Evaluation of attenuated bovine farcy vaccine; *M. farcinogenes* in zebu calves using serology, passive immunization and Plaque forming Cell Assay, Sudan.

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Aims: The aim of the present study was to prepare an attenuated bovine farcy vaccine and to evaluate its efficacy in zebu calves in South Darfur state, Sudan. Methodology: A total of 59 Mycobacteria isolates from cases of bovine farcy during 2007-2009 in South Darfur State, Sudan. The isolates were identified by conventional phenotypic tests in comparison to a 16S-23S rDNA PCR assay. *M. farcinogenes* (MF216) was serially attenuated 10 times 5 times in-vitro and 5 times in-vivo. the 10th passage was propagated in Souton’s broth and was considered as a candidate attenuated farcy vaccine (CAVF). The vaccine was tested in zebu calves and evaluated using serological, passive immunization and plaque forming cell assay. Results: Titration of circulatory antibodies induced by the live attenuated farcy strain MF216 in zebu calves using Heamagglutination test detected low antibodies titers after primary vaccination which clearly increased post the booster dose. ELISA cut-off values (P> 0.142) determined in this study also illustrated low circulating antibodies induced by the vaccine. Despite the low level of antibodies 2 of 3 quinea pigs passively immunized resisted challenge with fresh farcy strain MF304. Plaque forming cells (PFCs) were demonstrated microscopically using X40 lens as two cells for one reaction containing 20 µl effectors cells. Conclusion: An attenuated farcy vaccine produced low circulating immunoglobulins in zebu calves which had a significant protective role, hence it passively immunized quinea pig.

Keywords: *Live attenuated Mycobacterium farcinogenes*, bovine farcy, Acid fast filaments Darfur, Sudan.

INTRODUCTION

Control of mycobacterial infections in animals is applied in developed countries especially for bovine tuberculosis using Bacilli Calmette-Guerin (BCG) of cattle which offered the best long-term solution for controlling TB in National Herds (Hewinson *et al.*, 2003). More recent experimental studies with BCG have confirmed its potential to protect cattle to some degree against bovine TB by reducing disease severity and pathology (Wedlock *et al.*, 2003).
Intracellular immunogenic mycobacteria exhibit a cellular immunity which plays an important role in body defense (Thoen and Barlett, 2004).

Like other Mycobacterial infections of economical value, bovine farcy is an endemic non treatable disease in Africa caused by M. farcinogenes and M.sengelenses strains (Chamoiseau 1979 and El Sanousi et al., 1986). The pathological picture of bovine farcy resembles tuberculosis which leads to false carcass condemnations (Mohan, 1985 and El Sanousi et al., 1979a). Recently Farcy vaccine attempts were tried in zebu calves using an attenuated vaccine which showed a short duration period of antibodies titer elevated in 5th week post vaccination then declined (Eiman et al., 2005).

Guinea pigs are innately susceptible for mycobacterium infection because they have poor T-cell immunity, therefore pathogenic Mycobacteria produce disseminated caseation, hence they are considered the most suitable animal species for inducing iv-vivo attenuation of bovine farcy vaccine (Gupta and Katoch, 2005 and El Sanousi et al., 1979b and Tag Eldin et al., 1988).

METHODOLOGY

Identification of farcy field strains:

M. farcinogenes (MF) isolates from clinically infected animals and caseated bovine tissues collected from South Darfur abattoirs, were conventionally identified according to (Kent and Kubical 1985; Konemann 2005) and molecularly typed with M. farcinogenes (M39) reference strain (Hamid et al., 2002 and Roth et al., 1998). MF216, a field strain was used for preparation of a live attenuated candidate vaccine while, strain MF304, a fresh identified isolate was used for challenge of actively and passively vaccinated animals.

In-vitro antigen attenuation

Purified MF216 was subcultured in Tryptose Phosphate Broth (TPB) 2 times, recovered in LJ medium to check the purity of the cultures then passaged 3 times in the same medium for in-vitro attenuation.

