Efficiency of inactivated vaccines against heartwater in Burkina Faso: Impact of *Ehrlichia ruminantium* genetic diversity

Hassane Adakal[a], Frédéric Stachurski[a,b], Maurice Konkobo[a], Sébastien Zoungrana[a], Damien F. Meyer[c], Valérie Pinarello[c], Rosalie Aprelon[c], Isabel Marcelino[d], Paula M. Alves[d], Dominique Martinez[b,c], Thierry Lefrancois[c], Nathalie Vachiéry[c,*]

[a] CIRDES (Centre International de Recherche-Développement sur l'Elevage en zone Sub-humide), BP 454, Bobo-Dioulasso 01, Burkina Faso
[b] UMR 15 CIRAD-INRA « Contrôle des maladies animales exotiques et émergentes », TA 30/G, Campus international de Baillarguet, 34398 Montpellier Cedex 5, France
[c] UMR 15 CIRAD-INRA « Contrôle des maladies animales exotiques et émergentes », Site de Duclos, Prise d'Eau, 97170 Petit Bourg, Guadeloupe, France
[d] IBET (Instituto de Biologia Experimental e Tecnologica), Oeiras, Portugal

ARTICLE INFO

ABSTRACT

In order to identify the appropriate strains to use in vaccination trials against heartwater in Burkina Faso, the protective effect of Gardel and Welgevonden strains was assessed against local strains on sheep vaccinated by infection-and-treatment method: Gardel protected significantly against Burkina Faso strains tested (survival rate 59% for immunised sheep vs 13% for control sheep) while Welgevonden did not (survival rate 45% for immunised sheep vs 25% for control sheep). The efficacy of the ISA50 inactivated vaccine, produced under industrial process, was evaluated in sheep during field challenges in two successive years. During year 1, there was a limited protective effect of the Gardel vaccine with 65% of survival rate for the vaccinated group compared to 49% for the control group ($N = 153$, $p = 0.053$). During year 2, the vaccine containing Gardel and a local strain gave an increased protective effect compared to the first trial: 72% of the vaccinated animals survived compared to 47% of the naïve animals ($N = 173$, $p < 0.001$). There was an important genetic diversity of strains in the field with detection of 11 different *map1* genotypes in brains from control and vaccinated sheep post mortem. *Map1* genotyping of strains detected in brains from control sheep showed that genotype distribution varied according to time and study areas, which could explain the difference in efficacy of the vaccine.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Heartwater is a tick-borne rickettsial disease of wild and domestic ruminants caused by *Ehrlichia ruminantium* (*ER*) and transmitted by *Amblyomma* tick species. It is prevalent in Sub-Saharan Africa, in many islands of the Indian Ocean (Madagascar, Réunion, Mauritius and the Comoros) and in some Caribbean islands (Antigua, Guadeloupe and Marie Galante). The presence of heartwater in the West Indies constitutes a threat for North and South America where ruminants are susceptible and where potential vectors are present [1]. In Africa, heartwater has an important economic impact, decreasing livestock production and causing high mortality in susceptible herds [2].

Various but unsatisfactory means are available to protect susceptible animals in endemic areas. The control of the tick vectors is very expensive and usually inadequate or insufficient to prevent transmission. Vaccination methods include infection and treatment, attenuated, DNA and inactivated vaccines [3]. The only commercially available method, used in South Africa since decades, is the infection-and-treatment method which consists in inoculating intravenously virulent Ball 3 strain of *ER* (blood of an infected animal or tick homogenate) and subsequently treating with tetracycline. This method is risky (possible transmission of other pathogens and possible loss of animals), expensive (cold chain required for storage, close monitoring of animals during 2–3 weeks) and thus inadequate in low-input farming systems. Attenuated vaccine of the Senegal strain displayed adequate protection against homologous challenge but poor protection against heterologous strains or field challenge [4,5]. The possible reversion to virulence of the attenuated strain is one of the limitations for the use of such vaccines. Moreover, as for any live vaccine, cold chain storage in liquid nitrogen is necessary. However, live vaccines (attenuated and infection-and-treatment method) offer the advantage of low morbidity with no clinical signs or only transient fever following lethal challenge [6,7].

Inactivated vaccines have various advantages: they contain killed *ER* organisms and their storage conditions are compatible

* Corresponding author at: UMR 15 CIRAD-INRA, Domaine de Duclos, 97170 Petit Bourg, France. Tel.: +590 590 25 54 42; fax: +590 590 94 03 96.
E-mail address: nathalie.vachiery@cirad.fr (N. Vachiéry).

with field use (−20 °C or refrigerated). The inactivated vaccine using killed *ER* emulsified in oily adjuvant (ISA 50) was tested in experimental and field conditions [8–12]. It conferred a good protection of animals challenged with homologous and some heterologous strains. The protection against homologous lethal challenge was 80–100% on animals vaccinated with Gardel inactivated vaccine [11,12] and there was a two-fold reduction of mortality on animals immunised with Mbizi inactivated vaccine after field tick challenge in Zambia and Zimbabwe [9]. In contrast with live vaccine, inactivated vaccine requires two injections and induces a higher morbidity with a long period of hyperthermia after lethal infection.

Whatever the type of vaccine used (inactivated, recombinant or live vaccines), its efficiency is limited due to the genetic and antigenic diversity of *ER* [5,6,9,13]. Animals protected with a specific strain would not be systematically protected against all heterologous strains. The use of a cocktail of strains included in the inactivated vaccine would be a solution to improve the efficiency in the control of heartwater.

The protective dose of *ER* inactivated vaccine was recently markedly reduced from 1 mg to 35 μg of antigen [12]. At the same time, the optimisation of *ER* vaccine production in stirred tanks and identification of purification process, optimal buffer and storage conditions were successfully done, demonstrating the feasibility of the industrial production of the vaccine [11,14]. The results from both studies have led to a drastic reduction of the cost of the vaccine, making it suitable especially in developing countries in Africa.

One main question that remains to be answered is whether the inactivated vaccine produced using an industrial process is efficient in field conditions. Moreover, the impact of the *ER* genetic diversity on the efficiency of this vaccine has to be determined. For that purpose, two field trials using inactivated vaccine were implemented in successive years in South West of Burkina Faso (West Africa) in a heartwater-endemic area where the vector is *Amblyomma variegatum* [15]. Farmers from this region regularly attempt to introduce Sahelian sheep from the north of the country to improve the productivity of their herds. However, these attempts are unsuccessful due to important mortality caused by heartwater. The Gardel strain isolated in Guadeloupe, was the only strain used in the first trial, whereas, an additional local strain was included in the vaccine for the second year field trial. Simultaneously, genotyping of *ER* strains present in animals which died in both vaccinated and control groups, was carried out.

