RESEARCH

Using Montanide™ ISA 50 V2 as adjuvant for the formulation of the anti-tick Gavac® vaccine

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ABSTRACT

Infestations with cattle tick (*Rhipicephalus microplus*) have an economic impact by reducing animal weight gain and milk production. An alternative for tick control in cattle is the use of Gavac® vaccine, which contains the Bm86 antigen as active pharmaceutical ingredient. In this work, Montanide™ ISA 50 V2 was evaluated as adjuvant for Gavac® to homogenize the formulation process. The current adjuvant (mineral oil), the proposed Montanide™ ISA 50 V2 and the emulsion formed were subjected to physicochemical characterizations. The results confirmed that both adjuvants were physicochemically similar and the derived emulsions exhibited better characteristics when using Montanide™ ISA 50 V2. The three batches studied 24 months for stability showed results analytically consistent with the expected ones, confirming that Gavac® immunogen is stable for two years. Until October 2017, nine vaccine batches equivalent to 1.5 million doses were obtained containing Montanide™ ISA 50 V2 as a new variant of vaccine adjuvant formulation. Those batches were compared with historical data, showing similar qualitative and biological activity properties. Therefore, it was proposed that the use of Montanide™ ISA 50 V2 as adjuvant for the Bm86 antigen formulation of the Gavac® vaccine provides better qualitative physicochemical characteristics, to be corroborated in vaccination trials in cattle.

Keywords: Tick control, Gavac vaccine, adjuvant formulation, Montanide ISA 50 V2, physicochemical properties, cattle

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RESUMEN

Uso de Montanide™ ISA 50 V2 como adyuvante para la formulación de la vacuna Gavac® contra las garrapatas. Las infestaciones con garrapatas del ganado (*Rhipicephalus microplus*) causan pérdidas a la economía al reducir el aumento de peso de los animales y la producción de leche. Una alternativa para el control de las garrapatas en el ganado es el uso de Gavac®, vacuna que contiene al antígeno Bm86 como ingrediente farmacéutico activo. En este trabajo se evaluó la formulación de Gavac® con la inclusión de Montanide ™ ISA 50 V2 como adyuvante para homogeneizar el proceso de formulación. El adyuvante actual (aceite mineral), el Montanide ™ ISA 50 V2 propuesto y la emulsión formada fueron caracterizados según parámetros fisicoquímicos. Los resultados confirmaron que ambos adyuvantes eran fisicoquímicamente similares y las emulsiones derivadas exhibían mejores características cuando se utilizaba Montanide ™ ISA 50 V2. Los tres lotes a los que se les evaluó la estabilidad durante 24 meses mostraron resultados analíticamente consistentes con los esperados, lo que confirma que el inmunógeno Gavac® es estable por dos años. Hasta octubre de 2017, se obtuvieron nueve lotes de vacunas equivalentes a 1.5 millones de dosis que contenían Montanide ™ ISA 50 V2 como nuevo adjuvante en la formulación vacunal. Esos lotes se compararon contra los datos históricos, lo cual demostró que los lotes similares desde el punto de vista cualitativo y de actividad biológica. Por lo tanto, se propuso que el uso de Montanide ™ ISA 50 V2 como adyuvante para la formulación del antígeno Bm86 de la vacuna Gavac® proporciona mejores características fisicoquímicas cualitativas, lo cual deberá ser corroborado durante los ensayos de vacunación del ganado.

