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Review Article

Development of a Live Attenuated Vaccine for the Control of Bovine Babesiosis in Mexico

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Abstract

Bovine babesiosis is a hemoparasitic disease caused by intra-erythrocytic protozoa of the genus *Babesia*. It is mainly transmitted by *Rhipicephalus* (*Boophilus*) *microplus* ticks and cause of a hemolytic devastating disease with a high economic impact in the cattle industry, particularly in the tropical and subtropical areas of the world where the tick vector is endemic. Immunization with in vitro culture-derived parasites is thus far the best procedure that can be used for the prevention or control of bovine babesiosis. It has been proved that when the vaccine parasites are established in the animals, the induced immune response is protective, able to respond to natural challenge with parasites of high virulence in the field. With this methodology, a better control of possible contamination with other pathogens is also achieved. It is therefore reasonable to recommend the use of the vaccine in susceptible animals that could be introduced into endemic areas. This article outlines only some of the basic studies that support the possibility of favoring a mass production, possibly of the commercial type, of a live attenuated vaccine derived from the in vitro culture for the prevention of bovine babesiosis in Mexico.

ABBREVIATIONS

M199: Culture Media 199; CO_2: Carbon Dioxide; HEPES: 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid; TES: Tris Hydroxymethyl Methyl 2-Aminoethanesulfonic Acid; CENID-PAVET INIFAP: National Research Center for Veterinary Parasitology; PCV: Packed Cell Volume; IE: Infected Erythrocytes; UE: Uninfected Erythrocytes; PV: Post Vaccination

INTRODUCTION

Bovine babesiosis is a hemoparasitic disease caused by intra-erythrocytic protozoa of the genus *Babesia*. Among the main species of *Babesia* that cause bovine babesiosis are: *Babesia bovis*, *Babesia bigemina* and *Babesia divergens*. Other *Babesia* that can infect cattle include *B. major, B. ovata, B. occultans* and *B. jakimovi* [1]. The disease is transmitted by ticks of the family Ixodidae, genus *Boophilus* [2] now reclassified as *Rhipicephalus.* The main tick vectors for *B. bovis* and *B. bigemina* are *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus* (*Boophilus*) *annulatus*, which are widely distributed in tropical and subtropical regions of the world; however, *Rhipicephalus decoloratus*, *Rhipicephalus geigy*, and *Rhipicephalus evertsi* are also competent vectors [3].

In Mexico, the tick *Rhipicephalus (Boophilus) microplus* is the main vector and transmits both species, *Babesia bovis* and *Babesia bigemina*, which are the most important from an economic point of view [4,5]. The presentation of the disease may vary in severity, with characteristic clinical signs including; fever with a rectal temperature above 40°C, pale mucous membranes, hemoglobinuria, emaciation, anorexia, rectal tenesmus, and in the final phase death of the affected animals [6,7].

Bovine babesiosis is currently considered to limit the mobilization of genetically improved cattle producing milk and/or meat from tick-free areas to tropical and subtropical regions [8]. Livestock relocation is usually necessary to improve herd productivity. With the introduction of livestock, the enzootic stability condition of naive livestock is immediately impaired, because newly introduced cattle have never been exposed to the vector or to the babesial parasites [9]. In this situation, severe outbreaks can occur, with morbidity and mortality rates above 50% [10]. To better understand the magnitude of bovine babesiosis in Mexico, it is important to mention that out of several epidemiological studies carried out, seroprevalence rates have been estimated in different cattle regions; and an average prevalence above 50% has been determined and up to 96% prevalence rates have been found using the indirect fluorescence antibody test [4,8,11].

It is well known that there is interaction of three elements in

the epidemiology of bovine babesiosis: 1) The tick vector; 2) The parasite *Babesia* spp. and 3) The bovine host. This has suggested that, for the control of the disease, the integration of different methods of disease control should be applied as a strategy to achieve acceptable effectiveness and efficacy [12].

