Porcine cysticercosis: first evaluation of antigens from the liquid of cyst for the development of a serological test for Livestock

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Abstract

Background: In low-income countries, rapid discard of cysticercosis suspected pigs from the meat chain, is a key step to preserve customers. Unfortunately, in most place only rapid visual inspection of carcasses is practiced. Despite their fluctuant specificity serological tests performed in living animals before entry at slaughterhouse, could be a better and easier approach to promote. However, recombinant antigens must be developed and evaluated to improve accessibility of serological diagnosis. In this study, five recombinant antigens have been expressed and evaluated in Elisa in comparison with electro-immune transfer blot (EITB).

Results Antigens were expressed in a baculovirus-insect cell system, purified by affinity chromatography and evaluated on pig serum. Sensitivity, specificity, concordance, and positive likelihood ratio of each recombinant antigen were calculated in reference to EITB. Sensitivityvalues ranged from 43.9% (34.5 - 53.8) to 88.8% (80.8 - 93.7). Specificities ranged from 37.5% (28.5 - 47.5) to 65.6% (55.7 - 74.3). To improve performance of the tests, combination of antigens was used rising sensitivity between 90.8% and 94.9%. Compared to visual examination of carcasses as performed in slaughterhouses these combinations only missed 1% to 5% of infection. Specificities were lower but at the same level as the native antigen ELISA.

Conclusion: These recombinant antigens have sensitivity and specificity in the same range as EITB and can be now evaluated against the necropsy as gold standard. Using such tests, cysticercosis suspected animals could be treated with oxendazole before entering the market chain to improve the management of animals at the farm level.

1. Introduction

Swine cysticercosis is caused by the development of the *Taenia solium* larva in pigs after ingestion of eggs from contaminated human feces (Copado 2004). Swine cysticercosis is usually asymptomatic and overlap with the distribution of taeniasis one (Raghava 2010). It varies greatly with the quality of sanitation (i.e. artisanal latrines draining into rice fields, custom of open defecation...), pig rearing practices (animals left to roam), and dietary habits of the region (undercooked meat, artisanal sausages) (Jambou 2017). Consumption of water or eggs infected food is also a hidden source of contamination (Dorny 2009; Thompson & Conlan, 2011). Cysticercosis is a cosmopolitan zoonosis but with a higher prevalence in developing countries where poor husbandry and hygiene conditions are frequent (Gabriël 2012). Investigated mostly by serological methods, the sero-prevalence of porcine cysticercosis ranges in Africa, from 0.06–39.8%, with the highest rate observed in Chad (Assana 2001; Ngowi 2010; Porphyre 2016). This parasite is therefore responsible for a significant financial loss for pig farmers in endemic areas as carcass are supposed to be destroyed when found infected during inspection at slaughterhouses. Indeed, swine cysticercosis can be detected in live animals by palpation of the tongue, and by inspection of the meat after slaughter (Gonzalez 1990). Tongue probing is a very specific technique, but poorly sensitive, as only massively infected animals can be detected by well-trained prober (Phiri 2006). Carcass inspection, conducted when human resources are sufficient to organize it, is a retrospective diagnosis that can preserve consumer health but which does not prevent financial loss to the farmer. Serological tests practiced in the herd could therefor allow the detection of cysticercosis infected animals before slaughter, paving the way of a simple treatment of the animals (Deckers & Dorny, 2010; Jayashi 2012). Indeed, the main objective of a screening at farm gate or at the entry of slaughter houses, is not to do a real diagnostic of pig cysticercosis (which could only be confirmed by full necropsy of the animal), but to discard suspected animals and to propose treatment for them. Indeed, in low-income countries where pig rearing supports poor family incomes, destruction of carcass is not a socially accepted strategy, and leads to circumvention of controls.

Four biological approaches can be commonly used for the selection of potentially infected animals, the ELISA method and Western Blot for the detection of antibodies (Gekeler 2002; Furrows 2006; Arroyo 2018), ELISA detection of circulating antigens, and PCR detection of DNA in the blood. All these techniques need laboratory facilities not available at the farm. Lateral flow tests should be the easiest way to perform tests, even by farmers themselves. Moreover, most reference serological tests use native antigens extracted from the *Taenia solium* parasite, which is not widely available, impairing large production of test for herds. Serological methods are useful test to screen livestock, even if their sensibility and specificity can be poor. The immune electrotransfert blot (EITB) developed by Tsang et al using glycosylated native proteins is widely used as reference test despite it debating performance according to the number of cyst in the animal and of bands used for the diagnostic. Therefore, as a witness of contact between the parasite and the immune system of the animal, the antibodies can persist after disappearance of the cyst. Overall, the specificity of the IETB can be lower than 50% when parasite load is low (Jayashi 2013; Gavidia 2013).

