Diagnosis of Bovine Cysticercosis in Cattle by Milk ELISA

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Abstract: Bovine cysticercosis is coopolitan in its distribution, occurring in developing as well as in industrialized countries. Control of this parasite requires a good diagnostic test to identify infected animals carrying viable cysticerci. The currently applied meat inspection method has low sensitivity.WhileELISA protocols were developed for detection of circulating antigens or antibodies against *T. saginata*cysticerciin cattle. But, these assays have not been validated and applied in field conditions yet. Therefore, the aim of this study was to develop and optimize a milk Ab-ELISA protocol for the diagnosis of *T. Saginata* cysticerci in cattle. As no reference milk samples were available, the protocol was developed and optimized using milk spiked with reference serum samples. Series of tests were performed to develop and optimize the test. Finally, the protocol was established using sheep anti bovine IgG1 as conjugate, *T. saginata* metacestodeexcretory and secretory (ES) as antigen and 2% casein as blocking buffer. The test detected all reference positive samples as positive and negative samples as negative spiked in skim milk and cow milk. In a next step, both serum and milk samples should be collected from infected and non-infected dairy cows to confirm the use of the test. The results of this study showed that the protocol is promising test for diagnosis of *T. saginata* in dairy cattle.

Key words: Bovine cysticercosis · Antibody · Milk · Serum · ELISA · Helminth · *T. saginata* · Cattle

INTRODUCTION

Bovine cysticercosis is an infection of cattle caused by themetacestode larval stage, *Cysticercus bovis*, of the human intestinal cestode, *Taenia saginata* [1]. Humans are the final host and bovines are the intermediate host. Cattle become infected after ingestion of *T. saginata* eggs (proglottids) expulsed by infected humans. Once cattle are infected, cysticerci develop primarily in the muscles and subsequently become infective to humans after approximately 10 weeks [2, 3]. The final host acquires *T. saginata* tapeworm infection from eating raw or undercooked beef infected with viable cysticerci [1]. As cited byKandil,*et al.* [3] a person infected with a single *T. saginata* tapeworm is capable of contaminating the environment with up to half a million eggs per day over the course of infection, which, if left untreated, can persist for year(s). The environment can be contaminatedwith eggs via defecation or spontaneous discharge of proglottids can be disseminated by water and wind,

scavenging birds such as gulls feeding on raw sewage, oribatid mites, flies, earthworms, or fomites such as boots or farm machinery. Infective *Taenia* eggs can survive under a variety of environmental conditions such as in sewage and in sludge for up to several months. Moreover, the eggs are resistant to most conventional chemical and disinfecting agents [4].

Bovine cysticercosis is cosmopolitan in its distribution and occurs in developing as well as in industrializedcountries [5]. Based on slaughter houses surveys, the prevalence of cysticercosis varies in the European Union between 0.01 and 6.8% [6 - 8]. However, routinely used inspection methods are assumed to detect only relatively heavily infected animals. Different studies indicated the routine meat inspectionmethods underestimate the prevalence by a factor 3 to 10 [7, 8]. As indicated by Dorny *et al.* [9] , in the meat industry, economic losses due to bovine cysticercosis are associated with total condemnation of carcasses with generalized infestation and downgrading lightly infected

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of carcasses which are subjected to freezing, in addition to the cost of freezing and extra handling and transport [9]. Moreover,the diagnosis and treatment cost for human taeniosis and costs of manufacturing of drugs have a significant contribution in the estimation of economic losses [6]. For example, in England only, the estimated costs of freezing, handling and transport was 100euro per carcass, or 4.0 million euro annually [10].As cited by Kandil *et al.* [3] Africa suffers great losses due to bovine cysticercosis estimated to be $1.8 billion annually [3].

Cattle with cysticercosis are improbable to exhibit clinical signs.However, detectionof *C. bovis*is made during post-mortem carcass examination. During post carcass examination, inspection predilection sites, namely: external and internal masseter muscles, tongue, heart, oesophagus and diaphragm ismade for detection of the parasite [3, 11]. The routine meat inspection procedure is time consuming, insensitive and lightly infected carcasses can be easily missed and passed for human consumption [12].Different studies indicated the sensitivity of meat inspection will vary with the number of cysts in the muscles examined as well as the stage of cysts. The measure is rather subjective and will vary with the meat inspector [13, 8].The sensitivity of the current routine meat inspection procedure has been estimated at between 10% and 30% [7, 14].

The limitations of the currently applied meat inspection procedures result in significant challenges for regulators and diagnostics tasked with preventing zoonotic transmission of the parasite. This problem could be addressed if a reliable serological test, example; enzyme-linked immunosorbent assay (ELISA) was developed for use on live animals. The immune response against Taenia parasite is reported to be antibody-mediated. A positive antibody (Ab) ELISA indicates that the animals have been exposed to the infection, but may not necessarily have a current infection. However, it is a useful method for epidemiological studies to indicate the spread of the infection [15, 3].