The procedures hitherto used and recommended are; control of the tick vector by using ixodicides; controlled livestock mobilization to prevent asymptomatic and tick-infested carrier cattle from being taken to free zones; chemotherapy and chemoprophylaxis, both can be tactically included in a comprehensive program although they are costly and impractical by themselves; the use of resistant cattle has been used in some countries with results not at all satisfactory [12]. Thus, immunization is the procedure indicated for many years as the most appropriate way to prevent and control bovine babesiosis [1,13].

Of the great diversity of research on the development of immunogens, probably the first used was the premunition. It consisted of the subinoculation in susceptible cattle of blood from an asymptomatic carrier bovine, to induce the infection and to apply the specific treatment. This procedure has presented serious drawbacks such as the transmission of other diseases such as bovine brucellosis, leucosis, tuberculosis, etc. In addition, it can occasionally cause severe outbreaks in vaccinated herds, showing high rates of morbidity and mortality [14,15].

In the development of a possible vaccine against babesiosis, there have been studies using vaccines with dead biological material; one of them being from extracts of erythrocytes infected with *Babesia bovis*, lyophilized and added with incomplete Freund's adjuvant. In these studies there were adverse outcomes such as isoerythrolysis in newborn calves and a low level of protection even in animals challenged with homologous strains [16]. Other studies have used plasma obtained from cattle infected with *Babesia bovis*; the plasma also lyophilized and added with incomplete Freund's adjuvant induced variable results at challenge, in some cases there was acceptable protection, although in others the immunity was null. These types of vaccines have not been used outside experimental conditions [17,18,19].

So far, the only procedure that has provided favorable results in terms of protection and safety has been the use of live attenuated vaccines. Good examples of this procedure are vaccines used in Australia, which contain attenuated strains of *Babesia bovis* and *Babesia bigemina* [13]. These are prepared in splenectomized calves and have been used since 1964. They have shown some disadvantages in the degree of protection they can induce, as well as safety, viability, quality control and vaccine management. However, they exist at commercial level [13,20]. In other countries, such as Argentina, Brazil, Uruguay and Israel, these vaccines have also been produced by governmental laboratories and used [21,22].

IN VITRO CULTURE OF *BABESIA SPP*

A different living vaccine alternative to the biological material derived from serial passes of splenectomized calves has been the in vitro culture of *Babesia bovis* and *Babesia bigemina*. The development of in vitro culture of *Babesia* spp strains in a defined medium has been the basis for the beginning of a continuous source of whole parasites and exoantigens for a variety of studies on the biochemistry and immunology of babesiosis. In vitro cultivation of bovine *Babesia* spp generally include culture reagents consisting of bovine erythrocytes, buffered culture medium and adult bovine serum [23]. The first successful report of the in vitro cultivation of *Babesia bovis* used a 50% suspension of bovine erythrocytes in M199 culture medium in Earle's salt, supplemented with 10% fetal bovine serum (pH 7.4), and incubating at 37°C in an atmosphere of 5% CO_2 in air [24]. Subsequently, it was possible to establish the continuous cultivation with some modifications, maintaining the continuous growth of the parasites for up to 32 days. The original description indicated the use of a 50% erythrocyte suspension and M199 medium supplemented with 50% bovine serum. In addition, the culture was kept under constant stirring conditions [25].

On the other hand, *Babesia bigemina* and *Babesia rodhaini* were successfully cultivated at almost the same time, but only for extremely short periods of time (96 hrs), in which the infectivity of the parasites was demonstrated [26,27].

The establishment of the *in vitro* culture of *Babesia* spp maintained a continuity and refinement in the methodology. Thus, by replacing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) by TES (Tris Hydroxymethyl methyl 2-aminoethanesulfonic acid) as the buffer salt, preservation of inoculum was achieved for seeding cultures under laboratory conditions, and finding that *Babesia bovis* could be recovered for in vitro culture using a new cryopreservation procedure [28].