Facing the need of an easy to perform method for the discard of suspected animal from the meat chain, new antigens must be identified to develop a lateral flow test. During previous studies, antigens from the liquid of the cyst were selected using serum from *T. solium* infected pigs and then identified and cloned (Nativel 2016). As a preliminary step for the selection of new antigens usable at the farm gate, this study was conducted to evaluate the diagnostic value of five of these recombinant antigens in comparison with IETB according to Tsang et al. (1989). This non-inferiority approach conducted using ELISA method was design to selected antigens which could be used further for evaluation against necropsy. The sensitivity, specificity, positive and negative predictive values of the recombinant antigens were determined for antigens used separately and then the performance of combination of proteins was evaluated. This evaluation was performed using the ELISA method and a bank of sera from *T. solium* infected or not infected pigs randomly collected in slaughterhouses in Madagascar (N´dri 2022).

2. Materials and Methods

2.1 Type of study and pig sera

The study was performed as a retrospective analysis based on a serum library collected during a cross-sectional study conducted in three slaughterhouses in Antananarivo, the capital city of Madagascar (N´dri 2022). Animals arrived at slaughterhouses from different regions of the country to enter the local market of Antananarivo, without any previous selection. They follow the usual process applying in these structures, i.e. slaughtering and visual inspection of the carcass by veterinarians according to Goussanou et al. This method uses only incision of the heart, muscles of the neck, head, tongue and psoas muscles (Goussanou 2013) which is far from necropsy performance.

For each animal included, 5 mL of blood was collected in a dry tube at the time of slaughter and then placed in a cooling container. Blood samples were transported to the laboratory at the end of each day. Samples were then centrifuged at 3000 rpm for 5 minutes and sera were collected and stored at -20°C.

2.2 Antigens preparation

Total native antigens were produced from cysticerci collected from infected pigs (Tsang 1989). As previously described (Melki et al in press), five recombinant antigens have been produced in *E.coli* system (R93, R914, R915) and baculovirus (for the glycosylated T14 and T18). Derivate from the standard WB according to Tsang et al, the recombinant T14 and T18 antigens have been previously evaluated in the literature (Greene 2000; Assana 2007). Recombinant proteins R93, R914 and R915 have been selected by Nativel et al. from *T. solium* cyst fluid (Nativel 2016). All antigens were produced and purified by the Recombinant Protein Platform of the Institut Pasteur in Paris. The recombinant proteins purified by affinity chromatography.

Briefly *Spodoptera frugiperda* (Sf9, Invitrogen) insect cells were grown in monolayer or suspension cultures at 27°C, respectively, in Insect-XPress protein-free medium (Lonza, Switzerland) supplemented with 4 mM glutamine (Gibco-BRL). Viral stocks were produced in Sf9 cells cultured with 5% fetal calf serum and 50 µg/ml gentamycin and stored at 4°C. The synthetic gene codon optimized for insect and coding for T14 was combined with gp64 signal peptide and 6xHIS tag, then was cloned into PVL1393 transfer vector (Expression Systems, CA USA). The synthetic gene codon optimized for insect and coding for T18 was combined with gp64 signal peptide, the SUMOstar sequence and a 6xHIS tag, and was cloned into PVL1393 transfer vector. In both cases, a TEV site was introduced between the 6xHistag and the protein of interest.

The vectors were co-transfected with Bestbac2.0 (Expression Systems, CA USA) in SF9 cell line. After 5 hours, Sf9 was supplemented with 4 ml Insect Xpress 5% FCS and 50 µg/ml gentamycin. After 7 days, the culture was centrifuged at 2000 rpm for 10 minutes and a supernatant was kept for amplification. The recombinant virus particles were amplified in T25 flask containing $5x10^6$ sf9 supplemented with 5 ml Insect Xpress 5% FCS and 50 µg/ml gentamycin. After 6 days, the recombinant virus was isolated from the culture supernatant by end point dilution assay. One viral clone was amplified successively in T25, T150 and 500 erlen flasks. Viral stocks were produced in SF9 insect cells, titrated and stored at 4°C. SF9 cells (3.10^9 cells) were infected with recombinant baculovirus for 3 days and the supernatant, containing the recombinant proteins, was then harvested by centrifugation for 45 min at 8000 rpm at 4°C.

