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RESEARCH / INVESTIGACIÓN

Assessing HeberFast® Line Gavac, a lateral flow immunochromatographic system for the rapid detection of anti-Bm86 antibodies in Gavac vaccinated cattle

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Abstract: *Rhipicephalus Boophilus* microplus cattle tick is a scourge for livestock production. The infestations produced by this pathogen are incompletely contained by chemical treatments, with the associated environmental pollution risks. Vaccination against cattle ticks has emerged as a feasible and environmentally friendly strategy to control tick-borne diseases. In this setting, Gavac® vaccine has proven effective in decreasing cattle tick populations through antibody responses against the tick Bm86 antigen, as part of an Integrated Control Program. However, animal vaccination programs require easy and ready-to-use screening tests to follow up the immune response in vaccinated animals under field conditions. This study reports the evaluation HeberFast® Line Gavac, a lateral flow immunochromatographic system for the rapid detection of anti Bm86 antibodies in vaccinated cattle. The system was tested on 598 serum samples taken from immunized animals, arranged in three groups according to their anti-Bm86 antibody response in ELISA (209 high, 150 medium or 239 low and 100 samples from non-immunized animals. The HeberFast® Line Gavac system was assessed for sensitivity, specificity, and concordance against the ELISA reference test. Consistency was evaluated among production batches and inter-analyst reading-independent consistency at two moments: ten minutes after completing the test and after strip drying. The system showed high sensitivity (81.6%, 82.2%, and 81%), specificity (96.7, 94.6, and 93.3%), and agreement with the ELISA reference test (75%; 74%, and 71%) for high, medium and low anti-Bm86 sera, respectively. The effectiveness of the diagnosis was 87.6; 87.1; 85.9 for high, medium, and low antibody titers, respectively. Consistency among production batches and analysts was documented, and no significant differences between evaluation times were found. These results indicate that HeberFast® Line Gavac is a valuable tool for the serological surveillance of Gavac vaccinated cattle.

Key words: Cattle tick, vaccination, antibody screening strip, lateral flow immunoassay, Bm86, Gavac®.

Introduction

Rhipicephalus (Boophilus) microplus cattle tick is a scourge for livestock production in tropical and subtropical areas. Ticks cause significant losses due to their direct weakening effects in cattle and through tick-borne diseases, ultimately causing animal death[1]. In Cuba, an Integrated Management Program for cattle tick control has been implemented nationwide. This program comprises the combination of Gavac®, a recombinant vaccine against cattle tick, with chemical acaricide treatments, scheduled according to the infestation index, and other measures for the biological, mechanical, immunological, and genetic control of the plague. The application of the vaccine has effectively sparse the number of acaricide treatments required, thereby reducing the environmental pollution risks and diminishing a 98% the deaths rates caused by hemoparasites[2-4].

The active ingredient of Gavac® is the tick gut Bm86 protein. Anti-Bm86 antibodies induced upon vaccination will attach to the gut epithelium of suckling ticks, with deleterious effects on their populations and the size of their progeny[5,6]. Regular immunizations are required to maintain protective anti-Bm86 antibody titers in the blood of the animals[6-8].

Commonly, antibody titers in sera of immunized animals are monitored through antigen-specific Enzyme-Linked Immunosorbent Assays (ELISA) tests, high efficiency, and specificity. However, such assays are troublesome under field conditions, time-consuming, and require specialized laboratory facilities and qualified personnel to perform and evaluate the results objectively. Thus, a fast and easy-to-use screening test is needed to implement and evaluate ongoing, and future cattle tick vaccination campaigns under field conditions.

Therefore, this study aimed to evaluate the performance of HeberFast® Line Gavac, a fast chromatographic screening test for the detection of anti-Bm86 antibodies, and its concordance with the reference ELISA test used in the laboratory. The main parameters to compare were sensitivity, specificity, and consistency.

Materials and methods

Material and Reagents

Ethical statement

Blood was extracted from cattle following an experimental protocol elaborated according to the ethics guidelines for animal care[9] and approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology (CIGB).

Serum collection and measurement of anti-Bm86 antibody titers by ELISA

Positive and negative anti-Bm86 antibody blood was collected from animals in units either under tick control programs

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with Gavac® vaccination or without vaccination. Blood was taken from healthy cattle, regardless of age, sex, or reproductive categories, and the sera were stored at –20oC until use.