Similar procedures were applied in Mexico to establish the continuous in vitro culture of *B. bovis*; for which, 10% erythrocytes were used, medium 199 with Earle's salts supplemented with 40% bovine serum, and incubation at 37°C in an atmosphere of 5% CO_2 in air [29].

The persistence in this type of studies led to the establishment of the culture system in a stationary microaerophilic phase, with an increase in the invasion of *Babesia* to normal erythrocytes. Similar procedures were used with *Babesia bigemina* and an in vitro culture was established continuously, improving the cryopreservation method in liquid nitrogen [30,31]. Subsequently, modifications were made to the in vitro culture process and *Babesia bovis* and *Babesia bigemina* lines were obtained. In addition, an important advance was achieved, when the biological cloning of both species of *Babesia* was utilized with the limiting dilution process [32,33].

In general, the instrumentation of the in vitro culture has allowed to gather more knowledge about the metabolic and reproductive behavior of *Babesia* spp. *In vitro* cultivation of bovine *Babesia* spp. provides a process for the attenuation of the virulence of these parasites, and allows vaccine preparations, cheap maintenance of field strains for antigen characterization, drug testing, seroneutralization assays, production of transgenic variants, morphological studies, invasion assays and study of antigenic proteins for potential development of subunit vaccines [22,32,34-37]. In the present review, out of the advantages mentioned above, the study of live immunogens from attenuated strains derived from in vitro culture stands out.

Currently in Mexico there is no commercial vaccine available

to prevent or control bovine babesiosis. However, since the inception of the in vitro culture of *Babesia bovis*, it marked the beginning of the development of a vaccine. First, different isolates derived from field outbreaks of *Babesia bovis* and *Babesia bigemina* were collected, which were adapted to in vitro culture. Taking advantage of the progress of this methodology, particularly the biological cloning by limiting dilution and cryopreservation, attenuated subpopulations were obtained to be tested as possible immunizing agents. For this reason, numerous studies have been carried out on the pathogenicity and immunogenicity of different *Babesia* spp clones [21,22,30,31,33,38,39].

DEVELOPMENT OF AN ATTENUATED LIVE VACCINE

Detailed studies with two strains of *Babesia* have been carried out in INIFAP's CENID-PAVET laboratories for use as possible immunogens in susceptible cattle mobilized to farms located in endemic areas of babesiosis [38,39]. Both strains are derived from clinical cases diagnosed in Mexico. One strain is from *Babesia bigemina* and one biological clone from *Babesia bovis*; the former has been maintained alternately in culture and in liquid nitrogen (-186 ˚C) [30,31,33]. The second has been cloned, irradiated in a Cobalt-60 source and returned to in vitro culture [32,40]. Research has been carried out under controlled conditions with the two subpopulations as well as under natural conditions of high bovine babesiosis endemicity [41- 43].

In the first series of studies with these strains, the reduced virulence of the clones derived from in vitro cultured and cryopreserved parasites was evaluated when inoculated in susceptible animals [34]. With regard to the virulence of the strains applied to cattle and derived from the in vitro culture, it was noticed that some degree of attenuation had occurred, as assessed by the moderate clinical effect of babesiosis provoked in the recipient animals. With these experiences it was considered promising to design experiments in which the culture materials were incorporated as immunogens, to induce a protective immune response in animals never exposed to *Babesia* spp.

Thus, this article outlines only some of the basic studies that support the possibility of favoring a mass production, possibly of the commercial type, of a live attenuated vaccine derived from the in vitro culture for the prevention of bovine babesiosis.