Indirect ELISA formats have been established for the detection of circulating antibodies in serum against the larval stage of *T.saginata* cysticerci in cattle [14, 16]. Different studieshave reported that the serum indirect ELISA for specific antibody detection against *T.saginata* cysticerci in cattle based on excretory/ secretory (ES) *T. saginata* metacestode antigens showed the highest sensitivity and specificity with 81.6% and 96.3%, respectively [17].

Both serum and milk indirect ELISA formats have been developed for the detection of antibodies against some helminth infections in dairy cattle. As described by Pritchard [18] and Pritchard*et al.* [19] the use of milk samples for diagnosis and surveillance of different diseases in cattle has become routine and milk antibody testing now plays a significant role in cattle disease control and eradication programmes in many countries [18,19].Different studies have shown for many infections that there is generally a good correlation between milk and serum antibody titres [20, 21, 22] , but that milk sampling is easier, cheaper and non-invasive compared to blood sampling [23].

Different studies have developed Ab milk ELISA against *Dictyocaulus viviparous* in cattle based on recombinant major sperm protein (MSP) [24].In clinically diseased animals, individual milk samples allow an easy diagnosis of infection. Furthermore, if used in bulk milk, this ELISA offers a cost-effective method for epidemiological studies and herd monitoring programmes [24]. Many research findings havedescribed at the end of the housing period or beginning of the grazing period, positive bulk milk samples indicate previous exposure and perhaps protective immunity in some animals. Evidence of exposure may also indicate that this farm is endemic for *D. viviparus* and therefore may require routine monitoring and control measures, especially in first year grazing animals. During the grazing season, routine examination of bulk milk samples provides a relatively inexpensive method to monitor herd health and potentially to prevent disease and production losses [24, 25].

The antibody milk ELISA is suitable for routine veterinary diagnostic use as an alternative to testing sera in lactating animals. Milk ELISAs are an effective method for diagnostic and surveillance purposes as compared to serum ELISAs. They are more cost-effective since veterinarians are not required to collect milk samples and farmers can submit samples directly to regional laboratories [22]. Thus, the use of milk samples as a diagnostic specimen could be very useful for the control and surveillance of bovine cysticercosis at farm level. However,thepossible development and optimization of an antibody milk ELISA format for the detection of circulating antibodies in milk against *T. Saginata*cysticerciin cattle has not been investigated yet. Thus, the aim of this study was to develop a milk antibody-ELISA format as diagnostic tool for the diagnosis of bovine cysticercosisin cattle.

The Specific Objectives Were:

- To review the use of milk Ab-ELISA in other helminth infections in bovine
- To develop a milk Ab-ELISA based on the procedures of the existing serum Ab-ELISA (CC) and milk Ab-ELISA's from other helminths

MATERIALS AND METHODS

Serum and Milk Samples: Eighteen reference negative serum and 11 reference positive bovine serum samples were used. The reference positive samples were collected at the abattoir from confirmed infected cattle, as well as from experimental infections. Based on the results of the serum antibody ELISA, eight negative serum samples and six positive serum samples were selected based on their optical density values (different levels of positivity), for the spiking of skim milk and cow milk samples. Sixteen fresh milk samples were collected from individual cows at a Belgian dairy farm (Table 1).

Fresh Milk Sample Preparation: Sixteen milk samples were collected from individual cows at a Belgian dairy farm. Milk samples were centrifuged at 2000 g for 10 minutes. The fat layer was removed and the underlying supernatant was collected and tested. The remaining underlying supernatant was frozen at (-20°C). Fresh (full fat milk), refrigerated or previously frozen skimmed cow milk samples were tested.

Phosphate Buffer Saline (PBS) and Skim Milk (10%) Preparation: One tablet of PBS was added in 100ml of distilled water to provide a 100 ml of PBS buffer, pH 7.3. The skim milk (10%) was prepared in the laboratory from skim milk powder for microbiology purpose. Ten grams of skim milk powder was added in 100ml of distilled water to provide a skim milk (10%) solution.
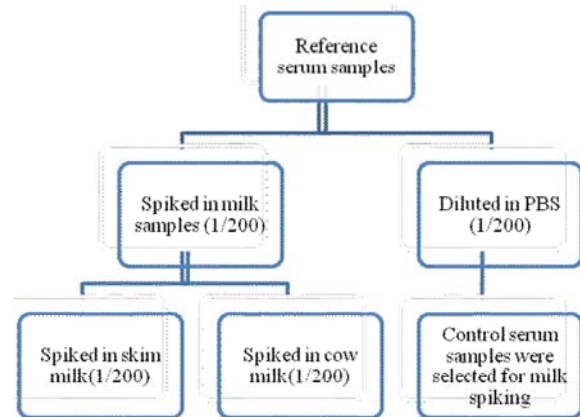