ELISA measured the level of anti-Bm86 antibodies in sera. Briefly, ELISA microplates (Polysorp, Nunc, USA) were coated with Bm86 protein at 2 µg/mL and incubated overnight at 4ºC. Plates were washed with PBS-Tween 20 (0.05%), and blocked with 1% skimmed milk for 1 h at 37ºC. Sera was next diluted 1:500 in PBS-Tween 20 (0.05%) and 100 µL added to each well, and further incubated for 1 h at 37ºC. Next, a rabbit anti-bovine IgG antibody conjugated to horseradish peroxidase was added for 30 min at 37ºC. Finally, the reaction was developed with 0.5 mg/mL ortho-phenylenediamine and 0.015 % H_2O_2, and the reaction stopped 10 min later with H_2SO_4 (1%). The optical density (OD) was measured at 492 nm in an ELISA plate reader (Sunrise, Austria). A standard curve made with five 1:2 serial dilutions of an anti Bm86 positive reference serum reference, starting with 1:640, was used to interpolate the OD values of each serum. The anti Bm86 antibody values were expressed as absorbance units (AU)/mL. Three groups were formed according to the results of this evaluation:

Group 1. High responders: 209 positive samples with anti-Bm86 antibody values higher than 1000 AU/mL.

Group 2. Medium responders (gray zone): 150 samples with anti-Bm86 antibody values between 530 and 1000 AU/mL (minimum protective titers).

Group 3. Low responders: 239 samples with non-protective (negative) antibody values lower than 530 AU/mL.

Another group of negative sera with 100 samples from non-immunized animals was conformed.

HeberFast® Line Gavac

Bm86 protein and its conjugation to colloidal gold

The active pharmaceutical ingredient in GAVAC® vaccine was supplied by its manufacturer, the Center for Genetic Engineering and Biotechnology of Camagüey, Cuba. The conjugation of the Bm86 protein to colloidal gold was carried out according to Bailes et al., 2012[10] using homemade 20 nm colloidal gold particles. The protein was solubilized in 0.005 mol/L of sodium chloride. The colloidal gold was also adjusted to pH 7 with 0.1 M K_2CO_3.

The following flocculation test first determined the amount of protein in relation to a fixed volume of gold: 500 µL of 20 nm colloidal gold at pH 7 were mixed with 100 µL of the protein solution in a range between 4 and 20 µg. The mixture was homogenized and incubated for 10 min at room temperature, and 500 µL of 10% sodium chloride solution was added to each reaction mixture. Finally, the conjugate content in the mixture was quantified by measuring the absorbance at 530 nm in a spectrophotometer (Thermo Scientific GENESYS 10S UV-Vis, Madison, USA).

Once the optimal amount of protein had been selected, the reaction was conducted for 30 min at -20 °C -25 °C, stirring at 300 rpm. The free sites on the colloidal gold particles were blocked with an adequate volume of 10% BSA, for a final concentration of 0.5 %. The final mixture was centrifuged at 16000 x g for 1 hour at 4ºC. The conjugate obtained (pellet) was suspended in 0.1 M TBS, BSA 1% pH 7.0, and diluted up to an absorbance of 40 at a wavelength of 530 nm.

Preparation of the immunoreactive strip

The strip components were nitrocellulose (NC) membrane of 15 µm (MDI, type CNPC-SS12-L2-H50, dimensions 7,5 x

26 cm, 1,5 cm of NC), pad for the antigen-gold conjugate (MDI, type PTR-5, dimensions 2,7 x 26 cm), and absorbent pad (MDI, type AP080, dimensions 3,5 x 26 cm) . The conjugate was diluted to an absorbance of 20, at a wavelength of 530 nm and dispensed onto the antigen-gold conjugate pad.

The Bm86 protein, dissolved at 4 mg/mL in phosphate buffer saline (PBS) was absorbed into the membrane in the test line. The mixture of the control line components consisted of a monoclonal antibody against Bm86 protein provided by the Center for Genetic Engineering and Biotechnology of Sancti Spíritus, Cuba at 4 mg/ml, and 0.2 mg/mL of poly L-lysine, dissolved in PBS.

Both the macroporous material and the nitrocellulose membrane were sprayed with the BioDotQuanti 2000, BioJet, England, and then dried in an oven at 37 °C, for 30 min. The NC membrane cards were blocked for 1h at room temperature in blocking solution (0.02% (v/v) Tween 20, 0.02% (w/v) sodium azide, 5% (w/v) sucrose, and 0.5% bovine seroalbumin (BSA), dissolved in PBS. Later they were dried in the oven at 37 °C for 1 h.