2. Material and methods

2.1. Animals

Sahelian sheep, 12–18 months old, purchased in the *A. variegatum*- and heartwater-free Dori area, in the North of Burkina Faso, were used for isolation of strains, cross-protection experiments and field vaccination trials. They were dewormed and vaccinated against *peste des petits ruminants* (produced and supplied by CIRAD, Montpellier) and pasteurellosis (Pastovax, Lanavet, Cameroon). All animals were tested for antibodies to *ER* using the indirect MAP1-B ELISA [16]. Only sero-negative sheep were included in the experiments. Cross-protection experiments and *ER* strain isolations were implemented in CIRDES facilities, located in Bobo-Dioulasso, Burkina Faso where animals were kept in cowshed preventing thus any tick infestation and natural *ER* infection.

2.2. Isolation of local Ehrlichia ruminantium stocks

The isolation of Burkina Faso *ER* strains was achieved as follows. Engorged nymphs collected in the study area during the beginning of the dry season were kept for moulting in controlled conditions at CIRDES. The study area was localised in the Banankeledaga research station (4.33°W, 11.32°N, 20 km North of CIRDES) and in four villages: Bekuy (3.91°W, 11.60°N), Sara (3.83°W, 11.71°N), Lamba (4.05°W, 11.66°N) and Bankouma (3.26°W, 11.88°N). After moulting at 27 °C with 85–90% of relative humidity, adult ticks (10 males and, 5 days later, 10 females) were placed to feed on susceptible Sahelian sheep. At hyperthermia onset (T > 40.5 °C), blood was collected from the sheep and cultured for *in vitro* isolation of *ER* on bovine aorta endothelial cells until the appearance of lysis as previously described [17]. Fresh blood from these sheep was also either immediately injected intravenously to animals for challenge (2 ml per sheep) or supplemented with 10% DMSO before snap freezing in liquid nitrogen for further use.

2.3. Cross-protection experiments in controlled conditions: evaluation of the heterologous protective effect of Gardel and Welgevonden strains

The protective effect of *ER* Gardel (from Guadeloupe) and Welgevonden (from South Africa) isolated as described elsewhere [18], was evaluated against Burkina Faso local strains. Gardel and Welgevonden strains were grown in bovine endothelial cells in Guadeloupe at CIRAD before sending to CIRDES. These strains have two different *map1* genotypes defined as Gardel and Welgevonden genotypes respectively. Infective material (fresh blood) was prepared from two Sahelian sheep inoculated with infected cell culture supernatants either from Welgevonden (passage 14) or from Gardel (passage 33) strains. Infected blood collected at hyperthermia was frozen in 10% DMSO and stored in liquid nitrogen. Whenever needed, it was then thawed and injected intravenously to sheep: 11 animals were infected with the Welgevonden strain and 12 with the Gardel strain. One or 2 days after the hyperthermia onset, animals were treated with long acting oxytetracycline.

The Burkina Faso local strains, Lamba 465, Sara 292, Bekuy 242, Bankouma 421, Sara 445 and Lamba 479, isolated as described above, were used to evaluate the protective effect of Gardel. Lamba 465, Bankouma 421 and Sara 445 were used for heterologous challenges on Welgevonden vaccinated sheep. These strains were recently *map1* genotyped by sequencing of *map1* PCR products [19]. All are Senegal *map1* genotype except Lamba 479 which is 97% identical to Kiswani *map1* genotype and they were all different from Gardel and Welgevonden *map1* genotypes.

Heterologous challenges were carried out 4–9 weeks later using fresh or stored infective blood from animals experimentally infected with local strains. Four control animals (naïve for *ER*) were simultaneously infected with each of these local strains.

2.4. Antigen preparation and vaccine formulation

Vaccine doses were produced by IBET in stirred conditions as previously described [14]. The vaccine used during the first field trial contained only Gardel strain, whereas equal proportion of Gardel and Bekuy 242 strains were present in the vaccine used for the second trial. Each dose contained 375 μg of *ER* antigens (in 1 ml of PBS) assessed using BCA technique (Bicinchoninic acid, Pierce, Brebières, France) according to the manufacturer's instructions. After overnight inactivation at 4 °C with 0.1% sodium azide, the vaccine was stored (−20 °C). Whenever necessary, the vaccine doses were thawed and emulsified 1:1 in Montanide ISA 50 (Seppic, Paris, France) in the field before subcutaneous injection [20]. PBS emulsified with ISA 50 was used to inoculate control animals.

Table 1
Field trials.

	Banankeledaga	Bekuy	Lamba	Sara
Year 1				
Vaccinated animals (*N*)	19	11	16	19
Control animals (*N*)	46	11	14	17
Year 2				
Vaccinated animals (*N*)	40	18	12	12
Control animals (*N*)	48	18	12	13

N: number of animals.

2.5. Field vaccination trials

2.5.1. Vaccination of Sahelian sheep

Sahelian sheep were vaccinated and boosted 4–5 weeks later with the inactivated vaccine in *Amblyomma* and heartwater-free Dori area. Table 1 summarizes the data regarding the number of sheep involved in the two trials. The primo-injection occurred on the 30th or 31th August 2002 (first trial) and on the 16th or 17th September 2003 (second trial). Two weeks after boosting, the vaccinated animals were dispatched to the Banankeledaga research station and to three villages (Bekuy, Sara and Lamba, described in Section 2.2) where they were kept with flocks of local Djallonke sheep. Vaccinated animals arrived in the study areas at the end of October, 2–3 weeks after the onset of *A. variegatum* nymph infestation period, and 1–3 weeks before the infestation peak. The studies were carried out during the first half of the dry season because it was previously noticed (unpublished observations) that sheep infestation by adult ticks during the rainy season is very low, preventing the vast majority of the animals from being exposed to the disease at that period.

2.5.2. Monitoring of sheep during vaccination trials

On the station as well as in the village flocks, when clinical signs appeared (reluctance to move or difficulty to stand up), rectal temperature of the sick animal was checked. Brains of the dead sheep were collected and part of each brain was used to prepare brain smears and part was stored at -20 °C or in ethanol for further molecular analysis of the *ER* strains.