For purification, the supernatant was concentrated 10-fold by tangential flow filtration on akta flux (Cytiva, Velizy France) using a 10kDA MWCO cassette.

The concentrate was then filtered through on a 0.2µm stericup filter (Millipore MA USA). The concentrated supernatant was injected onto a 1ml histrap column (Cytiva, Velizy, France). The column was then washed with 50mM phosphate buffer; 300mM NaCl; 10mM Imidazole; pH = 7.5. After that, the protein was eluted by a gradient of 50mM Buffer; 300mM NaCl; 500 mM Imidazole; pH = 7.5 from 0 to 100% during 10 column volume.

The fractions were analyzed on a 4–12% SDS PAGE gel (BIORAD, Marne-la-coquette France). Fractions containing the protein of interest were pooled and introduced into a dialysis device Slide-A-lyser, 3.5kDa MWCO with 1mg of TEV protease. The device was dialyzed overnight in 2 liters 50 mM phosphate buffer; 300mM NaCl; pH = 7.5. The content was then injected in a 1ml histrap column. The cleaved protein was not retained by the column. The flow-through fractions were analyzed on SDS PAGE gel and then frozen at -80°C.

2.3 Serological analysis

For ELISA-IgG analysis, recombinant antigens were coated on 96-well polystyrene plates (nunc imuno plate, maxisorp, Thermo Scientific) in a 1x PBS at a concentration of 1µg/mL for T18 and T14, and 0.1µg/mL for R93, R914 and R915. After dilution, 100µL of antigen were added to each well (one antigen/plate) and incubated at 4°C overnight. After this step, the plate wells were washed three times with the wash buffer (PBS 1X + 0.2% Tween20). The saturation of non-specific sites, the incubation of sera and of the conjugate were done at 37°C for 1 h. These three steps were interspersed with a 3-cycle and a 5-cycle wash, respectively. A PBS buffer 1X-0.2%tween20 + 5% none fat dry milk was used for saturation. Serum and conjugates (peroxidase labelled anti-pig IgG-FC, SIGMA-ALDRICH) were diluted to 1:500 and 1:20000, respectively. Substrate buffer (100µL at 0.48mg/mL OPD, 0.22% H_2O_2 and citrate buffer) was added to each well and incubated at room temperature for 20 minutes for R93, R914 and R91, and 10 minutes for T14 and T18. The reaction was stopped by adding 50 µL of H_2SO_4 (2.5N).

Native purified glycoprotein antigens were used for ELISA and Western Blot tests according to Tsang et al as previously described by Jambou et al (2020). Details of these protocols can be found at: www.protocols.isoxxx. The Western Blot method using native antigen was the reference test during this study (Tsang, 1989).

2.4 Data analysis

This study is based on a comparison of methods using a well none (even imperfect) test and not on an estimation of the disease of the animal. For all ELISA tests, results were obtained using only one antigen per well of the plate. For each plate, six negative serums (for animals living in cysticercosis non-endemic countries) were tested in duplicate to determine the threshold of positivity as follows: Threshold = 2x[Mean (OD negative samples) + 3x Standard deviation (OD negative samples)]. Samples with mean ODs greater than or equal to the positivity threshold were declared positive for the antigen.

However, to improve performance of the tests, during statistical analysis two strategies were performed to combine the results, an alternative combination (a sample was finally reported as positive when it is positive for at least one antigen) and a cumulative combination (a sample was finally reported as positive when it is positive for at least two or more antigens in the same time) (Fig. 1). The first strategy was intended to increase sensitivity, whereas the latter supported specificity. A descriptive statistical analysis was carried out with XLSTAT 2014.5.03 and the proportions of positive and negative serum were determined with a 95% confidence interval for each antigen. Sensitivity, specificity, predictive values and positive likelihood ratio of each antigen test were calculated using the EITB as reference with a 95% confidence interval. The same analysis was performed using the results of the combination of antigens.

In a second time, ELISA tests were compared with the result of the visual inspection of carcass, considered as the gold standard. Cohen's Kappa test was used to measure the agreement between the ELISA tests and the carcass inspection technique according to Khanaliha et al. (2014). The raw agreement between the ELISA tests using the recombinants and the reference test was calculated as [(TP + TN)×100/(TP + TN + FP + FN)], were TP, TN, FP, and FN represents the number of true positives, true negatives, false positives, and false negatives, respectively.