Test strip assembly

The NC membrane was coupled in the center of polyvinyl chloride support. The absorbent pad and the conjugate pad were glued by overlapping 1 mm at the top and bottom of the NC Membrane, respectively. The sample pad was then glued by overlapping 2 mm on the bottom of the conjugate pad. The master assembled card was cut into 4 mm wide strips using a CM5000-guillotine-cutter (BioDot, Irvine, USA).

Immunochromatographic assay

The rapid, one-step, and visual reading lateral flow immunoassay (LFIA), branded HeberFast® Line Gavac, consists of a strip of nitrocellulose (NC) membranes to detect antibodies against the Bm86 antigen in bovine serum. The membrane consists of two phases: a fixed phase composed of the Bm86 protein as a capture biomolecule for the specific immunoglobulins present in the serum and a mobile phase containing a protein-colloidal gold marker conjugate, which finally develops the reaction with a specific dye.

Each strip was introduced into 80 µL of bovine serum (previously diluted 1:8 in buffer). The diluted sera migrated by capillarity within the macroporous material, dissolving the Bm86 conjugate labeled with colloidal gold. Two independent reactions could be observed in the virtual window. If the sample was positive, the immune complex reacted with the Bm86 protein attached to the NC membrane, forming a first horizontal redline Positive Line (PL). For all samples, the free conjugate was captured in a second red horizontal line Control Line (CL) where a polyclonal anti-IgG murine antibody is fixed to the solid phase.

Performance evaluation

Strips were used to detect antibody responses in 698 samples of bovine sera, from598 vaccinated and 100 unvaccinated animals. The results of the samples were interpreted at two moments, at the end of the reaction 10 min after the sample was applied and once the strip dried. LFIA tested samples in triplicate. The specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), efficacy as well as kappa coefficient (K), were determined.

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Kappa coefficient (K)

The Kappa coefficient was calculated to assess the level of significance of the agreement between the ELISA and the LFIA. The following ranges and their respective performance criteria were defined: 0.81-1.00, almost perfect agreement; 0.61-0.80, reasonable agreement, 0.60-0.41, moderate agreement; 0.40-0.21, discrete agreement, 0.20-0.01 insignificant agreement, and lower than 0.01, no agreement[11].

Batch to batch consistency

The samples from all panels were all evaluated by the three batches of strips (Gavac1501, Gavac1502, and Gavac1503). Results were considered valid for samples with coincident results with two different strip batches.

Inter-analyst interpretation

Three analysts read each sample, and results were regarded as valid when two out of the three observers coincided.

Statistical analysis

The LFIA and the ELISA reference system results were statistically compared using the epidemiological analysis of tabulated data (EPIDAT) software, version 3.1[12]. Concordance, sensitivity, specificity, efficacy, positive and negative predictive values, batch to batch consistency, and inter-analyst interpretation were compared. Statistical significance was considered for a 95 % confidence interval.

Results

Evaluation of the panel of samples from unimmunized animals.

All sera from unimmunized animals were negative. Only one signal appeared in the diagnostic control line, which guarantees the validity of the test. This represented a 100% of specificity for the system. Four representative samples from this group are shown in figure 1.

Evaluation of samples from immunized animals

All the strips used were accepted as valid, as the control line indicating their quality and reliability was observed. The intensity of the test capture region line varied depending on the concentration of immunoglobulins in the sample.

The overall false positive rate fluctuated between 3.34% and 6.69%, while a range of 4.78%-5.26% was found for false-negative results for the different lots and times evaluated. As expected, the percentage of false negative and false positive values was highly dependent on anti-Bm86 antibodies in the samples. Samples from group 2 (medium responders) showed the greatest incongruities when compared to the reference ELISA (36% - 38.7% of false negatives) (Table 1). The percentage of false-negative values dropped to 4.8%-5.2% in the group of high responders. On the other hand, the percentage of false positives in the ELISA negative sera was only 3.4-7.1%. Figure 1 shows 4 representative samples from each of the three groups evaluated.

The general agreement of the immunochromatographic strips with respect to ELISA was good for the 3 batches evaluated based on the kappa coefficient (Gav1501, Gav1502, and Gav1503) (Table 2).

The results of sensitivity, specificity, efficacy (test validity index), positive and negative predictive values, and confidence intervals of the 3 batches in studies are shown in Table 3.