2.5.3. Tick counts

A. variegatum infestation was determined once a week (interval between two counts: 6–8 days) by counting all ticks attached to animals randomly chosen in the different flocks. During both field trials, 20 animals were examined at Banankeledaga station. In other areas, 6–12 sheep (year 1) and 4–8 sheep (year 2) were examined in the village flocks according to flock size. The surveys were ended when the infestation had become very low (less than one nymph per sheep during the first study and less than two nymphs per sheep for the second one). The total number of infesting nymphs was assessed taking into account the mean engorgement period of *A. variegatum* nymphs (6.25 days, unpublished data) and the interval between two successive tick counts. The increase of tick infestation per day (Δ) was calculated using the number of ticks counted at two successive counts (*I*) following the formula: $\Delta = [I(n+1) - I(n)]/[\text{Day}(n+1) - \text{Day}(n)]$. For each day (*x*) between *n* and *n* + 1, we calculated the number of ticks (Nt), $\text{Nt}(\text{day}(x)) = I(n) + x \times \Delta$. The total number of infesting nymphs was the sum of Nt calculated for each day divided by mean engorgement period (6.25 days).

2.5.4. Antibody response to vaccination and field challenge

During the first trial, sera were collected from all sheep before the primo-vaccination, one month after this injection and two weeks after the boost, when the animals were brought from the heartwater-free area. After 2 months of field exposure, sera were collected from surviving control animals. During the second trial, sera were collected before the primo-injection and two weeks after the boost.

Detection of MAP1 antibodies was done on serum samples using indirect MAP1-B ELISA with a cut-off point of 20% positivity [16].

2.5.5. Diagnosis of heartwater and Ehrlichia ruminantium strain typing

The diagnosis of heartwater was done by microscopical examination of Giemsa-stained brain crush smears for presence of *ER* rickettsiae in capillary endothelial cells [21] and by *pCS20* nested PCR on brain samples [22]. DNA was extracted from brain using the Qiamp DNA extraction kit following the manufacturer's instructions (Qiagen, GmbH, Hilden, Germany). The nested *pCS20* PCR was used as previously described [22]. Briefly, AB128 (5′-ACTAGTAGAAATTGCACAATCTAT-3′) and AB130 (5′-ACTAGCAGCTTTCTGTTCAGCTAG-3′) primers were used on 200ng/μl of total DNA for first PCR at annealing temperature of 50 °C, then AB128 and AB129 (5′-TGATAACTTGGTGCGGGAAATCCTT-3′) primers were used for second PCR at 55 °C on 1 μl of the first PCR product. All samples were also tested by *map1* nested PCR as previously described [19]. Digestion of PCR products using restriction enzymes including TaqI, RsaI and MspI, allowed the characterization of *map1* genotypes [22]. Some of the *map1* PCR products were sequenced and compared with *map1* sequences from known isolates. For some samples, we detected two or three strains per animal. Thus, the incidence of each *map1* genotype was calculated as a percentage of the total number of *map1* genotypes within the sample group.

2.6. Statistical analysis

Survival rates and *map1* genotype distribution between groups were compared using the Chi-square test. Difference was significant when *p* values ≤0.05.

3. Results

3.1. Protective effect of Gardel and Welgevonden strains against experimental challenge with Burkina Faso strains

Following the heterologous challenge with Burkina Faso strains belonging to the Senegal *map1* genotype, the survival rate was 45% (4/11) in the Welgevonden immunised group whereas 25% (3/12) of the control animals survived (*p* = 0.30) (Table 2). There was no significant difference in incubation period and intensity of fever between control and groups immunised by infection-and-treatment method ($T_{\text{mean}} = 41.4 \pm 0.5$ °C for control group and $T_{\text{mean}} = 41.2 \pm 0.6$ °C for immunised group). Welgevonden strain conferred a different level of protection against each heterologous strain (between 25% and 67% survival).

Results of protective effect of Gardel strain against heterologous challenges are presented in Table 3. The survival rate was significantly higher in the vaccinated animals (59%, 17/29) than in the control group (13%, 3/24) (*p* < 0.001). Depending on the strain used for challenge, the percentage of survival varied from 100% to 20% for immunised group whereas none to 25% of the control animals survived (Table 3). Gardel strain conferred the best protection against Sara 445, Bankouma 421 and Lamba 479 strains with more than 67% of survival. The level of protection against strains in the Senegal *map1* genotype cluster varied widely.

Table 2
Protective effect of Welgevonden strain against experimental challenge with Burkina Faso strains.

Challenge strain	map1 genotype	Control group Survival rate (%)[*]	Vaccinated group Survival rate (%)[*]
Sara 445		1/4 (25%)	2/3 (67%)
Bankouma 421	Senegal	1/4 (25%)	2/4 (50%)
Lamba 465		1/4 (25%)	1/4 (25%)
Overall survival		3/12 (25%)	5/11 (45%)

[*] Survival rate=Number of surviving/Total number of animals.

Table 3
Protective effect of Gardel strain against experimental challenge with Burkina Faso strains.

Challenge strain	map1 genotype	Control group Survival rate (%)[*]	Vaccinated group Survival rate (%)[*]
Sara 292	Senegal	0/4 (0%)	1/5 (20%)
Bekuy 242		0/4 (0%)	3/6 (50%)
Lamba 465		1/4 (25%)	2/4 (50%)
Bankouma 421		1/4 (25%)	3/4 (75%)
Sara 445		1/4 (25%)	4/4 (100%)
Lamba 479	97% Kiswani	0/4 (0%)	4/6 (67%)
Overall survival		3/24 (13%)	17/29 (59%)

[*] Survival rate=Number of surviving/Total number of animals.

3.2. Field vaccination trials

3.2.1. Protective effect of the inactivated vaccine during field challenge

Each field trial lasted four months with first deaths observed 14 (trial 1) and 19 days (trial 2) after introduction of the sheep in the flocks. Up to 82% of deaths occurred during the next 30 days while 18% were observed during the following month (data not shown). Of the total number of animals exposed to ER during the field trials, 72 of the 153 sheep died during the first field trial, and 71 of the 173 animals during the second one.

ER colonies were observed in the brain smears of 54 (75%) and 55 (77%) of these animals, for the two studies respectively. Seventy out of seventy-two brain samples were analysed by PCR during the first year. Sixty-eight out of seventy sheep (97%) were positive for pCS20 target region and 67 (96%) for map1 gene. During the second field trial, all the 71 brains were found positive when tested with both PCR tests. Altogether, these results suggested that at least 140 out of the 144 dead animals were infected by ER and died from heartwater. From one year to another, the percentage of survival in the control group overall was similar: 49% vs 47% ($p = 0.83$). However, some differences were observed between areas and a significant 5-fold decrease of the survival rate was observed in Bekuy village between the two successive years from 55% to 11% ($p = 0.01$).