3. Results

Serum used

For this study 194 serums have been analyzed (Table 1) among which, 98 were positive in EITB. For these positive serums, 87 were positive in ELISA-CS50 among which 71 were collected on animals with a positive carcass whereas 25 animals were negative in visual inspection of the carcass. For the 96 serums negative in EITB, 25 were positive in ELISA-CS50 and 28 were from animals with positive carcass (which confirms default in sensibility of EITB).

Recombinant antigens used in singleton

In comparison with EITB, the sensitivity values obtained for recombinant antigens used individually ranged from 43.9% (ELISA-R93) to 88.8% (ELISA-T18 and ELISA-CS50). These values ranged from 48.5% (ELISA-R93) to 97% (ELISA-CS50) when compared with carcass inspection. The specificity ranged from 37.5% (ELISA-T18) to 65.6% (ELISA-R915), and from 46.2% (ELISA-T18) to 72% (ELISA-R915) when compared with EITB and carcass inspection, respectively. The positive likelihood ratios were all low with 1.1 as the lowest value and 2.3 as the highest (Table 2).

Recombinant antigens used in combination

To improve the sensibility and specificity of the analysis, results of antigens were grouped in duplex or in triplex, either as cumulative or alternative methods as previously defined.

The results from the duplex combinations are shown in Table 3 in comparison with the EITB or with visual inspection of carcass. For all these combinations, the sensitivities ranged from 33.7% (C2 = T14 + R93) to 94.9% (A5 = T18 + R93) when determined against Western Blot, and from 41.6% (C2 = T14 + R93) to 99% (A6 = T18 + R914) when determined using carcass inspection as the gold standard. Specificities were also variable and ranged from 24% (A6) to 74% (C4) and 30.1% (A6) to 88.1% (C1) when Western Blot and carcass inspection were considered as reference respectively.

For triplex combinations (Table 4), sensitivities ranged from 50% (C20) to 94.9% (A11, A17 and A18), and from 57.4% (C20) to 99% (A12, A17 and A19) when Western Blot and carcass inspection were considered as reference tests, respectively. The specificity values from these combinations ranged from 21.9% (A19) to 61.5% (C18), and from 28% (A17 and A19) to 71% (C15) when determined against Western Blot and carcass inspection, respectively.

In order to define the performance of these combinations as a tool to discard infected animals from the meat chain, agreement tests were calculated for the best duplex combinations (sensitivity higher than 90%) in comparison with carcass inspection (Table 5). Cohen's Kappa

coefficient ranged from 0.299 to 0.344, with a good false negative fraction (ranging from 1–5%), but a medium false positive fraction (58.1–69.9%).

4. Discussion

The global project aims to develop a farm gate point of care test to discard cysticercosis suspected animals from the meat chain. The preliminary performances of five recombinant antigens (T14, T18, R93, R914 and R915) have been first evaluated during this study by ELISA in simplex and in combination (duplex and triplex) with EITBas reference method. During slaughtering, a visual inspection of the carcasses was been conducted according to the local veterinarian rules and antigens have been evaluated in comparison with this method in a non-inferiority approach.

Recombinant T14 and T18 antigens were derived from the native glycosylated membrane proteins used for EITB. As expected, ELISA results obtained with these antigens were the most related with the EITB ones with a sensitivity of 88.8% and 80.6 for T18 and T14, respectively. However, their specificity was lower (less than 50%) but in the same range as for the native antigens ELISA itself. This lower performance could be explained by a difference in the glycosylation, as these recombinants were produced in a baculovirus-insect cell system which ensures a partial glycosylation probably different from the parasite itself. Another explanation can also be the use of 6 bands in western blot instead of a single antigen.

The other antigens originated from soluble proteins from the liquid of the cyst and not from the parasite membrane. They harbored lower performances. Aside biochemical difference with the Tsang' glycosylated antigens, this difference could be due to a difference in the delay of appearance of the antibodies. Indeed, the contact of these proteins with the host immune system could appear later during the infection, when the cyst begins to crack. The immune response induced should therefore correspond to older parasitic infections and thus poorly correlate with the one detected using Western Blot.

However, the objective of a screening test should be *in fine* a rapid selection of suitable animals for slaughter at the slaughterhouse gate or at farm and the removal of contaminated pigs from the market chain. A screening test should therefore have a good sensitivity in order not to "miss" any positive animal even if the specificity is lower. Even more, in a non-inferiority point of view, a "acceptable" test should be as good as with the visual inspection of the carcass already practiced in low-income countries.