Palabras clave: Control de garrapatas, vacuna Gavac, formulación adyuvante, Montanide ISA 50 V2, características fisicoquímicas, ganado vacuno

*I*ntroduction

Rhipicephalus (*Boophilus*) spp. ticks are disseminated mainly in tropical and subtropical regions [1-3]. Among them, cattle tick (*Rhipicephalus microplus*) infestations have an economic impact in cattle by reducing animal weight gain and milk production. They are also carriers of pathogens causing significant diseases such as babesiosis and anaplasmosis [4]. In Australia, cattle losses by cattle tick Boophilus (Rhipicephalus) microplus were estimated to be US$ 62 million, while in Brazil they accounted for around US$ 3.24 billion per year [5]. In India, there were estimates of US$ 498.7 million per year [6]. Moreover, direct and indirect losses related with tick-borne disease are difficult to estimate. It was estimated that when crossbred Holstein-Zebu cows are infested with an average of 105 ticks, with a reduction in 23 % of

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milk yield per day expected [7]. Animals with an average of 40 ticks per day could lose weight up to 20 kg per year [6]. Economic losses, accompanied of the zoonotic danger by ticks affectation, foster research on new strategies for tick control [8].

Among the alternatives proven effective for tick control in cattle is vaccination. One of the vaccines available for tick control in cattle is Gavac® (Heber Biotec S.A., Cuba), which contains the *B. microplus* Bm86 protein as Active Pharmaceutical Ingredient (API) [9]. It has been proven effective for more than 20 years of use in Cuba, Venezuela and other Central America countries, as part of integrated pest management systems. In Venezuela, it has been applied in more than 38 000 farms, thereby reducing in 83.7 % the use of chemical acaricides equivalent to 260 000 kg [10].

Gavac® has also been evaluated in the boundaries of the United States and Mexico, for a possible establishment of integrated control. Miller *et al*. [11] showed that vaccination was able to control tick infestation in 99.9 % of animals after 5 weeks and 91.4 % at 5.5 months. Those results were widely validated at the CATVAC meeting [12]. The use of Gavac® have also shown significant protection levels in camels infected by H*yalomma dromedarii* and *H. a. anatolicum* [13].

However, new research is needed to enhance the immunogenicity of the Gavac® vaccine, to cope with increasing demands on effectiveness improvements. One strategy for vaccine improvement consists on adjuvant replacement. Particularly, Montanide™ ISA 50 V2 has been tested in various species of animals with excellent immunogenicity results [14-17]. Its effectiveness has been demonstrated equivalent to that of classical adjuvants such as Freund's, guaranteeing a memory immune response and reducing adverse effects [20]. Moreover, it enhanced the cellular cellular response against the antigens tested, also showing a higher homogeneity in the humoral response [19, 20]. It was shown that production processes including this adjuvant also displayed improved development and scale-up processes for the obtained injectable products [21].

Therefore, with this aim and following an agreement with SEPPIC, it was evaluated the replacement of the Gavac® vaccine adjuvant (a mix of mineral oil and Montanide™ 888 VG) with Montanide™ ISA 50 V2 adjuvant in the formulation, as part of the vaccine production process.

Materials and methods

Physicochemical characterization of adjuvants

The formulations of the Gavac® vaccine with either Montanide™ ISA 50 V2 or Montanide™ 888 VG-mineral oil mixture were characterized. Assessments were done jointly with the adjuvants' manufacturer (SEPPIC, France). The characteristics, methods and limits established by SEPPIC.

Then, the two adjuvants analyzed were formulated using a phase volume ration of 60 parts of the aqueous phase and 40 parts as oily phase (current ratio as established in the formulation of Gavac®). The mixture Montanide™ 888 VG-mineral oil was prepared at a 1:9 v/v ratio, and Montanide™ ISA 50 V2 was used as provided by the manufacturer.

Formulations were characterized by standardized tests by SEPPIC: viscosity, with a Brookfield viscometer with spindle 63 at 60 min^{-1} (mPa·s); syringeability, with a 10-mL syringe 21 Gauge × 1 inch length needle and time (s) for discharge of 10 mL of formulation; droplet size, by using a Malvern MasterSizer S granulometer (µm).

Statistical comparisons were established with the GraphPad Prism Software, and paired samples were compared by the Student's t test with Welch's correction and a 95 % confidence interval.