Fig. 1: Use of reference serum samples for developing milk Ab-ELISA protocol

Dilution and Spiking of Reference Serum Samples: The reference serum samples were diluted in PBS (1/200). The serum samples (non-diluted in PBS) were used to spike the skim/cow milk (1/200). The mean optical density values of spiked skim/cow milk (1/200) versus the same serum samples (as used for spiking) diluted in PBS (1/200) were measured and compared. Serial dilutions of the positive serum samples (R61 and R81) and monoclonal antibodies (B158C11A10 and B60H8A4) were made both in PBS and skim milk (10%). A serial dilution of one positive serum sample (R61) was also done in skim milk (10%) and cow milk during the development and optimization of the protocol. The use of reference samples were indicated in (Figure 2).

Serum Ab ELISA Protocol for Diagnosis of *C. Bovis* in Cattle: The serum Ab ELISA of the reference serum samples was performed as previously described in the protocols of the in house serum Ab ELISA of the Institute of Tropical Medicine (ITM) Manual, Eichenberger *et al*. [17] and Omnia [3]. The serum Ab ELISA was used as first basic and starting protocol for developing and optimizing

Table 1: List of reference serum and cow milk samples used for the development and optimization.

Negative serum samples	Positive serum samples	Milk samples	Reference serum samples selected for the spiking of skim milk and cow milk	
			Negative serum	Positive serum
B5,B6,B7,B8,	R25,R196	19,53,45,44	B 1,B2,B3,B4,B5	R61,R81, R25,
1,2,3,4,9,13,	R78, R79	67,62,50,18	B6, B7 and B8	R196,R78,R79
15,18,19, 20	R9,1528,	20,56,4		
B1,B2,B3,B4	2501, 7705	47,7,9,37		
	PC Dorien	Bulk milk		
	R61,R81			

initial reference protocol of milk Ab ELISA.The serum Ab ELISA of the reference serum samples was carried out as follow:the Polystyrene 96-microwell ELISA plates (Nunc® Maxisorp) were coated with 100 µl /well of 10 µg/ml of excretory and secretory (ES) *T.saginata* metacestode antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plateswereincubated for 30 min at 37°C. The wells were washed once with phosphate buffered saline (PBS) in 0.05% Tween 20(PBS-T20). The non-specific bindings were blocked with 150 µl 2 % bovine serum albumin (BSA) in PBS-T20. The plates were incubated for 15 min at 37°C. 100 µl of the diluted serum (1/200 in 2 % BSA +PBS-T20) was added and incubated at 37°C for 15 minutes while shaking. The plateswere washed five times with PBS-T20. 100 µl of goat anti bovine IgG conjugatelabeled alkaline phosphatase (AP) diluted 1/1000 in 2% BSA + PBS-T20 was added.The plates were incubated for 15 min at 37°C. Then, the wells were washed five times with PBS-T20. 100 µl of substrate solutionpara nitro phenyl phosphate (PNPP) prepared in RO-DI water was added. The plates were incubatedin the dark room for 15 min at 30°C. After 15 minutes,the plates were read with the help of an automated spectrometer (Thermo Lab systems/Multiskan EX ELISA reader) at wavelengths of 405 nm and 655nm. The cut off value was calculated based on the OD's of the negative reference serum samples using a variation of the students test [26].

Reference Protocol Used for the Development and Optimizing of the Milk Ab-ELISA: Based on existing milk antibody ELISA and serum antibody ELISA protocols for the diagnosis of *T. saginata* in cattle [16] and ITM manual, an initial protocol was designed.

Initial Reference Protocol: Flat-bottom, 96-well microplates (Nunc Maxisorp) were coated with 100 µl /well of 10 µg/ml of excretory and secretory (ES) *T.saginata* metacestode antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed once with phosphate buffered saline containing (PBS) in 0.05% Tween (PBS-T20). Non-specific binding sites were blocked by adding 150 µl of 2 % bovine serum albumin (BSA) in PBS-T20 per well. The plates were incubated for 30 minutes at 37 °C while shaking. Undiluted 100µl spiked skim milk samples were added to the wells. The plates were incubated for 1 hour at 37 °C. After five times washing, 100 µl rabbit anti-bovine IgG coupled to horseradish peroxidase (1/10000 in 2 % BSA in PBS-T20) was added as conjugate. The plates were incubated for 1 hour at 37 °C while shaking. After five times washing, 100 µl Ortho-phenylene diamin (OPD) including H2O2 was

added to all wells and incubated at 30 °C in the dark for 15 minutes. The reaction was stopped by adding 50 µl of Stop Solution (H2S04) to each well. The absorbance of the plate was read at 492 and 655 nm. The cut off valuewas calculated based on the OD's of the negative reference serum samples spiked in skim milk (10%) using a variation of the students test [26].