To improve the sensitivity of the recombinant antigens, combinations of the results were tested. Six duplex combinations harbored a better sensitivity ranging from 90.8–94.9%. They all included both antigens derivate from proteins of the membrane and of the liquid of cyst. However, they all showed a specificity below 50% in comparison with EITB which can also have low performance according to the parasite load. These tests should only be conducted in living animals, to discard them from the market chain and to treat them with oxfendazole.

Other antigens have been proposed in the literature with various sensitivities, such antigens proposed by Leon-janampa et al. in 2019 (83,33%, León-janampa 2019), by Kabululu et al. in 2020 (82.7%, Kabululu 2020), by Nhancupe et al. in 2019 (27.9%, Nhancupe 2019) and by Bustos et al. in 2019 (82.9%, Bustos 2019). Other tests as also good specificity with 83.78%, 96.8%, 71.7% and 86.3% respectively (Bustos 2019; León-janampa 2019; Nhancupe 2019; Kabululu 2020) which allows confirmation of the disease and culling of animals on the production line. However, these tests had lower sensibility, which compromises the capacity to accurately screen animals and protect customers.

Finally, to evaluate the usefulness of our recombinant antigen combinations for pork survey, serology results were compared with visual carcass inspection. The inspection carried out following the ministry recommendations was far from the experimental dissection of meat used during experimental studies, but was the go-no-go evaluation conducted in all slaughterhouses. Using recombinant ELISA, the false negative rates were very low, ranging from 1–5% depending of the combination used. This confirms the non-inferiority of these antigens and their usefulness as a replacement for carcass inspection. The use of a rapid test for the screening of animals could be a major improvement in the control of the meat chain, as very few veterinarians are available for such inspections, especially in outlying towns. The withdraw of putative infected animals before slaughter should be acceptable for farmers, as in contrast to post-mortem testing, positive animals can be treated and slaughtered later without economic loss.

The next step of evaluation of these antigens has to be the comparison with the full necropsy of the animals, to evaluate their real sensitivity and specificity for diagnostic, and evaluation of cross-reactivity and reproducibility of the serological tests based on these recombinant antigens.

Declarations

Ethics approval and consent to participate

This study has been approved by the Madagascar National committee on animal wealth and heath.

Consent for publication

Not applicable

Availability of data and materials

The dataset supporting the conclusions of this article is available upon request to the authors

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

NK performed the analysis and analyzed the data, wrote the first draft: RZN and TR collected the samples in the slaughterhouses in Madagascar ; RZN prepared the recombinants antigens in Paris; NM, DE and PS were in charge of the cloning and expression of the recombinant antigens; TOA and DAJ supervised the project, obtained funds and reviewed the text; JR coordinated the project and the analysis, obtained funds, supervised the writing and reviewed the final text of the article.

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Tables

Table 1. Characteristics of the serums used

		Carcass inspection		
WBCS50	**ELISACS50**	Negative	positive	Total
Negative	Negative	43	1	44
	Positive	25	27	52
		68	28	96
positive	Negative	9	2	11
	Positive	16	71	87
		25	73	98
Total		93	101	194

Table 2. Diagnostic values of recombinant antigens evaluated in singleton and of Elisa-CS50

	Western blot (gold standard)				Carcass inspection (gold standard)			
Tests	**Sensitivity (%)**	**Specificity (%)**	**Concordance (%)**	**PLR**	**Sensitivity (%)**	**Specificity (%)**	**Concordance (%)**	**PLR**
	(CI 95%)	**(CI 95%)**			**(CI 95%)**	**(CI 95%)**		
Elisa-CS50	88.8	45.8	67.52	1.63	97	55.9	77.3	2.2
	(80.8 – 93.7)	(36.2 -55.8)			(91.2 - 99.3)	(45.8 - 65.6)		
Elisa-T14	80.6	43.8	62.37	1.43	88.1	52.7	71.1	1.9
	(71.6 – 87.3)	(34.3 – 53.7)			(80.2 - 93.2)	(42.6 - 62.5)		
Elisa-T18	88.8	37.5	63.4	1.42	96	46.2	72.2	1.8
	(80.8 – 93.7)	(28.5 - 47.5)			(89.8 - 98.7)	(36.5 - 56.3)		
Elisa-R93	43.9	60.4	52.06	1.1	48.5	65.6	56.7	1.4
	(34.5 - 53.8)	(50.4 - 69.6)			(39 - 58.1)	(55.4 - 74.4)		
Elisa-R914	58.2	48.5	52.06	1.07	65.3	53.8	59.8	1.4
	(48.3 - 67.4)	(36.2 - 55.8)			(55.6 - 73.9)	(43.7 - 63.5)		
Elisa-R915	58.2	65.6	61.85	1.69	63.4	72	67.5	2.3
	(48.3 - 67.4)	(55.7 - 74.3)			(53.6 - 72.1)	(62.2 - 80.1)		