Consistency between batches

The agreement among the three batches of strips evaluated (Gav1501, Gav1502, and Gav1503) is shown in Table 4. All batches exhibited high kappa coefficients, which indicate a good, or almost perfect degree of concordance. These results demonstrated the consistency of the manufacturing process.

Independence of the analysts

The concordance among the three analysts that read the assays ranged from good to almost perfect as the concentration of immunoglobulins increased. The results are shown in Table 5. The samples evaluated in the two moments gave no significant differences.
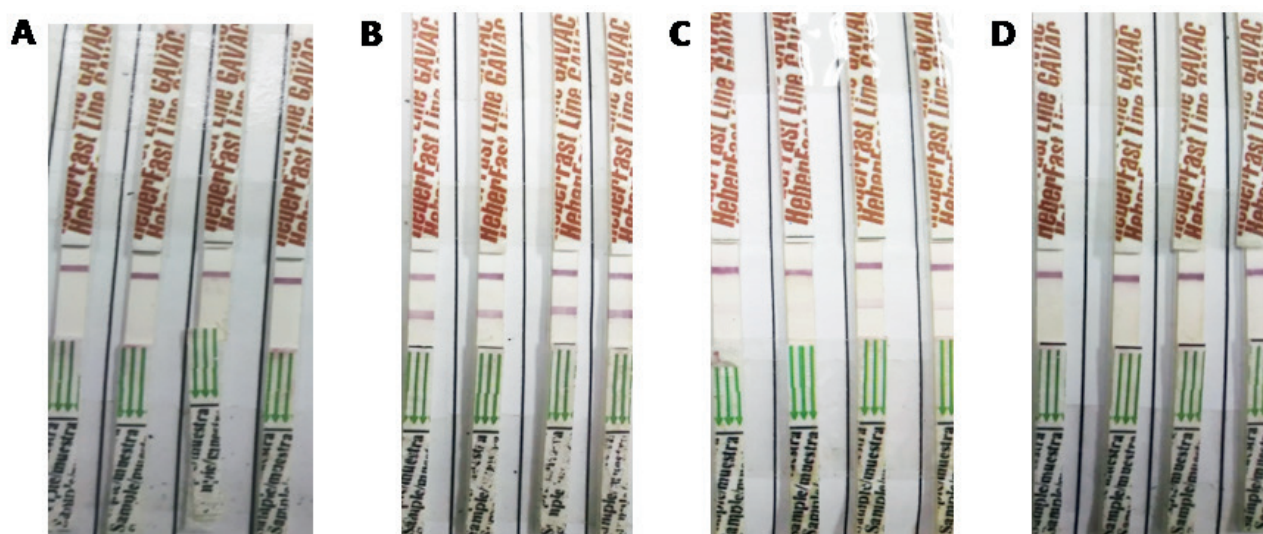

Figure 1. Evaluation of sera from Gavac immunizes animals and unvaccinated controls. Four representative samples per group were included. A: unvaccinated control group; B: high responders, (>1000 AU/mL); C: medium responders (530 - 1000 AU/mL); D: low responders (>530 AU/mL).

anti Bm86 ELISA values (n)	Lot Gav1501				Lot Gav1502				Lot Gav1503			
	reading 1		reading 2		reading 1		reading 2		reading 1		reading 2	
Group 1 > 1000 AU/mL (209)	P	N	P	N	P	N	P	N	P	N	P	N
	198	11	198	11	199	10	199	10	199	10	199	10
	94.8 %	5.2%	94.8 %	5.2%	95.2 %	4.8%	95.2 %	4.8%	95.2 %	4.8%	95.2 %	4.8%
Group 2 530-1000 AU/mL (150)	P	N	P	N	P	N	P	N	P	N	P	N
	95	55	96	54	96	54	97	53	92	58	92	58
	63.3%	36.7%	64%	36%	64%	36%	64.7%	35.3%	61.3 %	38.7%	61.3 %	38.7%
Group 3 <530 AU/mL (239)	P	N	P	N	P	N	P	N	P	N	P	N
	8	231	8	231	13	226	13	226	16	223	17	222
	3.4%	96.6%	3.4%	96.6%	5.4%	94.6%	5.4%	94.6%	6.7%	93.3%	7.1%	92.9%

Reading 1: reading done immediately after the end of the assay (10 min); reading 2: reading done after the strip was dried. The percentages represent the % of agreement of the positive or negative values with respect to the ELISA.