Assessment of batch formulation and stability

Three batches using the new adjuvant Montanide™ ISA 50 V2 were formulated. The Bm86 protein (protein purity higher than 95 %) was used as API, and a at 10-L batch formulation was prepared in a SD-41 emulsifier equipment (IKA, Germany), with droplet size adjusted to 1 µm. Batches were released and their stability monitored according to test procedures as established at the CIGB (Table 1). Stability was studied in the range of 2-8 ºC. Biological activity was determined in mice handled by following the National Institutes of Health guide for the care and use of laboratory animals [22].

Results and discussion

Physicochemical characterization of adjuvants

The physicochemical characterization of both adjuvants formulations analyzed shows that results comply with the value limits established by manufacturers (Table 2). Only the refractive index of the prior adjuvant formulation (mix of mineral oil and Montanide™ 888 VG) showed a value out of the established limits, a behavior caused by the type of mineral oil used in the old formulation. All this shows that

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Table 1. Characteristics and limits used by SEPPIC for physical-chemical characterization of adjuvant Montanide™ ISA 50 V2

Characteristics	Specifications
Organoleptic characteristics	White shiny liquid, oily consistency
Biological activity	Potency assay: DE50 ≤ 1 µg
General safety	EP: Pass the test
Sterility	USP: Pass the test
Droplet size distribution	Diameter of drop ≤ 5 µm for 80 % of drop or more
Thermal stability	Oil layer height/total height ≤ 0.1
Mechanical stability	Oil layer height/total height ≤ 0.2
Rheological behavior	
Flow consistency index (K)	≤ 1500 mPa · s
Flow behavior index (n)	< 1

Table 2. Characteristics and limits used by SEPPIC for physical-chemical characterization of adjuvant Montanide™ ISA 50 V2

Characteristics	Specifications	Montanide™ 888 VG-mineral oil	Montanide™ ISA 50 V2
25° C Appearance	Limpid - trouble/hazy	Opalescent	Opalescent
Acid value	0-0.5 mg KOH/g	0.1	0.1
Saponification value	16-19 mg KOH/g	16	17
Hydroxyl value	9-14 mg KOH/g	12	12
Peroxide value	0-4.0 mmol/kg	0.3	0.4
Iodine value	5.0-9.0 g I_2/100 g	7.6	7.3
Water content	0-0.5 %	0.07	0.03
10 % pH	4.5-6.5	5.7	4.7
25 °C refractive index	1.4560-1.4620	1.4642	1.4563
Gardner color	0.0-2.0 VCS	0.9	1.4

both adjuvant formulations meet the quality specifications attending to their physicochemical properties. In fact, both adjuvants show similar characteristics, an aspect essential for the approval given by the Cuban regulatory agency on using Montanide™ ISA 50 V2 for Gavac® formulation. Following these results, the emulsions were further characterized to evaluate the influence of both adjuvants.

As shown in table 3, there were no statistically significant differences between viscosity values of emulsions containing both adjuvants. The use of Montanide™ ISA 50 V2 induced a decrease of viscosity of about 300 mPa·s. This behavior guarantees that the inclusion of a new adjuvant in the production process provides a better applicability of the immunogen in the field. Noteworthy, it should be noted that the ratio used in the study is not the one suggested by SEPPIC (50 parts of aqueous phase and 50 parts of oily phase in the volume). Advantageously, the ratio used in the study supports a 10 % increase in the internal phase of the emulsion, what increases the viscosity of the system. It is emphasized that this w/o formulation ratio is the one used for the formulation of the Gavac® immunogen for the last 20 years, and proven in the field without associated adverse events.

The same improved behavior was found in syringeability, with a reduction in approximately 30 s the time required for the new adjuvant, further reducing the back pressure to be overcome during the administration of the vaccine. Values lower than 60 s are reported by SEPPIC for the 50 parts of aqueous phase and 50 parts of oily phase in volume. The improved syringeability is in agreement with the increased viscosity found, resulting from the raise in the relationship of the internal phase.