CI: Confidence interval; PLR: positive likelihood ratio

Table 3. Diagnostic values of recombinant antigens duplex combined

	Western Blot (gold standard)					carcass inspection (gold standard)				
Combination	**Sensitivity (%)**		**Specificity (%)**		**Concordance (%)**	**PLR**	**Sensitivity (%)**		**Specificity (%)**	**Concordance (%)**	**PLR**
C1	80.6	(71.6 - 87.3)	44.8	(35.2 - 54.8)	62.88	1.46	53.8	(43.7 - 63.5)	88.1 (80.2 - 93.2)	71.6	1.9
C2	33.7	(25.1 - 43.5)	71.9	(62.1 - 79.9)	52.57	1.2	41.6	(32.5 - 51.3)	80.6 (71.3 - 87.4)	60.3	2.1
C3	48	(38.3 - 57.7)	64.6	(54.6 - 73.4)	56.18	1.35	56.4	(46.7 - 65.7)	74.2 (64.4 - 82)	64.9	2.2
C4	48	(38.3 - 57.7)	74	(64.3 - 81.7)	60.82	1.84	55.4	(45.7 - 64.7)	82.8 (73.7 - 89.2)	68.6	3.2
C5	37.8	(28.8 - 47.7)	69.8	(59.9 - 78.1)	53.6	1.25	46.5	(37.1 - 56.2)	79.6 (70.1 - 86.5)	62.4	2.3
C6	53.1	(43.3 - 62.6)	59.4	(49.4 - 68.6)	56.18	1.31	62.4	(52.6 - 71.2)	69.9 (59.9 - 78.3)	65.9	2.1
C7	53.1	(43.3 - 62.6)	71.9	(62.1 - 79.9)	62.37	1.89	61.4	(51.6 - 70.3)	81.7 (72.5 - 88.3)	71.1	3.3
C8	39.8	(30.7 – 63.5)	63.5	(53.5 – 72.5)	51.55	1.09	46.5	(37.1 - 56.2)	71 (61 - 79.2)	58.2	1.6
C9	39.8	(30.7 - 49.7)	71.9	(62.1 - 79.9)	55.67	1.41	44.5	(35.2 - 54.3)	77.4 (67.8 - 84.7)	60.3	2
C10	48	(38.3 - 57.7)	70.8	(38.3 - 55.67)	70.8	1.64	51.5	(41.9 - 61)	75.3 (65.5 - 82.9)	63	2.1
A1	88.8	(80.8 - 93.7)	36.5	(27.5 – 46.5)	62.88	1.4	96	(89.8 - 98.7)	45.1 (35.4 - 55.27)	71.6	1.7
A2	90.8	(83.2 – 95.2)	32.3	(23.8– 42.2)	61.85	1.34	95	(88.6 - 98.1)	37.6 (28.5 - 47.8)	67.5	1.5
A3	90.8	(83.2 – 95.2)	25	(17.4 – 34.6)	58.25	1.21	97	(91.2 - 99.3)	32.3 (23.6 - 42.3)	66	1.4
A4	90.8	(83.2 – 95.2)	35.4	(26.6 – 45.4)	63.4	1.41	96	(89.8 - 98.7)	41.9 (32.4 - 52.1)	70.1	2
A5	94.9	(88.2 – 98)	28.1	(20.1 – 37.9)	61.85	1.32	98	(92.5 - 99.9)	32.3 (23.6 - 42.3)	66.5	1.4
A6	93.9	(86.9 – 97.4)	24	(16.5 – 33.5)	59.28	1.23	99	(94 - 100)	30.1 (21.7 - 40.1)	66	1.7
A7	93.9	(86.9 – 97.4)	31.9	(22.9 – 41.1)	62.89	1.36	98	(92.5 - 99.9)	36.6 (27.5 - 46.7)	68.2	1.8
A8	62.2	(52.3 – 71.2)	42.7	(33.3 – 52.7)	52.58	1.09	67.3	(57.6 - 75.7)	48.4 (38.5 - 58.4)	58.2	1.3
A9	62.2	(52.3 – 71.2)	54.2	(44.2 – 63.8)	58.25	1.36	67.3	(57.6 - 75.7)	60.2 (50 - 69.5)	63.9	1.7
A10	68.4	(58.6 – 76.7)	40.6	(31.4 – 50.6)	54.64	1.15	77.2	(68 - 84.3)	50.5 (40.6 - 60.5)	64.4	1.6