Protocol Optimization Test: Series of tests were performed by modifying the initial protocol described under initialreference protocol. The following factors were evaluated in the tests done for the development and optimization of milk antibody ELISA: blocking buffers, conjugates, substrates, monoclonal antibodies and excretory and secretory (ES) *T. saginata* metacestode antigen.

Blocking Buffers Used for the Optimization of the Test: Two % casein (w/v), 2 % bovine serum albumin (w/v), 3 % fetal calf serum (v/v) and 2 % fetal calf serum (v/v) in PBS, T20were evaluated for their use as the blocking buffer.

Conjugates Used for the Optimization of the Test: Three conjugates, namely: Sheep anti-bovine IgG1 (SAB-IgG1: 1/10000) coupled to horseradish peroxidase, rabbit anti-bovine IgG (RAB-IgG: 1/10000) coupled to horseradish peroxidase and alkaline phosphatase labeled goat anti-bovine IgG (GAB- IgG: 1/1000) were used in the tests done for the development and optimization of an antibody milk ELISA protocol for *T. saginata*. Sheep anti bovine IgG1 was evaluated for development of the protocol. A study conducted by [27] indicatedIgG1 is the predominant immunoglobulin in milk (representing about 80% of the total immunoglobulin content), which is transported by active receptors on mammary alveolar cells [7].

Substrates Used for the Optimization of the Test: Three substrates, namely:Peroxidase substrates (2, 2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate), ABTS and Ortho-phenylene diamin (OPD) and phosphatase substrate system (Para Nitro phenyl phosphate, PNPP) were evaluated.

Monoclonal Antibodies Used for the Optimization of the Test: Two monoclonal antibodies (MoAb), namely B158C11A10 and B60H8A4 were used in the tests. These MoAbs (B158C11A10 and B60H8A4) of the IgG1 isotype were produced against the secretion and excretion products (ES) of *T. saginata* cysticerci [7, 28]. Serial

dilution of monoclonal antibodies were made both in PBS and skim milk starting from 5µg up to 0.00 µg concentration to measure and compare the optical densities values of the monoclonal antibodies between PBS and skim milk (10%).

Antigen Used for the Optimization of the Test: Excretory and secretory (ES) *T. saginata* metacestode antigen was used as antigen to develop the milk Ab ELISA protocol. Different studies have compared the sensitivity and specificity of serum Ab ELISA using different antigens, namely: somatic larval antigen, isoelectric focused somatic larval antigen, larval excretory/secretory (ES) antigens, peptide HP6-2, peptide Ts45S-10 and pooled peptide solution [17]. The highest sensitivity (81.6%) and specificity (96.3%) wereobtainedusing Excretory and secretory (ES) *T. saginata* metacestode as antigen [17].

RESULTS

Serum Antibody Elisa for *C. Bovis*: The Ab serum ELISA was carried out on reference serum samples diluted in PBS (1/200) using goat anti bovine IgG and 2% BSA (Table 2).

Based on mean OD values, eight negative serum (B1,B2,B3,B4,B5,B6,B7,B8) and six positive serumsamples (R61,R81,R196,R79,R25,R78) were selected as reference serum samples for spiking of skim milk and cow milk samples.

Preliminary Testing: Initially, preliminary tests were carried out to evaluate whether antibodies against *C. bovis*could be detected in milk samples. Therefore, in a first test, monoclonal antibodies were compared in PBS and skim milk. Secondly, reference positive serum samples were used to spike skim milk. In a third phase, the reference samples were spiked in cow milk.

Detection of Monoclonal Antibodies and Positive Control Samples in Skim Milk (10%): Serial dilutions of two monoclonal antibodies (B158C11A10) and (B60H8A4) were evaluated in PBS and skim milk (SK) using rabbit anti mouse IgG peroxidase and 2% BSA (Figure 3).

Similar OD values of monoclonal antibodies (B158C11A10) and (B60H8A4) were measured both in PBS and skim milk (SK).

Detection of Positive Control Serum Sample in Cow Milk: In further step R61 was evaluated in skim milk (SK) and cow milk (CM) using goat anti bovine IgG and 3% FCS (Figure 4).

Protocol Optimization Tests
Conjugates: The performance of two conjugates, namely: goat anti bovine (GAB) IgG versus rabbit anti bovine (RAB) IgG were comparedfor the reference serum samples spiked in skim milk (1/200) using 3% FCS (Figure 5).

A better result was obtained using goat anti bovine IgG, and the further testing was continued using this conjugate.

Blocking Buffers for Goat Anti Bovine IgG: The performance of three blocking buffers (2% BSA, 3% FCS and 2 % FCS) were evaluated for the reference serum samples spiked in skim milk (1/200) using goat anti bovine IgG (Figure 6).