To precisely determine the dose for the experimental immunogen with the irradiated clone of *Babesia bovis*, different groups of cattle (comprised of at least 4 animals per group) were inoculated at increasing doses($1x10^5$; $1x10^6$;$1x10^7$;$1x10^8$;$1x10^9$) of infected erythrocytes (IE). The parameters that were evaluated were; the packed cell volume (PCV) determined by the microhematocrit method, the percentage of parasitemia and the clinical signs characteristic of babesiosis -fever, hemogloginuria, ictericia or mortality when it occurred. A group of cattle inoculated with normal uninfected erythrocytes (UE) was maintained as control group. In a similar manner, the challenge dose had previously been determined; applying increasing doses ($1x10^5$;$1x10^6$;$1x10^7$;$1x10^8$;$1x10^9$) of IE with a highly virulent strain obtained from a clinical case, cryopreserved in liquid nitrogen and reactivated in a splenectomized calf. From the results obtained it was suggested that the most adequate dose for challenge was $1x10^8$ IE. The selection procedure for the *Babesia bigemina* strain [39] was similar to that performed for *Babesia bovis* [38]. In this study it was found that after the application of different immunization doses, no animal showed clinical disease after challenge with a virulent field isolate. In contrast, animals in the control group showed severe decreases in the PCV, fever above 40˚C, and presence of parasites in blood smears. In addition, the control group cattle were treated to avoid death. It was inferred that the vaccine dose could be $1x10^7$ IE with the attenuated strains for both species [38,39].

Subsequently, the possibility of inducing cross-immunity between the two attenuated *Babesia* strains derived from in vitro culture was evaluated. In this study, groups of cattle (comprised of 4 animals per group) inoculated with the monovalent immunogen of *Babesia bigemina* or *Babesia bovis*, were compared to cattle immunized with the combined immunogen containing both parasite species, at a dose of $1x10^7$ IE of each strain. One control group received a similar dose with UE. At challenge with $1x10^8$ IE of the virulent strains, and based on the need to treat cattle with specific babesiacide, protection was estimated as 25%, 50% and 100%, for cattle immunized with monovalent *Babesia bigemina*, *Babesia bovis*, or combined *Babesia bigemina-Babesia bovis*, respectively [44]. In contrast, in the non-vaccinated group, all cattle were severely affected, one animal died, and the remainder were treated to avoid death. Therefore, concluding that cross-protection is insufficient, and to induce a solid immunity, the application of the combined *Babesia bigemina-Babesia bovis* immunogen is necessary [44].

In studies conducted using the fresh bivalent immunogen containing *Babesia bovis* and *Babesia bigemina*, experimental cattle were subject to a controlled challenge, and vaccine safety was demonstrated due to the moderate presentation of physiological alterations between days 7 and 21 post-vaccination (PV). The presence of parasites was detected with values lower than 0.01% in peripheral blood smears stained with Giemsa. When evaluating immunogenicity and protection at three-month PV challenge utilizing a virulent strain of each species, a slight decrease in PCV was observed, with no changes in rectal temperature and parasitemias of 0.01 to 0.06% for *Babesia bovis* and *Babesia bigemina*, respectively. Unlike the non-vaccinated group in which all animals had fever, a 29% decrease in PCV and a parasitemia of 0.5% for *Babesia bigemina* and 0.03% for *Babesia bovis*, with the need for specific babesiacidal treatment. Once again, the adequate protection induced by live attenuated, combined vaccine derived from in vitro culture was demonstrated [41].

Although the combined immunogen had been shown to be safe and possessed excellent potency for protection of up to 100%, this immunogen had been evaluated under controlled conditions. Thus, there was a need to know the behavior of the vaccine in susceptible cattle but immersed in a farm in an area of high endemicity for bovine babesiosis. Thus, experiments were needed to test the actual usefulness, and only then recommend its use in the field where animals permanently face various stressors, which may affect the immune status of the animals and / or the viability of the vaccine parasites.

For this, studies were carried out in cattle which had not been

previously exposed to ticks, nor to *Babesia* spp. Essentially, two groups of cattle (comprised of at least 4 animals per group) were conformed; to one group the combined vaccine was inoculated at doses of $1x10^7$ IE of each species and a control group to which UE were administered. Two months post-vaccination cattle were introduced to a farm where the seroprevalence to babesiosis had been previously estimated in 80%, in addition to maintaining high density of *Rh. (Boophilus) microplus* ticks. Under these conditions at day 18 post-introduction to tick-infested padocks, cattle from the vaccinated group showed fever and decreased hematocrit, but maintained their body condition. No treatment was given against *Babesia*. In contrast, all animals in the control group showed drastic signs of bovine babesiosis, including fever (41°C), presence of parasites in peripheral blood, and marked decrease in PCV (>50%). In this group all animals were treated to avoid their death and showed a detrimental effect on their general condition. These studies thus corroborated the usefulness of the vaccine in both, controlled and natural challenges [42,43].