C=Cumulative; C1=T14+T18, C2= T14+R93, C3= T14+R915, C4= T14+R915, C5= T18+R93, C6 = T18+R914, C7 = T18+R915, C8 = R93+R914, C9= R93+R915,

*C10= R914+R915.*A = Alternative; A1=T14+T18, A2= T14+R93, A3= T14+R914, A4= T14+R915, A5= T18+R93, A6= T18+R914, A7= T18+R915, A8= R93+R914,

A9= R93+R915, A10 = R914+R915 PLR: positive likelihood ratio

Table 4. Diagnostic values of recombinant antigens triplex combined

	Western Blot (gold standard)						Carcass Inspection (gold standard)					
Combination	Sensitivity (%)		Specificity (%)		Concordance(%)	PLR	Sensibility (%)		Specificity (%)		Concordance(%)	PLR
C11	84.7	(76.1 - 90.6)	40.6	(31 – 50.6)	62.88	1.43	93.1	(86.1 - 96.9)	50.5	(40.6 - 60.5)	72.7	1.9
C12	84.7	(76.1 - 90.6)	40.6	(31 – 50.6)	62.88	1.43	93.1	(86.1 - 96.9)	50.5	(40.6 - 60.5)	72.7	1.9
C13	85.7	(77.3 – 91.4)	40.6	(31.4 – 50.6)	63.4	1.44	94.1	(87.3 - 97.5)	50.5	(40.6 - 60.5)	73.2	1.9
C14	60.2	(50.3 – 69.3)	52.1	(42.2 – 61.8)	56.18	1.26	65.3	(55.6 - 73.9)	58.1	(47.9 - 67.6)	61.9	1.5
C15	63.5	(53.5 – 72.5)	60.2	(50.3 – 69.3)	61.85	1.6	66.3	(56.6 - 74.8)	71	(61 - 79.2)	68.6	2.3
C16	66.3	(56.5 – 74.9)	55.2	(45.2 – 64.8)	60.82	1.48	74.3	(64.9 - 81.8)	64.5	(54.36 - 73.5)	69.6	2.1
C17	61.2	(51.3 – 70.3)	49	(39.2 – 58.8)	55.15	1.2	66.3	(56.6 - 74.8)	54.8	(44.7 - 64.5)	60.8	1.5
C18	61.2	(51.3 – 70.3)	61.5	(51.4 – 70.6)	60.31	1.59	67.3	(57.6 - 75.7)	68.8	(58.8 - 77.3)	68	2.1
C19	68.4	(58.6 – 76.7)	52.1	(42.2 – 61.8)	60.31	1.43	76.2	(67 - 83.5)	61.3	(51.1 - 70.5)	69.1	2
C20	50	(40.3 – 59.7)	60.4	(50.4 – 69.6)	55.15	1.26	57.4	(47.7 - 66.6)	68.8	(58.8 - 77.3)	62.9	1.8
A11	94.9	(88.2 – 98)	28.1	(28.1 – 37.9)	61.85	1.32	98	(92.5 - 99.8)	32.3	(23.6 - 42.3)	66.5	1.4
A12	93.9	(86.9 – 97.5)	24	(16.5 – 33.5)	59.28	1.23	99	(94 - 100)	30.1	(21.7 - 40.1)	66	1.4
A13	93.9	(86.9 – 97.4)	31.3	(22.9 – 41.1)	62.89	1.36	98	(92.5 - 99.8)	36.6	(27.5 - 46.7)	68.6	1.5
A14	91.8	(84.4 – 96)	24	(16.5 – 33.5)	58.25	1.21	97	(91.2 - 99.3)	30.1	(21.7 - 40.1)	64.9	1.4
A15	91.8	(84.4– 96)	29.2	(21 – 39)	60.82	1.3	96	(89.8 - 98.7)	34.4	(25.6 - 44.5)	66.5	1.5
A16	91.8	(84.4– 96)	22.9	(15.9 – 32.4)	57.73	1.19	98	(92.5 - 99.8)	30.1	(21.7 - 40.1)	65.5	1.4
A17	94.9	(88.2 – 98)	22.9	(15.6 – 32.4)	59.28	1.23	99	(94 - 100)	28	(19.8 - 37.9)	64.9	1.4
A18	94.9	(88.2 – 98)	26	(18.3 – 35.7)	60.82	1.28	98	(92.5 - 99.8)	30.1	(21.7 - 40.1)	65.5	1.4
A19	93.9	(86.9 – 97.4)	21.9	(14.8 – 31.2)	58.25	1.2	99	(94 - 100)	28	(19.8 - 37.9)	64.9	1.4
A20	71.4	(61.7 - 79.4)	38.5	(29.4 - 48.6)	55.15	1.16	77.2	(68 - 84.3)	45.2	(35.4 - 55.3)	61.9	1.4