The highest mean OD value was obtained using 2% BSA. For further optimization tests, 2% BSA was selected

IgG1: Sheep Anti Bovine IgG1: For further optimization, the use of IgG1 as a conjugate was evaluated. The OD values of the reference serum samples spiked in skim milk (1/200) versus the samples diluted in PBS (1/200) were compared using sheep anti bovine IgG1 and 2% BSA (Figure 7).

Table 2: Results of mean optical density values of reference serum samples diluted in PBS (1/20

Controls	Serum samples			OD values						
SC	B1	R196	R78	R9	-19	0.046	0.087	0.731	0.664	0.861	0.144
SC	B2	R196	R78	R9	-19	0.042	0.097	0.723	0.603	0.853	0.139
CC	B3	R79	1528	4	-15	0.054	0.084	0.918	0.31	0.377	0.138
CC	B4	R79	1528	4	-15	0.054	0.101	0.891	0.324	0.348	0.144
R61	B5	R41	2501	7	-17	2.04	0.092	0.858	0.802	0.929	0.108
R61	B6	R41	2501	7	-17	2.077	0.117	0.859	0.832	0.995	0.104
R81	B7	R25	7701	8	-13	1.415	0.098	0.827	0.423	2.093	0.104
R81	B8	R25	7701	8	-13	1.443	0.115	0.838	0.483	2.259	0.102

Serum cut off =0.183

Mean OD negative serum =0.11, Mean OD positive serum =0.933

Table 3: Mean OD values of reference serum samples in PBS and skim milk using OPD and ABTS substrates

Mean OD of serum samples spiked in skim milk (1/200)		
Substrate used	Negative serum	Positive serum
ABTS	0.025	0.076
0PD	0.026	0.283
Mean OD of serum samples diluted in PBS (1/200)		
Substrate used	Negative serum	Positive serum
ABTS	0.019	0.08
OPD	0.028	0. 264

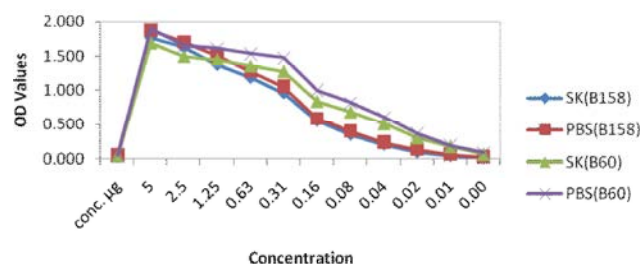Better results were measured using OPD substrate both in PBS and skim milk.

Fig. 2: Serial dilutions of monoclonal antibodies in PBS and skim milk (SK) Similar OD values of monoclonal antibodies (B158C11A10) and (B60H8A4) were measured both in PBS and skim milk (SK).
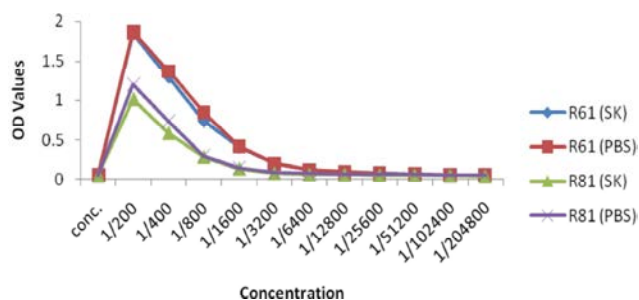

Fig. 3: Serial dilutions of two serum samples (R61, R81) in PBS and skim milk (SK) Similar OD values were measured both in PBS and skim milk (SK) for both control serum samples.
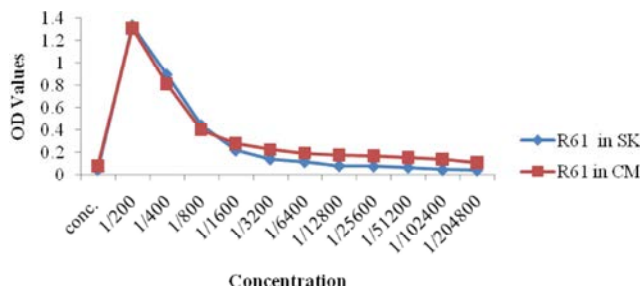

Fig. 4: Serial dilutions of serum sample (R61) in skim (SK) and cow milk (CM) Similar OD values of R61 were measured both in skim milk (SK) and cow milk (CM).

Similar results were measured both in skim milk and PBS.But lower Mean OD values of the positive samples were measured than with GAB IgG.

Substrates: The performances of two substrates (ABTS versus OPD) were evaluated for the reference serum samples diluted in PBS and spiked in skim milk using sheep anti bovine IgG1 in 2% BSA (Table 3).