Another relevant property evaluated was droplet size (Table 4). The results of Dv50 did not show statistically significant differences between the characterized groups. Nevertheless, there was a trend to increase drop size when using the old adjuvant. This provides 50 % of the droplets with a size lower than 1 µm. In the case of Dv90, a significant statistical difference was detected, with a reduction in 0.5 µm with the use of the new adjuvant formulation. In all cases, 90 % of the droplets were smaller than 2 µm. Overall, decreasing droplet size of the emulsion with the new adjuvant can reduce the sedimentation rate that derives in its enhanced stability [23]. Moreover, small droplet size may favor biological activity by the slow release of the active principle and its enhanced bioavailability [24, 25].

Table 3. Viscosity and syringeability values of obtained emulsions containing the Montanide™ ISA 50 V2 or Montanide™ 888 VG-mineral oil adjuvants for the Gavac® vaccine*

Adjuvants	Viscosity (mPa.s, Spindle 63/60 rpm) Values	Means ± SD	Syringeability (s) Values	Means ± SD
Montanide™ 888 VG-mineral oil	1028	1057.0 ± 83.94	121	130.3 ± 9.018
	1152		139	
	992		131	
Montanide™ ISA 50 V2	778	745.3 ± 28.45	95	100 ± 5.000
	732		100	
	726		105	
p value	0.0155		0.0132	

* Values were compared with the Student's t test with the Welch's correction and 95 % of confidence interval.

Table 4. Droplet size values of emulsions formed with the two adjuvants characterized for the formtulation of the Gavac® vaccine*

Adjuvants	Droplet size (µm) Dv50	Means ± SD	Dv90	Means ± SD
Montanide™ 888 VG-mineral oil	0.91	0.957 ± 0.057	1.88	1.98 ± 0.087
	0.94		2.03	
	1.02		2.03	
Montanide™ ISA 50 V2	0.80	0.880 ± 0.092	1.40	1.55 ± 0.138
	0.98		1.67	
	0.86		1.58	
p value	0.2978		0.0151	

* Values were compared with the Student's t test with the Welch's correction and 95 % of confidence interval.

Table 5. Physical-chemical characterization of three batches of Gavac® vaccine formulated with adjuvant Montanide™ ISA 50 V2

Characteristics	Batch 1	Batch 2	Batch 3
Organoleptic characteristics	Pass the test	Pass the test	Pass the test
Biological activity	0.56 µg	0.36 µg	0.13 µg
General safety	Pass the test	Pass the test	Pass the test
Sterility	Pass the test	Pass the test	Pass the test
Droplet size distribution (diameter ≤ 5 µm for ≥ 80 %)	100 %	100 %	100 %
Thermal stability	0.01	0.01	0.01
Mechanical stability	0.03	0.03	0.03
Rheological behavior			
Flow consistency index (K)	629.73 mPa·s	826.64 mPa·s	403.46 mPa·s
Flow behavior index (n)	0.757	0.710	0.792

Batch release for the stability study

The qualitative characteristics evaluated for batch release in all cases passed the test (Table 5), highlighting the importance of the general safety and sterility. On the contrary, some variations were found in the biological activity and rheological behavior. Changes in biological activity commonly derive from the use of different API batches, with animal models also influencing its variability. For viscosity, variability may be caused by fluctuations in operating parameters coming from the reduced production scale. Even so, the observed variability did not determine significant changes in product's quality. The droplet size distribution, mechanical and thermal stability showed favorable results, well above the established limits as for the formulation of Gavac® immunogen with the old adjuvant. It was seen that all evaluated properties met the specifications, ensuring that the produced batches were released and could be used for the stability study.

Stability study of the Gavac® immunogen formulated with Montanide™ ISA 50 V2

Produced batches were checked for the stability parameters evaluated upon release. Thermal and mechanical stability values of the accelerated stability tests were lower than 0.05 for the six-month period, with no further information provided due to its short duration.