C=Cumulative, C11=T14+T18+R93, C12=T14+T18+R914, C13=T14+T18+R915, C14=T14+R93+R914, C15=T14+R93+R915, C16=T14+R914+R915,

C17= T18+R93+R914, C18 = T18+R93+R914, C19 = T18+R914+R915, C20 = R93+R914+R915.

A=Alternative, A11=T14+T18+R93, A12=T14+T18+R914, A13 = T14+T18+R915, A14 = T14+R93+R914, A15 = T14+R93+R915, A16 = T14+R914+R915,

A1 = T18+R93+R914, A18 = T18+R93+R914, A19 = T18+R914+R915, A20 = R93+R914+R915. PLR: positive likelihood ratio

Tableau 5. Level of agreement between combination results and carcass inspection

		Carcass inspection				
		Pos	**Neg**	**FNF(%)**	**FPF(%)**	**K**
A2	**Pos**	96	**58**	5	62,4	0,333
	Neg	**5**	35			
A3	**Pos**	98	**63**	3	67,7	0,301
	Neg	**3**	30			
A4	**Pos**	97	**54**	4	58,1	0,344
	Neg	**3**	30			
A5	**Pos**	99	**63**	2	67,7	0,311
	Neg	**2**	30			
A6	**Pos**	100	**65**	1	69,9	0,299
	Neg	**1**	28			
A7	**Pos**	99	**59**	2	63,4	0,354
	Neg	**2**	34			

Pos = positive, Neg =negative, FNF = false negative fraction, FPF = false positive fraction,

K = Cohen's kappa coefficient.

Figures

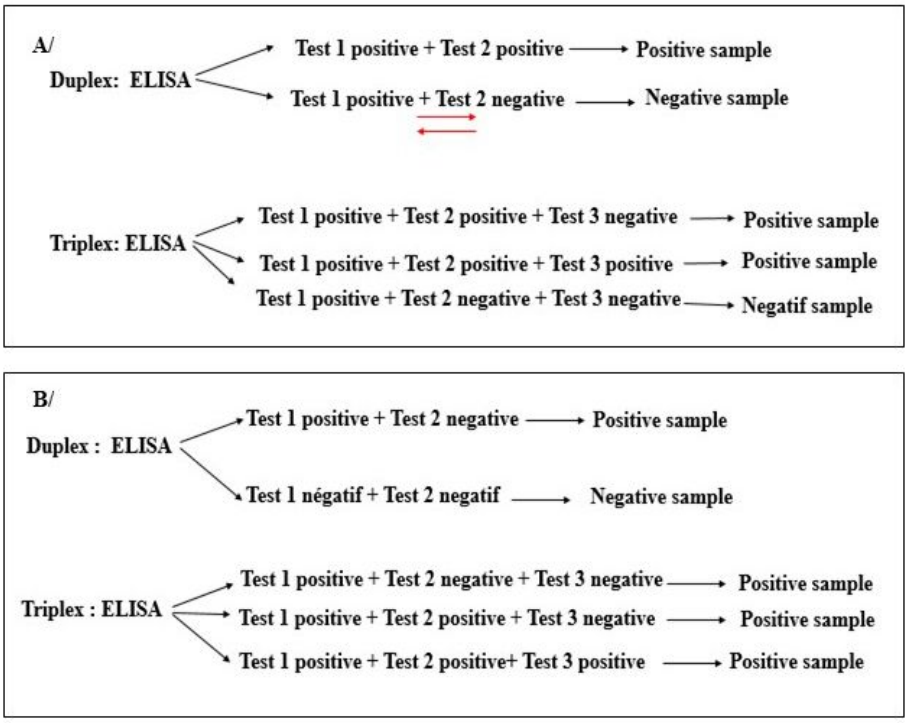

Figure 1

Figure 1

Different formulas for combining the results of different IgG-ELISAs with recombinant antigens

A. Cumulative duplex and triplex combinations

B. Alternative duplex and triplex combinations