Table 1. Evaluation of the diagnostic strips with a panel of positive anti-Bm86 sera.

Parameters	Reading	Gav1501	Gav1502	Gav1503
Kappa	1	0.75	0.74	0.71
	2	0.74	0.74	0.71
CI	1	0.70-0.80	0.69-0.79	0.66-0.77
	2	0.69-0.80	0.69-0.80	0.66-0.77
Agreement	1	Good	Good	Good
	2	Good	Good	Good

CI, confidence intervals

Table 2. General agreement between the immunochromatographic assay and the reference ELISA.

Parameters	Reading	Gav1501	Gav1502	Gav1503
Sensitivity	1	81.6 (77.5-85.8)	82.2 (78-86,3)	81 (76.9-85.2)
	2	81.9 (77.8-86.3)	82.4 (78.4-86.5)	81 (76.9-85.2)
Specificity	1	96.7 (94.2-99.2)	94.6 (91.5-97.6)	93.3 (89.9-96.7)
	2	95.4 (92.5-98.3)	94.1 (91-97.3)	92.9(89.4-96.4)
Efficacy	1	87.6 (84.9-90.3)	87.1 (84.4-89.9)	85.9 (83- 88.8)
	2	87.3 (84.5-90)	87.1 (84.4-89.9)	85.8 (82.9- 88.7)
PPV	1	97.3 (95.4-99.3)	95.8 (93.4-98.2)	94.8 (92.2-97.4)
	2	96.4 (94.2-98.6)	95.5 (93-98)	94.5 (91.8-97.2)
NPV	1	77.8 (72.9-82.7)	77.9 (73-82.9)	76.6 (71.6-81.7)
	2	77.8 (72.9-82.7)	78.1 (73.2-83)	76.5 (71.5-81.6)

Average (95 % confidence interval). PPV: positive predictive value, NPV: negative predictive value.

Table 3. Performance of the immunochromatographic system.

Discussion

Tick mitigation strategies are part of a long tradition and focus on the local application of acaricides or the search for biological products that reduce the impact of the problem[13]. The use of the Gavac® immunogen within a control program decreases the reproductive parameters of the tick through the specific antibodies developed in the immunized animals[14]. Moreover, the fast development of anti-Bm86 antibody levels guarantees herds immunity and the effective control of tick populations[4].

In this setting, the development of rapid, specific, sensitive, and economically feasible diagnostic methods such as LFIA is of paramount relevance in veterinary medicine. They support the massive screening of samples in a shorter time under field conditions and the early detection of pathogens, further providing specific treatments and monitoring for the control epidemiological units[15].

In this study, the performance of the HeberFast® Line Gavac immune screening test was evaluated for detecting anti-Bm86 antibody titers in sera of immunized cattle. The sensitivity and specificity of the immunochromatographic system for the three batches under study were in the 81-82% and 92-95% ranges, respectively. Similar results have been previously reported for the use of LFIA strips for massive screening. For instance, the direct Sensitive Membrane Antigen Rapid Test

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Batches	Positivity criteria	Reading	Parameters			
			Kappa	IC 95 %	P. estad. z	Consistency
Gav150 1	Positive	1	0.9252	0.8177-1.0000	23.1678	Almost Perfect
		2	0.9321	0.8350-1.0000	23.3405	
	Gray zone	1	0.8782	0.8135-0.9429	18.6291	Almost Perfect
		2	0.8866	0.8236-0.9495	18.8067	
	Negative	1	0.7459	0.5548-0.9368	19.9723	Good
		2	0.7221	0.5500-0.8941	19.3360	
Gav150 2	Positive	1	0.8915	0.7640-1.0000	22.3240	Almost Perfect
		2	0.8915	0.7640-1.0000	22.3240	
	Gray zone	1	0.9226	0.8697-1.0000	19.5719	Almost Perfect
		2	0.9319	0.8818-0.9820	19.7690	
	Negative	1	0.9494	0.8768-1.0000	25.4224	Almost Perfect
		2	0.9526	0.8847-1.0000	25.5071	
Gav150 3	Positive	1	0.9661	0.8959-1.0000	24.1902	Almost Perfect
		2	0.9661	0.8959-1.0000	24.1902	
	Gray zone	1	0.9344	0.8862-0.9826	19.8218	Almost Perfect
		2	0.9443	0.9001-0.9886	20.0325	
	Negative	1	0.8925	0.7972-0.9877	23.8984	Almost Perfect
		2	0.8981	0.8079-0.9883	24.0491	

1: reading immediately after the end of the assay (10 min); 2: reading after the strip was dried

Table 4. Consistency in production.