The qualitative properties evaluated for the 24 months of the full stability analysis met product specifications. No changes were observed either in color or appearance of the product. Favorable general safety profiles were evidenced throughout the study, guaranteeing the safe use of the product. Sterility was evaluated upon release and 24 months thereafter. It was determined that in this interval of time the

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container-closure system was effective and that none of the critical components contributed to microbiological contamination of the vaccine.

The performance of biological activity for the 24 months of analysis is depicted in figure 1. Biological activity is regarded as protective when 50 % of vaccinated mice are able to seroconvert at doses lower than 1 µg. Immunization assay were conducted in female Balb/C, *H2d*, *q* haplotype, the variations seen maybe arising from the limited number of animals used (n = 10) and the animal model tested [26]. Values were all within the specified limits for all data points, thus ensuring that the product maintains its biological activity for the next 24 months after the manufacturing date.

Additionally, the average frequency of the droplet size in the dispersed phase was evaluated at every evaluation period in the stability study (Figure 2). As shown, there were no droplets with values above 5 µm. In fact, 80 % of the droplets were below 2 µm. The distribution obtained guarantees a favorable stability of the Gavac® immunogen within 24 months after the manufacturing date. Similar profiles were obtained in all the evaluated points.

Thermal and mechanical stability values were lower than 0.05 in all evaluated points, for all produced batches. Tests were performed only during the first six months, and the observed behavior provided physically stable emulsions for up to 24 months in the range 2-8 ºC.

Regarding the rheological behavior, this is one of physical characteristics directly affecting the application of the Gavac® immunogen. The values for parameters of Flow consistency index (K) and Flow behavior index (n) are shown in figure 3. All determinations were within the established limits ($K \leq 1500$ mPa·s and $n < 1$).

Implementation and use of the Montanide™ ISA 50 V2 for Gavac® formulation

All the quality specifications for the Gavac® immunogen were fulfilled for the parameters evaluated in the stability study. Moreover, results were documented and presented to the Cuban regulatory authorities for veterinary drugs, supporting their approval on the use the of new adjuvant formulation of the immunogen Gavac® vaccine. Up to 9 batches were formulated with the new adjuvant, equivalent to more than 1.5 million vaccine doses, their properties detailed in Table 6.

The batches produced with the new adjuvant were also compared with historical data of the old adjuvant in the previous year. No significant differences in biological activity were observed, despite each batch was formulated with a different API. Moreover, no relevant reports were found in the scientific literature which could be relevant as to be contrasted to. For thermal and mechanical stability, statistically significant differences were detected, the values obtained in the batches produced with the new adjuvant formulation found better than historical data. In the rheological behavior, significant differences were observed between the values of flow consistency index, with a reduction in 170 mPa·s, corroborating the behavior observed during the characterization of the obtained emulsions. No significant differences were found in the flow behavior index between formulations.

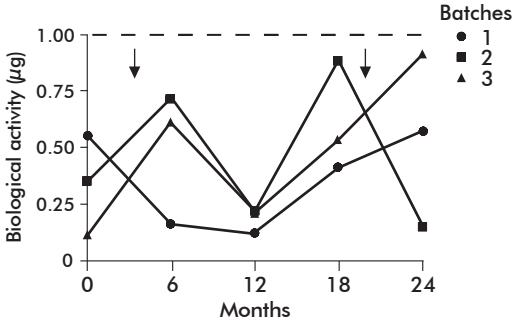

Figure 1. Biological activity during 24 months in the stability study in three batches of Gavac® vaccine formulated with Montanide™ ISA 50 V2 as adjuvant. Female Balb/C, *H2d*, *q* haplotype, were immunized with Gavac® vaccine (n = 10). Depicted are mean values per animal group. Arrows indicate immunization timepoints. The dashed line stands for the limit of biological activity (1 µg).