During year 1, the overall survival rate was 65% (42/65) for vaccinated animals whereas 49% (43/88) of the control animals survived ($N = 153$, $p = 0.053$). The protective effect of the vaccine was significant in Lamba village (50% of survival rate for vaccinated animals compared to 14% survival for control group; $N = 30$, $p = 0.04$) and in Banankeledaga station (89% of survival for vaccinated sheep compared to 63% for control sheep; $N = 65$, $p = 0.03$) (Fig. 1).

During year 2, the protective effect of the vaccine containing both Gardel and Bekuy 242 strains was significant with an increase of the overall survival rate of the vaccinated sheep (72%) in comparison with the control animals (47%; $N = 173$, $p < 0.001$). There was a significant protective effect of the vaccine in Bekuy village ($N = 36$, $p < 0.01$) and in Banankeledaga station ($N = 88$, $p < 0.05$) with
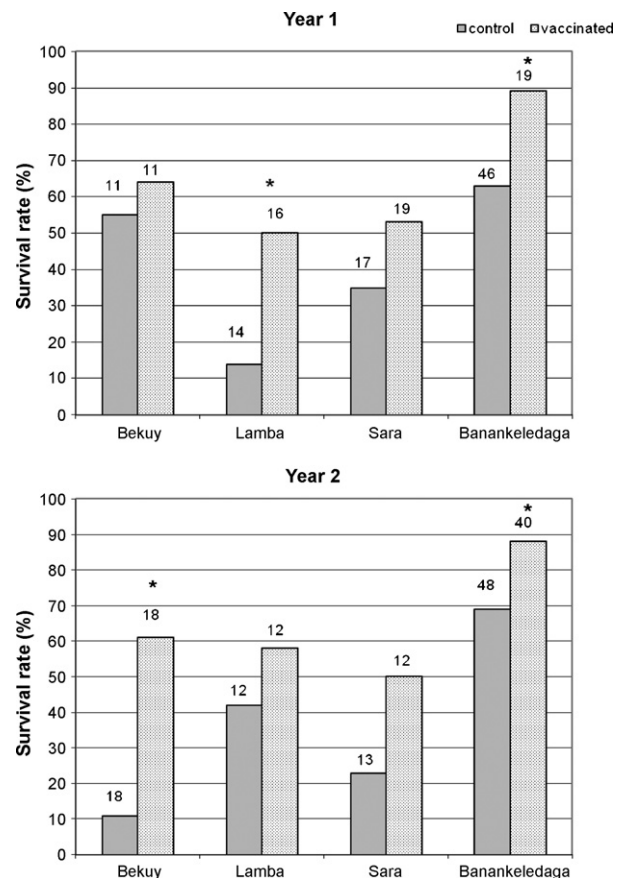

Fig. 1. Effect of single-strain and double-strains inactivated vaccine on the survival rate of animals in four areas in Burkina Faso. Number above the columns corresponds to the number of animals within control and vaccinated groups for each area. *Significant protective effect of the vaccine ($p < 0.05$).
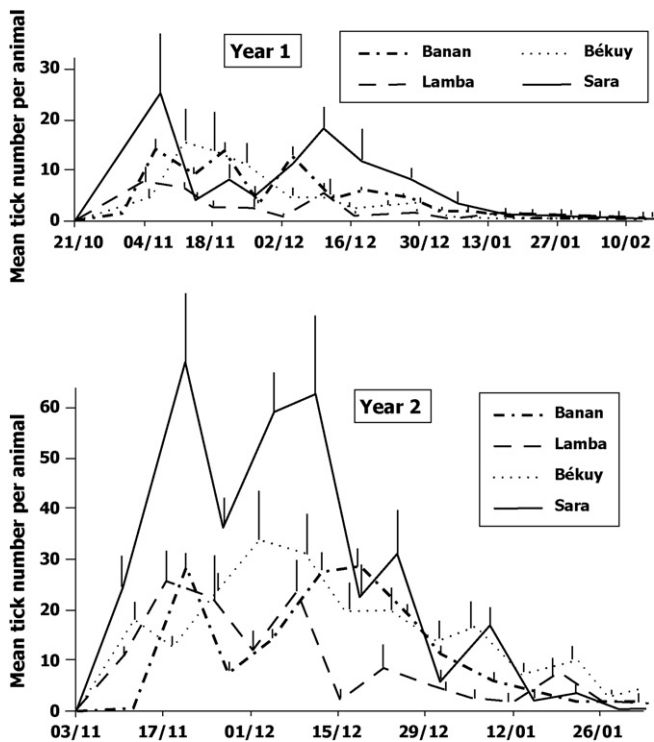
Fig. 2. *Amblyomma variegatum* nymph infestation during the two field studies. Vertical bars indicate standard error.

a marked increase of survival in vaccinated groups compared to control groups (Fig. 1). In the two other villages, Lamba and Sara, the survival rate differed between control and vaccinated groups from 42% to 58% ($p = 0.41$) and from 23% to 50% ($p = 0.16$).

3.2.2. Tick infestation and MAP1 seroconversion

Sahelian sheep were infested by *A. variegatum* nymphs as soon as they were placed in the different flocks. During year 1, the infestation peak was observed during the first (in Sara and Lamba villages) or the second (in Bekuy village and Banankeledaga station) tick count (Fig. 2). During year 2, the infestation peak was observed 1–3 weeks later compared to first year (Fig. 2). In year 1, the average sheep infestation peaked between 8 and 20 ticks, according to the flock; it reached 26–62 nymphs per animal during the second field trial. After two months, the infestation steadily decreased and at the end of the trial, each sheep was infested with less than two nymphs. On average, each animal was infested by 84 ($\pm$38) nymphs during the monitoring period of the first field trial, and by 237 ($\pm$115) nymphs during the second study. In Sara and Lamba villages, the mean number of ticks per animal was almost three-fold higher at infestation peak in year 2 than in year 1 whereas it doubled in Bekuy village and Banankeledaga station. The infestation varied dramatically according to the flock and the animal. For the Banankeledaga flock for example, the nymph burden varied from 3 to 32 ticks among sheep examined at the infestation peak of the first trial, and from 7 to 83 ticks at the infestation peak of the second one. Nineteen *A. variegatum* adults were counted in all on the 40 animals monitored in Banankeledaga during the two trials (i.e. less than 0.5 adult per sheep for the 3.5–4 month survey) and 14 adults for the three villages during the two surveys.