(SMART®; New Horizons Diagnostics Corp., USA) and the direct Pathogen Detection Kit® (PDK; Intelligent Monitoring Systems, USA) were reported to display 100 % sensitivity for cholera diagnosis in diarrheal and semi-formed stool samples, and in intestinal contents of corpses. Furthermore, 100 % specificity was also attained with SMART®, while the same parameter for direct PDK® ranged between 77.4% and 85.7%, depending on sample type[16]. In immunochromatographic studies to detect Giardia spp. and Cryptosporidium spp. in feces, 58-97.2 % sensitivity and 99-100 % specificity were reported[17,18].

In summary, diagnostic tests must perform with high specificity and sensitivity to avoid false positives or false negative results. This is more relevant when diagnosing severe illnesses or other conditions able to compromise herd health and integrity and devoid of any contingency measures other than animal sacrifice. A misdiagnosis can lead to serious economic losses in those situations, as forum treatable pests such as Foot and Mouth Disease, African Swine Fever, Classical Swine Fever, and similar[19,20].

Since the HeberFast® Line Gavac strip will be applied to determine the immune status of animals vaccinated with Gavac®, the specificity of this test is more critical than its sensitivity. Consequently, the essay must exhibit a low fraction of false positives when assessing post-vaccination immunity. The assay's specificity was in the range 92.9-95.4% for the three batches tested, well above its sensitivity values (81-82.4%).

In veterinary medicine, lateral flow immunoassays have been developed for various purposes; detection of diseases in companion and farm animals to achieve better results in the care and development of animals, for example, Parvovirus[21], Newcastle[22], Avian infectious bronchitis virus[23], African swine fever[24] and Babesiosis[25], The rapid detection of these diseases reduces the economic losses. Immunochromatographic methods have also been developed to detect antibiotics and analogs in milk[26], which is greatly important for food safety.

In general, this test offers advantages and benefits, especially in the field, due to its fast and safe use and relatively low production costs. Moreover, it does not require equipment for readouts, and the results are easily interpreted with the naked eye, with high specificity and sensitivity in just 10 min. Finally, this immunoscreening test has been developed to work with serum, a sample that can be easily collected and processed.

HeberFast® Line Gavac is the first rapid kit worldwide for the detection of antibodies against the Bm86 protein. Its introduction in the field could facilitate the follow-up of the immune response against any vaccine based on this antigen and determine the level of immunity achieved in the vaccinated herds.

Analyst	Positivity criteria	Reading	Parameters			
			Kappa	IC 95 %	P. estad. z	Consistency
Analyst 1	Positive	1	0.8643	0.7185-1.0000	21.6410	Almost Perfect
		2	0.8643	0.7185-1.0000	21.6410	
	Gray Zone	1	0.7113	0.6189-0.8037	15.0891	Good
		2	0.7008	0.6068-0.7947	14.8658	
	Negative	1	0.6283	0.4695-0.7871	16.8247	Good
		2	0.6283	0.4695-0.7871	16.8247	
Analyst 2	Positive	1	0.8184	0.6665-0.9703	20.4936	Almost Perfect
		2	0.8184	0.6665-0.9703	20.4936	
	Gray Zone	1	0.7164	0.6238-0.8089	15.1969	Good
		2	0.7157	0.6227-0.8088	15.1828	
	Negative	1	0.6665	0.4843-0.8487	17.8478	Good
		2	0.6558	0.4799-0.8316	17.5602	
Analyst 3	Positive	1	0.8950	0.7657-1.0000	22.4101	Almost Perfect
		2	0.8950	0.7657-1.0000	22.4101	
	Gray Zone	1	0.7150	0.6223-0.8078	15.1684	Good
		2	0.7041	0.6096-0.7985	14.9362	
	Negative	1	0.7221	0.5414-0.9028	19.3360	Good
		2	0.6746	0.5014-0.8478	18.0639	

1: Reading once the technique

Table 5. Analyst Interpretation.

Conclusions

The HeberFast® Line Gavac immunochromatographic system allows the determination of the anti-Bm86 serological status quickly and straightforwardly to a large number of samples in field conditions with minimal effort. The introduction of this test would significantly improve the follow-up of the antibody response to GAVAC in all the country as part of the Integrated Control Program for cattle tick control.

Acknowledgements

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