077315	1.28E-08	0.408626	0.722231
	3rd week	-0.058428571	0.077315	0.454735	-0.21523	0.098374
	5th week	-2.003285714*	0.077315	0	-2.16009	-1.84648
	38th week	0.365142857*	0.077315	3.49E-05	0.20834	0.521945
	52th week	0.442428571*	0.077315	1.63E-06	0.285626	0.599231
38th week	Zero week	0.200285714*	0.077315	0.013751	0.043483	0.357088
	3rd week	-0.423571429*	0.077315	3.46E-06	-0.58037	-0.26677
	5th week	-2.368428571*	0.077315	0	-2.52523	-2.21163
	17th week	-0.365142857*	0.077315	3.49E-05	-0.52195	-0.20834
	52th week	0.077285714	0.077315	0.324167	-0.07952	0.234088
52nd week	Zero week	0.123	0.077315	0.120379	-0.0338	0.279803
	3rd week	-0.500857143*	0.077315	1.6E-07	-0.65766	-0.34405
	17th week	-2.445714286*	0.077315	0	-2.60252	-2.28891
	17th week	-0.442428571*	0.077315	1.63E-06	-0.59923	-0.28563
	38th week	-0.077285714	0.077315	0.324167	-0.23409	0.079517
*	The mean difference is significant at the 0.05 levels.					

Discussion

Chapter IV

Discussion

The treatment and control of bovine farcy in cattle by cattle owners was not successful. Messairiya thought that the veterinary services had mange major epidemic expect ticks and bovine farcy (M. Hamid, unpublished participatory Epidemiology data). Isolation and slaughtering of clinically affected cattle with hygienic precautions and suitable husbandry measures has been unsuccessful. Moreover, the tedious and laborious diagnosis of this disease notably the pulmonary form, has led us to investigate the possibility of an effective vaccination method.

The aim of this study was to attenuate *M. farcinogenes,* to test its virulent after attenuation and to monitor humoral antibodies in calves and guinea pigs by ELISA technique.

The challenge strain (*M. farcinogenes* SD117), which has been isolated from a case of bovine farcy, was examined for its colonial morphology and the mycolic acid pattern in TLC, and was found to be similar to the reference *M. farcinogenes* strain.

The vaccine strain *M. farcinogenes* A24 produced lesion in 8% of the vaccinated guinea pigs while 25% of the vaccinated calves developed local nodular lesions. This variation in guinea pigs and calves was attributed to the number of the animals in each group. The number of guinea pigs was higher than that of calves. Both calves and guinea pigs are susceptible to bovine farcy but we do not know the degree of susceptibility of each of them. Frerichs (1973) found that the concentration of the antigen determines the size

of the local reaction. In our experiment the vaccine dose for guinea pigs was half that of calves while Frerich (1973) gave 1/3rd the dose of cattle when he vaccinated guinea pigs with *M. jahnei.* These lesions were also seen in some mycobacteria such as *M. bovis* which produced tuberculin hypersensitivity reaction when injected intradermallay (William, 2001). These findings agreed with Saxegaarad (1985) who noticed local infiltration at the site of inoculation in goats vaccinated against paratuberculosis. In the present study on challenge infection we noticed that all the non-vaccinated calves and guinea pigs showed local nodules at the site of inoculation. The size of nodules was bigger comparable to that of the vaccinated group; this is because the non-vaccinated animals had not been exposed before to the antigen and hence severe local reaction occurred when subjected to the challenge infection.

Seventy five percent (75%) of the calves vaccinated with *M. farcinogenes* strain A24 were protected when exposed to challenge infection. These results do not agree with Hines *et al.* (1998), who used killed bacterin of *M. bovis* in swine, and found that the bacterin did not prevent infection but it reduced the severity of both gross and microscopic lesions. The same authors used conjugated MIF-A3 subunit vaccine, but the vaccine also failed to prevent infection and development of lesions.

Complement fixation test (CFT) and enzyme linked immuno-sorbent assay (ELISA) were used successfully as serological methods to test *M. paratuberculosis* (Babiker *et al.* 1983, El-Hussein, 2001). For this reason we used ELISA to detect antibody titres, because it is more sensitive and more specific. The sample was taken once from the vaccinated and non-vaccinated guinea pigs. The optical density (OD) was determined at 450nm according to

El-Hussein (2001), and was found to be 0.15 and 0.05 in vaccinated and non-vaccinated guinea pigs, respectively. This indicated that there was significant increase in antibody titre when guinea pigs were inoculated with *M. farcinogenes* A24 strain.

Sera samples were taken from experimental calves during a period of one year. The results showed that there was a considerable increase in the titre, which reached its maximum in the 5^{th} weak, and then it started to decline. This result does not agree with Gilmour *et al* (1971) who found that the peak of antibody titre in cattle infected with *M. avium* reached in the 8^{th} weeks. We think that this difference might be due to the use of different animal species and of course *M. farcinogenes* different in its antigenic and virulent factor from *M. avium.* Statistically, there was a significant difference between vaccinated and non-vaccinated calves using the T-test. ($P \leq 0.05$) (Table 3). This finding came in line with El Hussein (2001) who used humeral antibody titre to diagnosis bovine farcy.