During the first field trial, the average antibody level (percentage of a positive control) of the vaccinated sheep increased from 4% ($\pm$1%) before the primo-injection, to 63% ($\pm$6%) before the boost, and to 89% ($\pm$5%) 2 weeks later. Two out of the 65 animals from vaccinated group were still sero-negative after the first vaccine injection but they sero-converted two weeks after the boost. The

antibody level varied after boosting from 26% to 134% depending on animals. Before the field challenge, the control sheep were sero-negative ($4 \pm 1\%$). Seven weeks after the beginning of the field challenge, 85% of the surviving control sheep (12/16 in the villages; 11/11 in Banankeledaga station) had sero-converted. During the second field trial, the average antibody level of the vaccinated group increased from 2% ($\pm$1%) before the primo-injection to 51% ($\pm$3%) two weeks after the boost. The control group was sero-negative before vaccination ($0 \pm 0\%$) and before field challenge ($3 \pm 1\%$).

3.2.3. Distribution and typing of ER strains during vaccination field trials

The *ER* strains present both in control and vaccinated groups during the two field challenges were identified by *map1* genotyping of brain samples. Eleven different *map1* genotypes were detected overall the four areas. Percentages of *ER* genotypes in each group
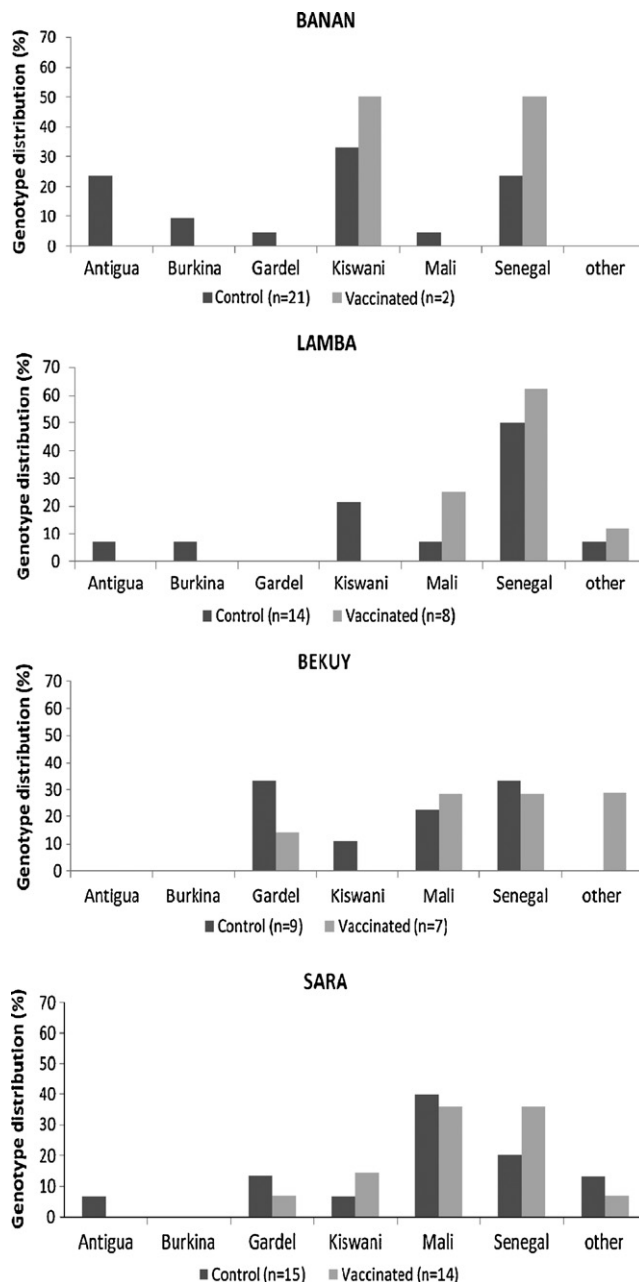

Fig. 3. Distribution of *ER map1* genotypes in brains from control and vaccinated groups during year 1 of vaccination trial.
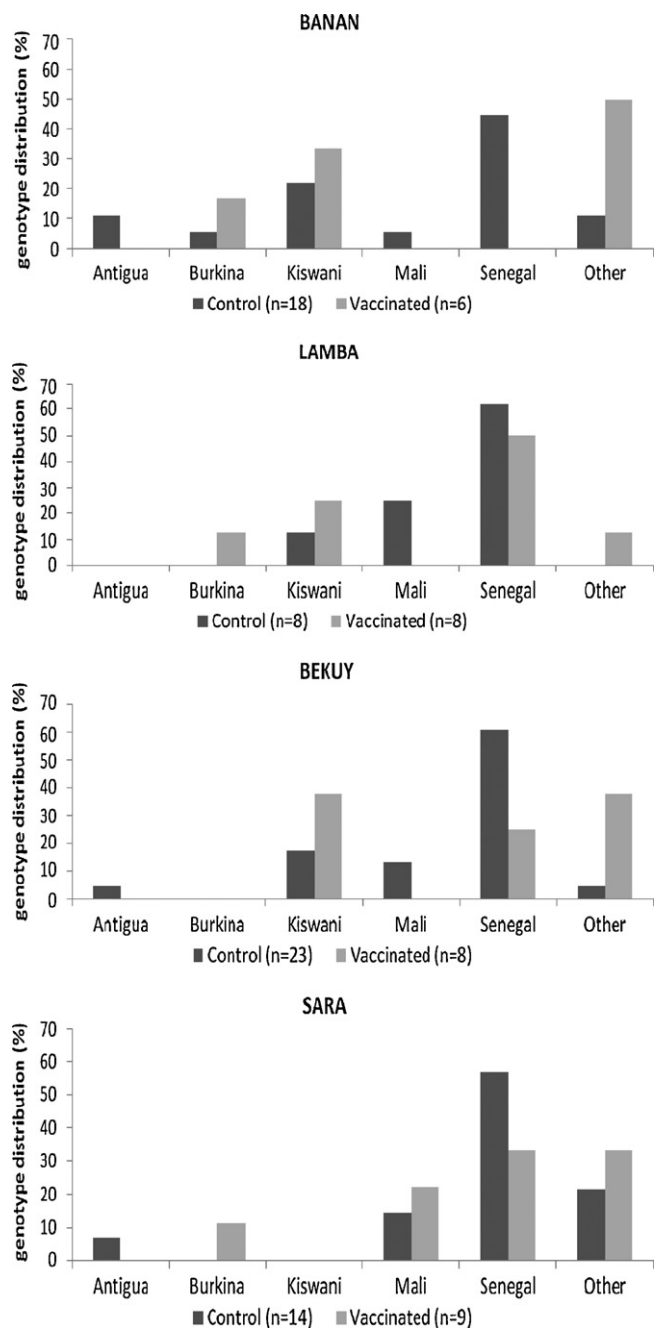
Fig. 4. Distribution of *ER map1* genotypes in brains from control and vaccinated groups during year 2 of vaccination trial.