However, despite of the live attenuated vaccine showing favorable results in terms of its safety and potency, its recommendation was limited to being fresh biological material. Due to their short half-lives, once it had been produced it should be applied to the experimental animals in a short period, to assure the parasite viability. For this reason it was necessary to determine the optimal dose of a frozen combined vaccine. Using cryopreserved material would facilitate the storage, handling and would have a better quality control. For this purpose, different doses ($1x10^7$, $5x10^7$, $1x10^8$, $5x10^8$) IE of each parasite strain were evaluated and compared against the $1x10^7$ dose of fresh combined vaccine of *Babesia bovis-Babesia bigemina*. It was observed that out of the material maintained in liquid nitrogen, the dose of $1x10^8$ IE of each species was the most effective in conferring protection at challenge with virulent field isolates [45]. Thus, there is a reduction in attenuated parasite viability of approximately 90% due to the freeze-thawing process.

Currently, the standard procedure for the in vitro culture of *B. bovis* and *B. bigemina* has made it possible to have a source of biological material useful as a vaccine antigen. This has involved the characterization of attenuated parasites as potential immunogens. By using the in vitro culture system of *B. bovis* and *B. bigemina*, different validation studies of an attenuated vaccine have been carried out in Mexico. It has been demonstrated the induction of protection in at least 80% of susceptible cattle when $1x10^7$ or $1x10^8$ infected fresh or frozen erythrocytes are applied, respectively [38,39,42,43,45]. In addition, protection has been demonstrated in native cattle maintained under conditions of endemicity and enzootic instability [8]. With the gamma radiation of the substrate, the viability of viral and bacterial type adventitious agents has also been significantly reduced [46].

However, it would be highly desirable to avoid the addition of bovine serum in order to have a chemically defined growth medium. This could probably eliminate possible growth inhibitory factors for in vitro cultivation of *Babesia* spp, and also to reduce the risk of dissemination of pathogens. Achieving this objective will improve the effectiveness of the process, which will be a major advance in scientific, technical and economic terms. In addition, eliminating serum from the conventional *in vitro* culture would reduce the use of donor animals, thus favoring animal welfare. Economically, the cost of in vitro culture would be reduced because fewer facilities, feed and personnel associated with animal care would be required and less risk of adventitious infectious agents would be faced. In this direction, the successful continuous cultivation of *Babesia bovis* in a bovine serum free-culture medium has been reported very recently [47,48]. Likewise, *Babesia bigemina* has been successfully adapted to continuous cultivation in a bovine serum free-culture medium [49] and parasites derived from both culture systems have been tested in experimental animals without detriment on their immunoprotective capacity once immunized cattle are exposed to tick-transmitted babesia infection under field conditions [50].

CONCLUSION

In summary, immunization with the in vitro culture-derived vaccine is thus far the best procedure that can be used for the prevention or control of bovine babesiosis. It has been proved that when the vaccine parasites are established in the animals, the induced immune response is protective, able to respond to natural challenge with parasites of high virulence in the field. With this methodology, a better control of possible contamination with other pathogens is also achieved. It is therefore reasonable to recommend the use of the vaccine in susceptible animals that could be introduced into endemic areas. However, scaling up in production for commercialization and mass release must be considered. On the other hand, it is important to maintain basic research for the development of subunit or recombinant vaccines that allow the activation of the T-cell subpopulations associated with protection against *Babesia* spp to have an ideal vaccine, which would most probably eliminate the concept of permanent exposure from the host to the agent for the maintenance of a long protective immunity.

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