To the best of my knowledge, there are no report in literatures concerning usage of vaccine against bovine farcy, but there is some debates in vaccination against some other mycobacteria. The killed bacterin of *M. avium* used by Hines (1998) in controlling swine paratuberculosis failed to prevent infection but it reduced the severity of gross and microscopic lesions. The same author used conjugate macrophage inhibitory factor –A3 (MIH-A3) subunit vaccine but it did not prevent infection or lesion development. However, the adjuvant vaccine with live *M. tuberculosis* offered a high degree of protection against paratuberculosis in goats (Saxegaard and Fodstad, 1985). It is well known that vaccination against paratuberculosis

sensitises animals both to mammalian and avian tuberculosis (Hole 1958, Blood *et al*., 1983). On the other hand the Bacilli-Calmette and Geurin (BCG) vaccine gives a positive tuberculin reaction in individuals when tested for several years after vaccination, this fact limits its usage for diagnosis. (William 2001), beside it is not recommended to be given to people with AIDS or other immuno suppressed individuals.

The present study concluded that *M. farcinogenes* A24 is potential vaccine, which can be used in the field given the 75% protection level. It is not yet known the duration of immunity due this vaccine therefore it is recommended to carry out field trial to determine the extent of the protection offered by this vaccine.

Conclusions

The present study concluded that:

- *M. farcinogenes* A20 was successfully attenuated in guinea pigs and lost its virulence to this animal after 4-5 passages.
- *M. farcinogenes* A24 protect calves from challenge infection with virulent *M. farcinogenes* SD117.
- Humeral antibodies, as detected by ELISA, monitor the immune response which have shown its maximum level at the 5^{th} week post vaccination.

Recommendations

1- Improvement of the quality of the vaccine by using some adjuvant.

2- Determination of the duration of the protective immunity of the vaccine in calves.

3- Determination of the shelf life of the vaccine.

4- Performance of a field trial for this vaccine.

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Appendices

Appendix (A)

Media

1-Lowenstein-Jensen Medium

Mineral Salt Solution:

KH_2SO_4	2.4g
$MgSO_4.7H_2O$	0.2g
Glycerol	120ml
Distilled water	600ml

Dissolve the ingredients in the water and autoclave at 110°C for 15 minutes.

Malachite green solution

Malachite green	0.2g
Distilled water	10ml

Mix and incubate at 37°C or place in water bath at 50-60°C to dissolve. Autoclave at 115°C for 10 min or sterilize by membrane filtration.

Egg mixture

Immerse twenty hen's eggs in 70%ethanol and leave for 15min, remove to a clean tray and allow drying. Break into a sterile jar containing glass beeds and shake well to mix the white and yolks. Strain through sterile gauze into a sterile beaker and add the mineral salt and malachite green solution .Mix

thoroughly and distribute in 5.0ml volumes in 30ml screw- capped (Universal or McCartney) bottles.

Screw- capped tightly, place the bottle almost horizontally in a hot-air oven and inspissate by a single heating, raise the temperature to 75°C and hold it between 75°C and 85°C for 45 minutes.

2-Glucose Yeast Extract Agar (GYEA) Medium (Gordon & Mihm, 1962).

- This medium was prepared by dissolving 10g powder of yeast extract, 20g powder of agar in 900ml of distilled water, and then boiled to dissolve completely by steaming at 100 °C.
- The pH was adjusted to 7.2± 2, then autoclaved at 121°C under 15 Ibs/in² for 15 min.
- 10g of glucose was suspended in 100ml distilled water, and adjusted to 7.2±2 and sterilized by autoclaved at 121°C under 5 Ibs/in² for one second added glucose to the previous medium and shaken gently and poured in 20ml amounts in sterile Petri dishes. And incubated in the incubator for 24 hours and then used.

Appendix (B)

Stains

1-Ziehl-Neelsen's Method (Barrow& Felthm, 1993).

-Ingredients:

-Staining method:

1. Flood the slide with strong Carbol fuchsine and heat until steam rises (but do not boil).
2. After 3-4 min apply more heat steam rises again, do not let the stain dry on the slide.
3. About 5-7 min after the first application of heat washes the slide thoroughly under running water.
4. Decolorize in acid-alcohol until all traces of red have disappeared from the film. Decolourization should not be attempted in one stage; there should be intermittent washings in water and re-application of acid-alcohol.
5. Wash well in water when Decolourization is complete.
6. Counter stain with Loeffler's methylene blue or0.5% malachite green for 1 minute.

Gram's stain

Gram's stain was done according to the method described by Barrow and Feltham (1993).

1. Fixed slides were put in the staining rack and flooded with Crystal Violet sfor ½ minute, then washed with water, iodine was poured and washed after one minute For decolourization, acetone was used for (2-3) seconds only, then the slide was flooded with water.

2.Slides were flooded again with dilute Carbol fuchsin for ½ minute, then with water and dried with filter papers, the dried slides were examined under the microscope using the oil immersion objective lens.

Appendix (C)

Reagents

Phosphate buffer saline (PBS)

PBS was used as diluents for both Antigen and sera, and as solvent for the washing solution. PBS, ready-made tablets (ICN Biomedical, Inc. 1263 South Chillico the Road, Aurora, Ohio 44202, 1-800-). Each tablet was dissolved in 100 ml sterile distilled water.

Tween 20

0.05 concentration of Tween 20 in PBS was used as washing solution.

Substrate p- nitro phenyl phosphate (p-NPP)

p-NPP was of choice for use with alkaline phosphates in ELISA procedures.It produces an insoluble yellow coloured product that can be read by spectrophotometer at 450 nm.

Diethanolamin buffer

Diethanolamin buffer was used as diluents for the p-NPP tablet (BDH Chemicals, Pool, England).

Anti–Bovine IgG

Anti-bovine IgG was prepared by adding 100µl of conjugate (No. A- 8025, Sigma) to 100ml of PBS.