In-vivo attenuation in Guinea pigs models

A loop full of MF216 colonies attenuated invitro was scraped, emulsified in 5 ml sterile normal saline containing about 0.5 g of sterile glass beads and vortexed for 5 minutes for homogenization. The homogenate was aseptically inoculated intraperitoniealy into Guinea pigs. Animals were kept and observed up to a month till dead or euthanized for necropsy and re-isolation according to (El Sanousi et al., 1979). At necropsy of Guinea pigs, injection sites, abdominal cavity, serous membranes, liver, spleen, lungs and kidneys were examined for the presence of tuberculous or caseaous lesions. Histopathology, microscopy and culture techniques were applied as described by Hewitson and Darby (2010) and Kent and Kubica (1985). After the 5th in-vivo passage, MF216 was recovered in pure culture, identified, characterized and repropagated in Souton’s broth and considered as a candidate attenuated vaccine.

Vaccination trials in zebu calves.

Seven zebu calves of 1.5 year age were selected from disease –free area (Khartoum area) and grouped into group A of 5 calves and B of 2 calves and were subcutaneously injected with 2ml of the candidate vaccine and NS as in table (1). Sera were collected for serological tests weekly up to 8th week post vaccination.
Evaluation of Live Attenuated Farcy Vaccine (LAFV)

Heamagglutination test (HAT)

Heamagglutination test was done according to method described by Ridell et al. (1982). In addition, twofold dilutions were made in buffered saline to titrate the sera with homologous reference strain antigen (M39). Agglutination was judged after 3 to 4 hr at 37°C and checked after overnight refrigeration.

Coating of Ag to Sheep RBCs (SRBCS)

SRBCs were collected in Alsever solution as 10% volume, washed three times in PBS (PH 7.4). Formalin killed Antigen (M39) was also washed in PBS (pH, 6.4) and an equal volumes of Ag and SRBCS were mixed and incubated in 37°C for 20 minutes before use.

HAT Procedure

1- 100µl sterile normal saline were dropped in dry cleaned glass test tubes.
2- 100 µl of test serum were serially diluted twofold in the normal saline up to 10 times and from the last tube 100 µl was discarded.
3- 100 µl of coated SRBCS were added to the diluted sera
4- The mixture was incubated at 37°C for up 4 hours then overnight in the refrigerator and examined visually or microscopically for RBCs agglutination.

Enzyme Linked Immunosorbent Assay (ELISA)

The test followed the method described by EL Hussain et al. (2009).

Control positives and negatives sera

Positive serum samples were collected in Nyala abattoirs from natural bovine farcy infected cattle which had clinical signs. The five negative serum samples were collected from Frsian cross breed calves from Khartoum north; a disease free-area. The samples were used for determination of cut-off point of ELISA to compare OD values of the tested sera.

ELISA test requirements

1- Polystyrene Microtiter plates (96 well) were coated with M. farcinogenes reference strain M39 in PBS.
2- 0.05 M carbonate and bicarbonate buffer (pH 9.6) was used as coating buffer for immobilization of Ag.
3- PBS-Tween-20 consisted of 0.2 M PBS (pH 7.2) containing 0.05% tween-20 was used as washing buffer.
4- Horse radish peroxidase enzyme conjugated with rabbit antibovine IgG was used.
5- Hydrogen peroxide (H₂O₂) was used as substrate to conjugate with TMB.

ELISA procedure

1- M39 antigen was suspended in carbonate bicarbonate (pH 9.6) coating buffer.
2- ELISA plates were coated with the antigen (disintegrated in PBS) and incubated overnight at 4°C.
3- Antigen-coated plates were washed extensively with washing buffer then blocked with 2% skimmed milk for 1 hour.
4- Serum samples diluted in PBS-Tween-20 (1µl:100ml) were then added to the coated wells and incubated for 1 hour at room temperature then washed extensively with PBS-T.
5- Rabbit anti-bovine conjugate diluted 1 µl per 1ml PBS-T was added to plates to bind with antibodies and incubated for 1 hour at 37C.
6- After washing 100 µl of substrate solution (TMB) were added to each well and the plates incubated for another 30 minutes at room temperature.
7- The reaction was stopped by addition of 100 µl sulphuric acid and measured by ELISA reader.