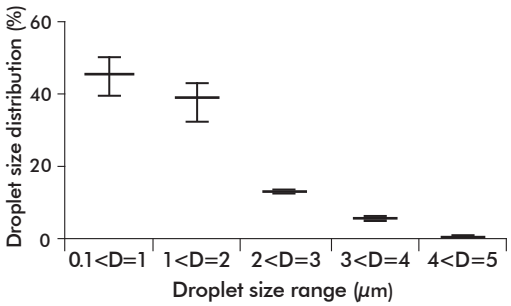

Figure 2. Average droplet size distribution of three batches of Gavac® vaccine formulated with Montanide™ ISA 50 V2 as adjuvant.

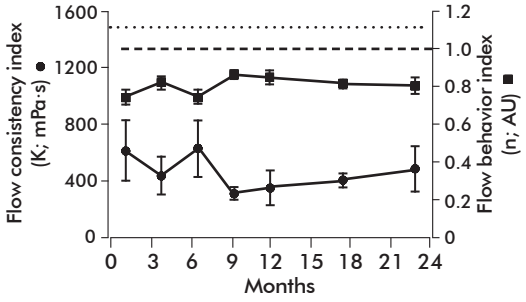

Figure 3. Biological activity during 24 months in the stability study in three batches of Gavac® vaccine formulated with Montanide™ ISA 50 V2 as adjuvant. Female Balb/C, *H2d*, *q* haplotype, were immunized with Gavac® vaccine (n = 10). Depicted are mean ± SD per animal group. Arrows indicate immunization timepoints. The dashed line stands for the limit of biological activity (1 µg). Dotted and dashed lines stand for the upper limits of K and n, respectively.

Conclusions

Despite overall similar results, the use of Montanide™ ISA 50 V2 reduced viscosity of the Gavac® vaccine formulation in about 300 mPa·s and 30 s in syringeability, compared the previous formulation using the Montanide™ 888 VG-mineral oil mixture as adjuvant. Droplet size values were similar, although the

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new formulation set a trend of droplet size reduction in Dv50 and Dv90. During the stability study, none of the evaluated quality characteristics were outside the limits for the product Gavac®. Thus, it is demonstrated that from the physical, chemical and biological points of view, the new formulation containing Montanide™ ISA 50 V2 as adjuvant meets the limits approved for the assay. In comparison with historical data, better behavior was achieved with the new adjuvant attending to the quality properties among batches, confirming preliminary results obtained when characterizing the emulsions formed with both adjuvants. These results confirm the adequate use of Montanide™ ISA 50 V2 as adequate for the formulation of Gavac®. This will reduce the dependence on multiple providers for mineral oil as previously required, thereby reducing that variability source during formulation among batches and, therefore, guaranteeing greater consistency during the vaccine production process.

Table 6. Results of the produced batches with the Montanide™ ISA 50 V2 adjuvant in production scale compared to historical data the Montanide™ 888 VG-mineral oil*

Characteristics	Batches		P
	Montanide™ ISA 50 V2	Montanide™ 888 VG-mineral oil	
Organoleptic characteristics	Pass the test	Pass the test	
Biological activity	0.56 ± 0.36 μg	0.43 ± 0.22 μg	0.2272
General safety	Pass the test	Pass the test	
Sterility	Pass the test	Pass the test	
Droplet size distribution (diameter ≤ 5 μm for ≥ 80 %)	100 %	100 %	
Thermal stability	0.01	0.0169 ± 0.001	< 0.0001
Mechanical stability	0.02	0.0233 ± 0.001	< 0.0001
Rheological behavior			
Flow consistency index (K)	635.9 ± 197.8 mPa·s	801.6 ± 289.7 mPa·s	0.0457
Flow behavior index (n)	0.767 ± 0.036	0.765 ± 0.039	0.9305

* Data was statistically compared by the Sudent's t test with Welch's correction and the 95 % confidence.

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