are shown in Figs. 3 and 4. There were three main *map1* genotypes, Kiswani, Senegal and Mali, and three minor genotypes, Antigua, Burkina Faso and Gardel. Other strains, corresponding to Lamba 350, Bekuy 070 and SenKBD strains, were included in the category "other genotype". Lamba 350, Bekuy 070 and SenKBD *map1* genotypes (originated from Senegal) were described in a previous study [19]. Only Bekuy 070 and Lamba 350 were detected during year 1 whereas in year 2, the additional SenKBD was detected and was present in the four villages. During the first year, there was a predominance in the control group of Kiswani (in Banankeledaga station), Senegal (in Lamba village), both Gardel and Senegal (in Bekuy village) and Mali *map1* genotypes (in Sara village). Except for Bekuy village, the percentage of Gardel *map1* genotype on the total *map1* genotypes detected was very low (0–13%) and this *map1* genotype was not detected during year 2 either in vaccinated or

in control group. Overall, there was a significant difference of the percentage of Senegal *map1* genotype in the control group from one year to another ($N = 122$, $p = 0.005$) from 31% to 56%. In year 2, 44–63% of *map1* genotypes belonged to Senegal depending on the areas.

During year 1, there was a significant difference of Antigua *map1* genotype distribution between vaccinated and control groups: 13% of *map1* genotypes was Antigua *map1* genotype in brains from control group whereas none was detected in vaccinated group ($N = 91$, $p = 0.05$). There was no difference of distribution of the Senegal and Mali *map1* genotypes between control and vaccinated groups during the first year. However, in year 2, a significant diminution of the distribution of *map1* Senegal genotype was observed between control and vaccinated animals from 56% to 29% ($N = 94$, $p = 0.015$). In Banankeledaga station, there was no Senegal genotype detected in vaccinated group whereas 44% of *map1* genotype was the Senegal type in control samples. In Bekuy, only 25% of *map1* genotypes were Senegal for vaccinated group compared to 61% for control group.

4. Discussion

In this study, we first analysed the comparative protective effect of Gardel and Welgevonden strains against Burkina Faso local strains, in order to choose the appropriate vaccinal strain for the first year field trial. We observed a difference of protective effect between strains due to antigenic diversity which has been demonstrated previously in other studies for strains such as Gardel, Senegal, Welgevonden, Mbizi and Kerr Seringe independently of the nature of the vaccine [5,6,9]. We showed that Gardel had a significant protective effect against the Burkina Faso strains tested while Welgevonden strain did not. For three out of six strains, the survival rate of Gardel immunised sheep was superior to 50% after Burkina Faso strain challenge. Thus, Gardel strain was more suitable for field trials in Burkina Faso and was chosen as a master strain. Conversely, the lack of protection of Gardel inactivated vaccine against the Kerr Seringe strain from Gambia has been described previously [6]: there was 43% of survival for Gardel vaccinated group after challenge with Kerr Seringe strain. On the other hand, Welgevonden strain had been demonstrated to be a good candidate for attenuated vaccine as it protected against several strains from South Africa (Ball 3, Blaauwkraans, Mara 87/7) [7]. These results highlighted the variability of cross-protection and the importance of choosing the appropriate vaccine strains for a specific area.

The survival rate in control animals varied from one year to another and depending on the areas except in Banankeledaga station. During the two field trials, the infestation by adult tick was very low (<0.5 adult tick per sheep for the 4 month study) and sheep were mainly infested by nymphs. Even though the nymph infestation level was higher during the second field trial, it was not correlated with the mortality due to heartwater. In Banankeledaga station, the survival rate of the control group was similar during the two years whereas the mean number of nymphs per animal doubled from year 1 to year 2. In the other areas, the cumulative number of nymphs was three-fold higher whereas the survival rate increased in Lamba village and decreased in Bekuy and Sara villages. The MAP1 sero-conversion of 85% of the tested control animals observed after 7 weeks of natural tick challenge during the first study demonstrated that the majority of the surviving sheep had already been infected by *ER* at that moment. Moreover, 67–90% of the nymph infestation had already occurred depending on the flock at this period. Three of the four sero-negative animals were observed in the flocks where the cumulative infestation was still low (25–33 nymphs). In the flocks where the infestation had reached 40 nymphs at that moment, all but one animal had seroconverted or were already dead of heartwater. Since the infestation

during the second year was 2- to 3-fold higher, it is assumed that all sheep were infected during the study, unless the percentage of tick infection by ER dramatically decreased during the second field trial. The strategy chosen (bringing the animals to the field just before the peak of nymph infestation) was proven to be appropriate to allow ER infection of nearly all the animals. The approximate 50% survival rate observed in control animals during both studies is therefore not due to lack of infection of part of the flocks. It may be due to variation of virulence among ER strains present in the areas, or to variation of the number of bacteria inoculated by the infected ticks, mimicking in certain cases an infection with a sublethal but immunising dose, protecting sheep against most of the further infections. In Gambia, all animals from control group died during the field trial whereas the level of infestation was similar to our observations in year 1 (12–27 nymph ticks per animal at post mortem examination) [6]. In Burkina Faso, there is no data available concerning the ER infection rate in nymphs. However, the ER prevalence in adult ticks had been measured during rainy season in nine regions including Lamba, Bekuy and Sara villages using pCS20 nested PCR: 9% of Amblyomma ticks were infected with ER [23]. The ER infection rate in adult ticks in Gambia (Kerr Seringe) was similar (9.8%) to Burkina Faso [24]. These results strengthen our hypothesis suggesting a difference of virulence between strains. A weak field challenge had been described previously during a vaccine assay using DNA vaccine in South Africa. However, the level of tick infestation was not evaluated in that study [13]. Our study shows for the first time a broad picture of heartwater in the field from tick infestation to the mortality of animals in Burkina Faso. Therefore, it would be interesting in the future to address the question of the role of ticks in the dynamics and distribution of ER strains.