Determination of cut-off value

The absorbance at 450nm was measured using an ELISA reader (Bio-Rad, USA). The ELISA cut-off value which would serve as threshold between the positive and negative sera samples was determined either by the mean value obtained when testing negative control sera plus two-fold standard deviation.

Passive immunization of guinea pigs

Antibodies concentration using Poly Ethylene Glycol (PEG):

1- A twenty centimeter dialysis membrane (MWCO12-14000 daltones, from Medical International Ltd, London.) was flooded with warm Disteld Water (DW) for 1/2 hour.
2- The membrane tube was washed three times with sterile DW and 20 ml of sera collected from vaccinated calves were pipeted into the dialysis tube. The membrane extremities were well tied, lubricated with polyethylene glycol (extremely covered), placed on clean dry tray and incubated at refrigerator for up to 2 hours.
3- Concentrated antibodies were aseptically harvested in sterile tube and immediately 2ml were inoculated into 3 guinea pigs intraperitonealy.

4- Animals were challenged with 1 ml of new isolated *M. farcinogenes* (MF304), the animals were kept and observed for the development of any pathological lesions for up to one month.

5- Finally the animals were euthanized, necropsied and careful searched for naked-eye lesions which were collected for conventional Mycobacteriology and histopathology studies.

Plague forming cell assay (PFCA):

The test done according to Jerne et al., (1963) and Nsreen, (2009). SRBCs were collected in 10% Alsever’s solution washed three times in PBS (pH 7.4) and once in balanced salt solution BSS "Hanks Solution". SRBCs were diluted (1: 3 PBS) and washed three times with PBS and resuspended as 20% volume. Equal volumes of 10% SRBCS and tannic acids solution (5mg tannic acid in 100ml PBS) were mixed and incubated at 37°C for 20 minutes. The combination was then centrifugated three times in NS containing 0.5% heat inactivated normal rabbit serum at (56°C) for washing. Spleen effectors cells were prepared and counted from a vaccinated calf weighted spleen. The cells were leaked through wire mesh to obtain splenic cells. The cells were washed three times in BSS and resuspended as 10% volume in BSS. Guinea pigs serum was diluted in (1:3 BSS) as complement source.

**Procedure**

Three hundred microliter of agarose (0.5% in BSS), 20 µl of Ag coated TSRBCs, 100 µl spleen cell suspension and 10 µl diluted guinea pigs serum (1:3 BSS) were mixed and poured on microscopic slides, incubated at humid incubator overnight and examined microscopically under 10X lens for observation of PFC. 300 µl of agarose.

**RESULTS**

Pathogenecity of *M. farcinogenes* (MF216).

Intraperitonieally inoculated Guinea pigs with MF216 were observed for up to 30 days. The necropsy findings are recorded in table (2) and Fig: 1 (A,B,C,D and F). The causative bacterium was microscopically demonstrated from the lesions using ZN stain as acid fast branching filaments. Culturing of the lesions reproduced slow growing yellow colonies which contained acid fast filaments that were identified as *M.farcinogenes* MF216 Figure 2. The 10th passage of MF216 (in-vitro and invivo) on Souton’s broth produced luxurient growth, microscopically confirmed as pure acid fast filamentous and considered as a candidate life attenuated farcy vaccine; CAFV.

**Calves vaccination**

**Vaccine efficacy in calves**

Local immune response of CAFV in all 5 calves which had been vaccinated showed local swelling of the inoculation skin site from the first week (day1) of vaccination, while 2 calves which received normal saline did not show any local skin reactions Figure (4).