Blocking Buffers for Skim Milk and Sheep Anti Bovine IgG1: Three blocking buffers (2% BSA, 3% FCS and 2 % casein) were evaluated for the
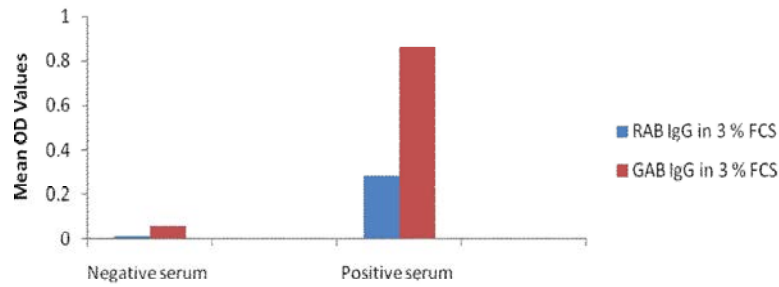
Fig. 5: Mean OD values of spiked skim milk (1/200) using Goat anti bovine (GAB) IgG in 3% FCSversus rabbit anti bovine (RAB) IgG in 3% FCS
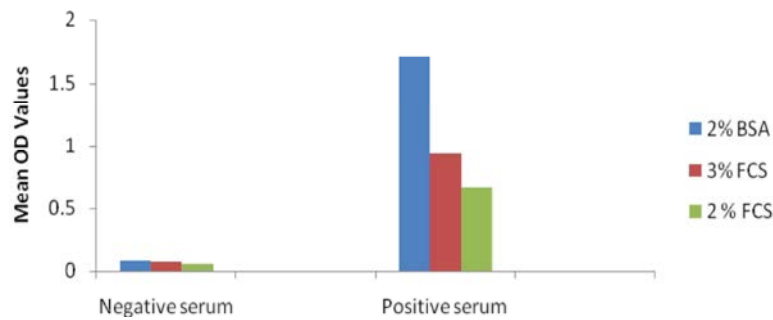

Fig. 6: Mean OD values of spiked skim milk (1/200) using three blocking buffers (2 % bovine serum albumin, 3 % fetal calf serum, and 2% fetal calf serum)
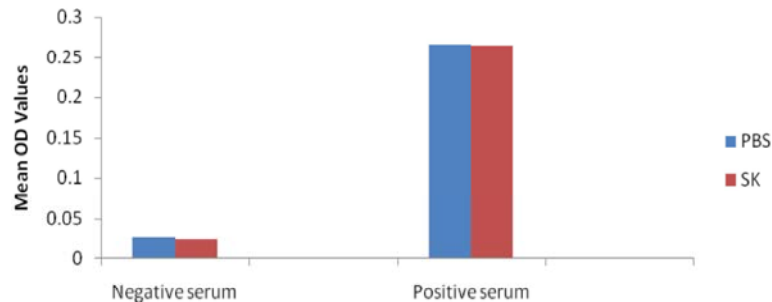

Fig. 7: Mean OD values of reference serum samples in PBS and skim milk (SK) using IgG1 Similar results were measured both in skim milk and PBS.But lower Mean OD values of the positive samples were measured than with GAB IgG.
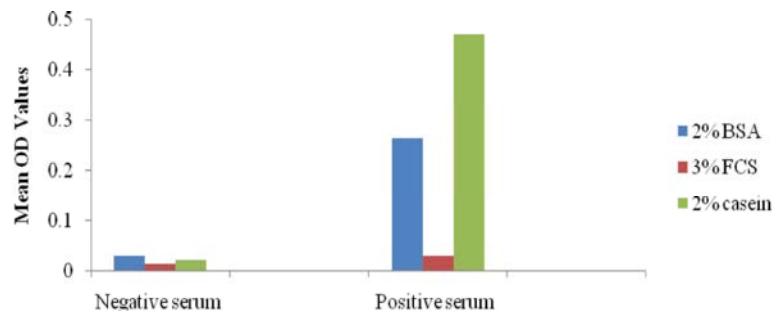

Fig. 8: Mean OD values of spiked in skim milk (1/200) using three blocking buffers (2 % BSA, 3 % FCS and 2% casein) and sheep anti bovine IgG1

reference serum samples spiked in skim milk (1/200) using Sheep anti bovine IgG1 (Figure 8).

In further testing, two blocking buffers (2% BSA and 2% casein) wereevaluated for reference serum samples spiked in cow milk (1/200) using Sheep anti bovine IgG1 (Figure 9).

Better results were obtained using 2% casein for both spiked skimmilk and spikedcow milk samples. 2% casein was selected for further optimization tests.

Final Comparison of Sheep Anti Bovine Igg1 with Goat Anti Bovine IGG:

Reference Serum Samples Spiked in Skim Milk (10%): The performances of two conjugates (sheep anti bovine IgG1 versus goat anti bovine IgG) were evaluated for spiked skim milk (1/200) (Table 4).