During the first field challenge, there was a marked increase of the survival rate for vaccinated group compared to control group in Banankeledaga station. In Lamba village, the increase of survival rate between control and vaccinated groups (14% vs 50%) was similar to results obtained during field challenge using inactivated vaccine in South Africa and in Zimbabwe with an increase of survival from 16% to 52% and from 19% to 58% respectively [9]. In the two other villages, the vaccine offered only a slight protection probably because of the low cross protective effect of Gardel strain towards circulating strains.

Map1 genotypes detected in brains of control sheep post mortem corresponded to the strains circulating during the vaccination trials whereas those found in vaccinated animals corresponded to breakthrough strains. Surprisingly, the same map1 genotypes were found both in control and vaccinated groups. However, even if map1 genotypes were close or identical, this did not mean that the strains were antigenically identical [19]. For example, the protection was widely different for sheep immunised with Gardel strain (Gardel map1 genotype) and challenged with two different strains of Senegal map1 genotype: Sara 292 or Sara 445. As demonstrated previously, map1 gene is not linked with protection since strains which are genetically distant for map1 gene could cross protect. For example, Senegal strain (Senegal map1 genotype) protected against Kerr Seringe (98.6% identical to Sankat 430 map1 genotype) [25] and Gardel protected against Sara 445 strain (Senegal map1 genotype). The absence of the Antigua map1 genotype in the brain of dead vaccinated animals suggested a possible effect of the Gardel vaccine against this genotype. Conversely the Gardel vaccine did not appear to limit infection due to the strains of Senegal and Mali map1 genotypes. During the second field challenge, the addition of Bekuy 242 strain to the vaccine induced a significant protective effect in Bekuy village and Banankeledaga station. Even though there was not a complete protection by the vaccine, there was an improvement of its efficiency by the addition of the local strain: 72% of survival for vaccinated group and 47% for control group. Overall, there was a significant effect of the addition of Bekuy 242 strain to the vac-

cine on the distribution of strains belonging to the Senegal map1 genotype: Senegal map1 genotype represented 56% of total map1 genotypes identified in control animals and 29% in vaccinated animals. Senegal map1 genotype was found in 73% of the dead control sheep and 39% of the dead vaccinated animals. Conversely, during year 1, the Senegal map1 genotype was present similarly in the vaccinated group (present in 57% of the dead animals) and in the control group (found in 40% of the sheep). Thus, Bekuy 242 strain appeared to give partial protection against strains of the Senegal map1 genotypes.

New vaccination approaches using DNA vaccines were recently tested [3,13]. They were efficient against homologous challenge but they did not give satisfactory results during field tick challenge. For DNA vaccination, the use of gene gun was also necessary to induce a protective effect. An attenuated vaccine with the Welgevonden strain conferred good protection in controlled conditions against homologous and four different strains but was not yet tested in field conditions [7,26]. Moreover, depending on breeds, the attenuated vaccine showed an unexpectedly high degree of virulence [26]. Another attenuated vaccine with the Senegal strain showed a better efficiency during field challenge (75% of protection) with none of the animals reacting clinically [6]. Compared to our results on the inactivated vaccine protection, the Senegal attenuated vaccine was more efficient during field challenge: the survival rate was similar for vaccinated groups but the challenge was stronger for Senegal attenuated field trial with 100% mortality rate for control group. However, results from Gambia suggested that mortality was mainly due to Kerr Seringe strain. Kerr Seringe map1 genotype was found in all controlled animals and another strain was typed in the three dead vaccinated animals. This suggested that the genetic diversity in the Gambia experimental area was lower than in Burkina Faso villages where 11 different map1 genotypes were found. In the context of high antigenic diversity in the field, the use of several attenuated strains included in a same vaccine would be necessary. Conversely, only three in vitro attenuated ER strains, Gardel, Senegal and Welgevonden are available and the achievement of attenuation to additional strains is uncertain (unpublished data, [7,27]).

In this study, we showed that an inactivated vaccine which has several advantages compared to other vaccines (cost, no cold chain required, no reversion to virulence), gave promising protective results during field challenge. In addition, we showed for the first time that an inactivated vaccine produced using an industrial process was efficient in field experiments. However, two injections of inactivated vaccine were necessary to induce protective effect and the vaccinated animals should be protected from tick infestation during the immunisation period (at least 2 months) inducing supplementary expenses. One solution would be to synchronize the vaccination process with the period of low tick infestation for example, at the end of rainy season after the pick of adults or at the end of dry season after the nymph peak. Moreover, the inactivated vaccine did not prevent the disease: vaccinated animals reacted clinically and displayed fever both after homologous or heterologous infections [6,11,12]. In West Africa and particularly for Burkina Faso, vaccination would be essential on breeding stock of high value (i.e. Sahelian sheep) moved from a heartwater-free area to a heartwater-endemic area. As regards sheep and goats born and raised in endemic areas like Djallonke sheep, they benefit age-related resistance during few months after birth and become resistant to heartwater if they were exposed to ER during this period. However, there are important losses during the first year of their life due to heartwater (i.e. 30% of mortality for Djallonke sheep up to 6 months old, unpublished data). This is probably due to a short period of innate immunity and/or low level of tick infestation and ER natural challenge at that time. In this context, the vaccination would be useful also for indigenous sheep and goats.

We demonstrated that the Gardel strain was more appropriate than Welgevonden strain for its use in Burkina Faso as inactivated vaccine. The addition of a second local *ER* strain to the Gardel strain vaccine improved protection against heartwater in field experiment. In the context of high genetic and antigenic diversity of *ER* which had been described in different geographical zones [4,19,22,23,28], the difficulty for vaccine design is to select the appropriate protective strains with the widest protective effect against regional circulating strains. The choice of a cocktail of strains would be an alternative if any reliable genetic marker related to cross-protection could be defined. Several approaches of genotyping using multi locus analysis (MLST/MLVA) of strains in relation to cross -protection data have been tested [29] and could allow identification of markers associated with cross-protection. Furthermore, it is possible that some of these markers could be involved in the protective immune response.