Challenge of vaccinated and none vaccinated calves with 2 ml of fresh cultured *M. farcinogenes* (MF304) at the opposite middle neck site produced a slight skin thickening at the inoculation sites while, the non vaccinated two calves exhibited obvious skin swelling during the first week of challenges and the swelling increased in size day by day in contrast to the vaccinated calves Figure (5).

**Serological tests**

**Heamagglutination test (HAT)**

The antibodies titer of vaccinated calves using Heamagglutination test was well visualized throughout the vaccination period and clearly increased post the booster doses as shown in Table (3). Slide HAT confirmed the clumping of antigen coated sheep red blood cells (ARBCs) and serum antibodies of serial two fold dilutions Figure (6).

**ELISA OD values of immunoglobulines-G raised in zebu calves**

The CO value was obtained from OD values of control negative sera from disease-free area (Khartoum North) and the value was determined as 0.142. In this study the sero- positive titers were considered above the cut-off value. The OD values of immunoglobulin-G of vaccinated, non vaccinated calves, control positives (natural farcy infections) and control negatives from farcy free area sera are shown in table (4). The cut-off values (P> 0.142) determined in this study illustrated low circulatory antibodies induced by live attenuated farcy strain MF216.
Figure 1: Necropsy, microscopy and histopathology of the infected guinea pigs with MF216 strains.
A: showing skin suppuration and abdominal mass, passage No1.
B: showing mesenteric and portal lymphatics tubercles in passage No.1.
C: showing multiple liver lesions observed in passage No.2.
D: showing more severe granulations in the diaphragm in passage No.2.
E: acid fast filamentous bacteria prepared from lesion No.B
F: liver histograph showing diffused granulomations and giant cell formation

Table (2). Necropsy findings following inoculation of attenuated MF216 in guinea pigs

<table>
<thead>
<tr>
<th>Anim.No.1</th>
<th>Necropsy findings</th>
</tr>
</thead>
</table>
| GP₁       | Skin and abdominal suppuration  
Tubercles in: omentum, peritoneum, mesenteric lymph nodes  
Liver and diaphragm |
| GP₂       | Abdominal granulomations & suppuration  
Tubercles in:  
Omentum & peritoneum  
Diaphragm**,  
mesenteric LNs***  
Kidney  
Multi-tubercles in liver*** |
| Gp₃       | Skin and abdominal suppuration (10 days)  
Visceral tubercles* |
| GP₄       | Skin suppuration (14 days) without any other visceral lesions. |
| GP₅       | Week1: skin swelling and suppuration  
Week3: tests swelling  
Week4: death. |

Key: Gp: Guinea pig passage No.  
MF: M. farcinogenes strains  
AFF: Acid Fast Filaments  
***: Severity of infection compared to passage (1) lesions.
Figure 2: *M. farcinogenes* (MF216) 5\(^{th}\) in-vivo passage culture and acid fast filaments from the culture.

Figure (4). Vaccinated calves A, B & C: animal No 2, 3 & 5 vaccinated calves showing local swelling at injection sites. D: animal No 7; control group; no local swelling was observed at injection site (normal saline inoculum).

Figure (5). A&B: animal No 7 & 8 non vaccinated calves showing local swelling at challenge sites. C&D: animal No 2 & 4 vaccinated calves showing slight skin thickening at challenge sites.

- Arrow No 1: initial vaccine dose (Picture C)
- Arrow No 2: booster vaccine doses (Picture C & D)
- Arrow No 3: challenge dose (all pictures)
Table (3). Mean antibody titers of HAT of two fold serum dilutions from vaccinated calves.