The test detected all positive serum samples spiked in skim milk (cut off serum =0.106) and non-spiked cow milk as control (cut off cow milk =0.151) using sheep anti bovine IgG1. Sheep anti bovine IgG1 was selected for further optimization test

Reference Serum Samples Spiked in Cow Milk: In further test the performances of two conjugates (sheep anti bovine IgG1 versus goat anti bovine IgG) were also compared for spiked cow milk (1/200) (Table 5).

In final protocol optimization test, the test detected all reference positive samples spiked in cow milk using serum negative samples spiked in cow milk as control(cut off serum=0.147) and non-spiked cow milk as control(cut off milk =0.331) using sheep anti bovine IgG1. This final test resulted in the selection of sheep anti bovine IgG1 for the protocol.

Table 4: Result of milk antibody ELISA on spiked skim milk and non-spiked cow milk samples using two conjugates: sheep anti bovine IgG1 versus goat anti bovine IgG

Sheep anti bovine IgG1								
				OD value				
Controls*	Controls**	Serum***	Milk****					Od Ratios
SC	Bovine 1	R25	47	0.006	0.012	0.152	0.023	R61=6.5
SC	Bovine 2	R25	9	0.008	0.014	0.152	0.064	R81=8.5
CC	Bovine 3	R196	37	0.016	0.015	0.179	0.044	R25=1.4
CC	Bovine 4	R196	19	0.018	0.023	0.185	0.082	R196=1.7
R61	Bovine 5	R78	53	0.687	0.025	0.356	0.052	R78=3.5
R61	Bovine 6	R78	45	0.699	0.013	0.383	0.055	R79=4.8
0R81	Bovine 7	R79	44	0.952	0.015	0.515	0.058	
R81	Bovine 8	R79	47	0.918	0.056	0.501	0.043	

Cut off serum=0.106; cutoff milk=0.151

Goat anti bovine IgG								
				Od Value				
Controls*	Controls**	Serum***	Milk****					Od Ratios
SC	Bovine 1	R25	47	0.035	0.068	0.701	0.113	R61=8.3
SC	Bovine 2	R25	9	0.031	0.097	0.702	0.241	R81=6.3
CC	Bovine 3	R196	37	0.055	0.069	0.698	0.231	R25=3.1
CC	Bovine 4	R196	19	0.063	0.111	0.657	0.372	R196=3.0
R61	Bovine 5	R78	53	1.894	0.134	0.535	0.252	R78=2.4
R61	Bovine 6	R78	45	1.878	0.081	0.528	0.241	R79=4.1
R81	Bovine 7	R79	44	1.434	0.077	0.893	0.233	
R81	Bovine 8	R79	67	1.434	0.106	0.958	0.143	

Cut off serum= 0.22; cut off milk =0.673

**** Non-spiked cow milk samples

*** Reference positive serum samples spiked in skim milk (1/200)

** Negative serum controls spiked in skim milk (1/200)

* Positive serum controls (R61 and R81) spiked in skim milk (1/200)

OD rations are calculated based negative serum samples spiked in skim milk as negative controls

Cut off serum=0.106

Cut off milk=0.151

Table 5: Results of milk antibody ELISA on spiked cow milk (1/200) and non-spiked cow milk samples using two conjugates: sheep anti bovine IgG1 versus goat anti bovine IgG

Controls*	Controls**	Serum***	Milk****	Sheep anti bovine IgG1 OD value				Od Ratios
SC	Bovine 1	R25	47	0.005	0.099	0.337	0.031	R61=6.8
SC	Bovine 2	R25	9	0.005	0.111	0.337	0.112	R81=8.1
CC	Bovine 3	R196	37	0.027	0.103	0.396	0.085	R25=2.3
CC	Bovine 4	R196	19	0.024	0.108	0.340	0.174	R196=2.5
R61	Bovine 5	R78	53	1.011	0.110	0.541	0.097	R78=3.6
R61	Bovine 6	R78	45	0.999	0.121	0.501	0.113	R79=6.1
R81	Bovine 7	R79	44	1.174	0.108	0.805	0.114	
R81	Bovine 8	R79	67	1.205	0.101	0.992	0.109	

Cut off serum=0.147; cutoff milk=0.331

Controls*	Controls**	Serum***	Milk****	Goat anti bovine IgG OD value				Od Ratios
SC	Bovine 1	R25	47	0.033	0.155	0.665	0.133	R61=7.7
SC	Bovine 2	R25	9	0.038	0.159	0.616	0.252	R81=4.5
CC	Bovine 3	R196	37	0.049	0.151	0.582	0.255	R25=2.6
CC	Bovine 4	R196	19	0.049	0.160	0.605	0.380	R196=2.4
R61	Bovine 5	R78	53	1.872	0.155	0.506	0.283	R78=2.1
R61	Bovine 6	R78	45	1.862	0.172	0.522	0.272	R79=3.1
R81	Bovine 7	R79	44	1.070	0.149	0.736	0.285	
R81	Bovine 8	R79	67	1.115	0.192	0.778	0.191	