Acknowledgements

References

[1] Barré N, Uilenberg G, Morel P-C, Camus E. Danger of introducing heartwater onto the American mainland: potential role of indigenous and exotic *Amblyomma* ticks. Onderstepoort J Vet Res 1987;54:405–17.
[2] Provost A, Bezuidenhout JD. The historical background and global importance of heartwater. Onderstepoort J Vet Res 1987;54(3):165–9.
[3] Collins NE, Pretorius A, Van Kleef M, Brayton KA, Zweygarth E, Allsopp BA. Development of improved vaccines for heartwater. Ann N Y Acad Sci 2003;990:474–84.
[4] Jongejan F, Thielemans MJC, Briere C, Uilenberg G. Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. Rev Vet Sci 1991;51:24–8.
[5] Jongejan F, Vogel SW, Guèye A, Uilenberg G. Vaccination contre la cowdriose avec des *Cowdria ruminantium* atténuées *in vitro*. Rev Elev Méd Vét Pays Trop 1993;46(1–2):223–7.
[6] Faburay B, Geysen D, Ceesay A, Marcelino I, Alves PM, Taoufik A, et al. Immunisation of sheep against heartwater in The Gambia using inactivated and attenuated *Ehrlichia ruminantium* vaccines. Vaccine 2007;25(46):7939–47.
[7] Zweygarth E, Josemans AI, Van Strijp FM, Lopez-Rebollar L, Van Kleef M, Allsopp BA. An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge. Vaccine 2005;23:1695–702.
[8] Mahan SM, Kumbula D, Burridge MJ, Barbet AF. The inactivated *Cowdria ruminantium* vaccine for heartwater protects against heterologous strains and against laboratory and field tick challenge. Vaccine 1998;16(11–12):1203–11.
[9] Mahan SM, Smith GE, Kumbula D, Burridge MJ, Barbet AF. Reduction in mortality from heartwater in cattle, sheep and goats exposed to field challenge using an inactivated vaccine. Vet Parasitol 2001;97(4):295–308.
[10] Martinez D, Maillard JC, Coisne S, Sheikboudou C, Bensaid A. Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. Vet Immunol Immunopathol 1994;41:153–63.
[11] Marcelino I, Vachiéry N, Amaral AI, Roldão A, Lefrançois T, Carrondo MJT, et al. Effect of the purification process and the storage conditions on the efficacy of an inactivated vaccine against heartwater. Vaccine 2007;25: 4903–13.
[12] Vachiéry N, Lefrançois T, Esteves I, Molia S, Sheikboudou C, Kandassamy Y, et al. Optimisation of the inactivated vaccine dose against heartwater and in vitro quantification of *Ehrlichia ruminantium* challenge material. Vaccine 2006;24:4747–56.
[13] Pretorius A, van Kleef M, Collins NE, Tshikudoa N, Louwa E, Faber FE, et al. A heterologous prime/boost immunisation strategy protects against virulent *E. ruminantium* Welgevonden needle challenge but not against tick challenge. Vaccine 2008;26:4363–71.
[14] Marcelino I, Sousa MFQ, Vérissimo C, Cunha AE, Carrondo MJT, Alves PM. Process development for the mass production of *Ehrlichia ruminantium*. Vaccine 2006;26:1716–25.
[15] Walker JB, Olwage A. The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus Amblyomma) and their distribution. Onderstepoort J Vet Res 1987;54(3):353–79.
[16] van Vliet AHM, Zeijst VD, Camus E, Mahan SM, Martinez D, Jongejan F. Use of a specific immunogenic region of *C. ruminantium* MAP 1 protein in a serological assay. J Clin Microbiol 1995;33:2405–10.
[17] Smith GE, Anderson EC, Burridge MJ, Peter TF, Mahan SM. Growth of *Cowdria ruminantium* in tissue culture endothelial cell lines from wild African mammals. J Wild Dis 1998;34:297–304.
[18] Frutos R, Viari A, Ferraz C, Morgat A, Eychenie S, Kandassamy Y, et al. Comparative genomic analysis of three strains of *Ehrlichia ruminantium* reveals an active process of genome size plasticity. J Bacteriol 2006;188(7): 2533–42.
[19] Raliniaina M, Meyer DF, Pinarello V, Sheikboudou C, Emboule L, Kandassamy Y, et al. Mining the genetic diversity of *Ehrlichia ruminantium* using map genes family. Vet Parasitol 2010;167(2–4):187–95.
[20] Martinez D, Perez JM, Sheikboudou C, Debus A, Bensaid A. Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. Vet Parasitol 1996;67:175–84.
[21] Purchase HS. A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry,1926) in heartwater brains. Vet Rec 1945;57:413–4.
[22] Martinez D, Vachiery N, Stachurski F, Kandassamy Y, Raliniaina M, Aprelon R, et al. Nested PCR for detection and genotyping of *Ehrlichia ruminantium*: use in genetic diversity analysis. Ann N Y Acad Sci 2004;1026:106–13.
[23] Adakal H, Bada-Alambedji R, Stachurski F. Etude de la variabilité antigénique d'*Ehrlichia ruminantium* dans la région sud-ouest du Burkina Faso en vue de l'application d'une vaccination. Rev Afr Santé Prod Anim (RASPA) 2004;2(2):132–7.
[24] Faburay B, Geysen D, Munstermann S, Taoufik A, Postigo M, Jongejan F. Molecular detection of *Ehrlichia ruminantium* infection in *Amblyomma variegatum* ticks in The Gambia. Exp Appl Acarol 2007;42(1):61–74.
[25] Faburay B, Jongejan F, Taoufik A, Ceesay A, Geysen D. Genetic diversity of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks and small ruminants in The Gambia determined by restriction fragment profile analysis. Vet Microbiol 2008;126(1–3):189–99.
[26] Zweygarth E, Josemans AI, Steyn HC. Experimental use of the attenuated *Ehrlichia ruminantium* (Welgevonden) vaccine in Merino sheep and Angora goats. Vaccine 2008;26(Suppl. 6):G34–9.
[27] Jongejan F. Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro* attenuated rickettsiae. Infect Immun 1991;59:729–31.
[28] Vachiéry N, Jeffery H, Pegram R, Aprelon R, Pinarello V, Kandassamy Y, et al. *Amblyomma variegatum* ticks and heartwater on three Caribbean Islands. Ann N Y Acad Sci 2008;1149:191–5.
[29] Adakal H, Meyer DF, Carasco-Lacombe C, Pinarello V, Allegre F, Huber K, et al. MLST scheme of *Ehrlichia ruminantium*: genomic stasis and recombination in strains from Burkina-Faso. Infect Genet Evol 2009;9(6):1320–8.