<table>
<thead>
<tr>
<th>Titration/days</th>
<th>D₀</th>
<th>D₇</th>
<th>D₁₄</th>
<th>D₂₁</th>
<th>D₂₈</th>
<th>D₃₅</th>
<th>D₄₂</th>
<th>D₄₉</th>
<th>D₅₆</th>
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</thead>
<tbody>
<tr>
<td>Mean A</td>
<td>6</td>
<td>7</td>
<td>28</td>
<td>32</td>
<td>22</td>
<td>18</td>
<td>30</td>
<td>44</td>
<td>72</td>
</tr>
<tr>
<td>Mean B</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

Figure (6). Diagram of Heam agglutination test showing agglutination of antigen coated sheep RBCs against antibodies from vaccinated calves serum of day 56. Slide 2, 4 represent vaccinated group.

Table (4). ELISA titers of diluted sera (1/100) of zebu calves vaccinated with CAFV.

<table>
<thead>
<tr>
<th>No</th>
<th>Control sera</th>
<th>Vaccinated group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C+ve</td>
<td>C-ve</td>
</tr>
<tr>
<td>1</td>
<td>0.146</td>
<td>0.089</td>
</tr>
<tr>
<td>2</td>
<td>0.132</td>
<td>0.119</td>
</tr>
<tr>
<td>3</td>
<td>0.157</td>
<td>0.124</td>
</tr>
<tr>
<td>4</td>
<td>0.146</td>
<td>0.130</td>
</tr>
<tr>
<td>5</td>
<td>0.133</td>
<td>0.108</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: C+ve: sera from Natural farcy infected cattle.
C-ve: sera from farcy-free cattle.

No internal lesions were observed at necropsy of vaccinated and non vaccinated calves two month post vaccination.

Passive immunization of guinea pigs

Tow of 3 Guinea pigs passively immunized with the concentrated antibodies resisted challenge with fresh bovine farcy isolate (MF304). The third one showed a small pyogenic lesion at the site of injection without any internal lesions fig. (7).

Plaque forming cells assay

The Plaque forming cells (PFCs) were demonstrated microscopically using (X40) lens as two cells for one reaction containing 20 µl effectors cells because most of gel had dried Fig. (8). Hence the total plaque (plasma) cells were estimated as 45X10³ PFC/ spleen and the total nucleated spleen cells were estimated as 132X10³/ml³.
DISCUSSION

Serial 10th in-vitro and in-vivo passages of farcy field strain (MF216) revealed severe pathogenic strain fatal for Guinea pigs in the last passage without any visceral suppurations except testicular swelling. Our results were come in line with El Sanousi et al. (1979a) who described assending pathogenecity of farcy agent by serial passage in guinea pigs. Preparation of LAFV in Souton’s broth was found to produce luxurient growth which showed local immune response and this may refer to good growth of CAFV strain achieved in such broth when compared with the response in claves inoculated with normal saline as control group.

The booster doses injected to vaccinated group at day 14 showed slight swellings or skin thickening at the sites of injection less than the swelling produced by initial dose. Challenge of vaccinated calves with fresh isolated farcy strain (MF304) revealed slight reaction or swelling less in size than the initial CAFV inoculation sites and this might be indicating for arising resistance due to previous sensitzation of the immune system in comparison with the response of non vaccinated calves which showed increasing swelling weekly at the sites of challenges inoculation.

Titration of the circulatory antibodies of the vaccinated calves using Heamagglutination and ELISA tests showed moderate level of antibodies titers throughout the vaccination period. The present results to some extent differ from that obtained by Manal et al. (2005) whom reported high antibodies titers in 5th week of vaccination then the antibodies decreased and completely disappeared in the 10th week post vaccination and this may refer to the booster dose we applied after 2week of the initial dose. Inspite of low titers of circulatory antibodies in vaccinated calves PFC demonstrated in this study resulted in circulating immunoglobulins in vaccinated calves justified the resistance of Guinea pigs passively immunized with concentrated immunoglobulins and challenged with fresh MF304 field strain. These finding may drew attention to the role of humoral immunity against the mycobacterial challenge in using laboratory animals and the guinea pigs per se as a model. Moreover, the cellular immunity which is induced by intracellular immunogenic organism plays the important role in body defense against pathogenic mycobacteria (Thoen and Barlett 2004).

REFERENCE

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