Cut off serum=0.243; cutoff milk=0.671

**** Non-spiked cow milk samples

*** Reference positive serum samples spiked in cow milk (1/200)

** Negative serum controls spiked in cow milk (1/200)

* Positive serum controls (R61 and R81) spiked in cow milk (1/200)

OD rations are calculated based negative serum samples spiked in cow milk as negative controls

Cut off serum=0.147

Cut off milk=0.331

DISCUSSION AND CONCLUSION

The diagnosis of *T. saginata* cysticercosis in cattle is based on routine meat inspection method [8]. However, the main limitation of routine meat inspection is its lack of sensitivity and objectivity. As reported by Geysen *et al*. [8] the procedure is restricted to inspection of predilection sites (heart, masseter muscles, diaphragm, tongue and oesophagus in all bovines older than 6 months [8]. The success of this method is highly dependent on the expertise of the inspector as well as on the stage of development of the cysts [8].

The routine meat inspection is used to eliminate aesthetically unacceptable, heavily infected carcasses from the market. However, lightly infected carcasses, which represent a high percentage of the infections, very often escape detection because of the inherently inaccuracy and insensitivity of the meat inspection procedure. A characteristic of *T. saginata* cysticerci is its huge biotic potential so that lightly infected carcasses

escaping into the food chain are quite sufficient, to ensure not only the continuation of the life cycle, but also the maintenance of high prevalence of the parasite. Thus, the routine current meat inspection method cannot markedly help control of bovine cysticercosis. Another major problem of this method is that diagnosis of the cysticerci occurs during post-mortem carcass examination, when it is too late to make decisions about possible control measures at the infected farm origin level. Thus, alternative diagnostic methods could be used for epidemiological surveys of bovine cysticercosis at farm level to take appropriate control measures [11].

Different immunodiagnostic tests, such as serum antigen and antibody ELISA protocols were developed for detection of circulating antigens or antibodies against *T. saginata* cysticercosis in cattle [3, 7, 17, 16]. But, these assays have not been validated and applied at field conditions yet. Different studies have described milk antibody ELISA formats for the detection of antibodies against some helminth infections in dairy cattle [23, 24,

29].The use of milk antibody ELISA format for diagnosis of *T. saginata* cysticercosis in cattle has not been published yet. In order to develop and optimize milk antibody ELISA protocol for *T. saginata*, we have used reference serum samples for spiking skim milk (10%) and cow milk. Moreover, to develop the protocol, we have tried different procedures step by step.

Molina*et al*. [30] have developed Ab milk ELISA for detection of antibodies against *Teladorsagia circumcincta* in goat milk (both individual and bulk milk samples [30]. A good correlation between specific IgG levels was observed in serum and milk samples as has also been observed in dairy cows [30, 31].As indicated by [30] the possible dilution produced by antibodies locally produced in mammary glands do not significantly affect the detection of IgG against *T. circumcincta* from milk samples in infected goats [30]. However, serological cross-reactions have been observed in goats infected with *T. circumcincta* and *Haemonchus contortus* [30]. Other studies indicated by [29] an acute mastitis in cattle causes a flow of specific and non-specific antibodies from serum to milk with a subsequent increase in the *O. ostertagi* ODR values [29].

Different studies have indicated that the relationship between serum, individual milk and bulk milk samples is complex. A study in Sweden indicated that median Optical Density Ratios (ODRs) of bulk milkwaslow ascompared serum. But, high median ODRs were measured using bulk milk ELISAthan individual milk samples [32]. The individual and bulk milk ELISA ODRs have been accessedfor two dairy herds in Normandy over a one year period [20, 25].Higher bulk milk ODRs were measured than mean individual milk ODRs.Charlier*et al*. [20] explained that a greater contribution of ODRs for the bulk milk tank is contributed by individuals with high antibody titres. However, the application of the bulk milk ELISA against *Ostertagia ostertagi*is further complicated by the fact that the crude antigen assay may cross-react with other bovine helminthes, such as *Cooperia oncophora* and *Fasciola hepatica* [33].Other studies by Molina*et al*. [30] indicated the quantitative relationship between the mean of individual milk antibody levels and those in the bulk milk against *T. circumcincta*in dairy goat are similar to those described byCharlier*et al*. [20] for *O. ostertagi* in cattle, which is the values in bulk milk typically exceed those calculated from the mean of individual samples that contribute to the bulk milk [20, 30]. Molina *et al*. [30] explained the relatively higher contribution of specific antibodies to the bulk milk from animals with higher relative units [30].

Different studies have described the most important management factor affecting specific antibody levels in the bulk milk tank against *O. ostertagi* in dairy cattle is the extent to which animals have outdoor access to pasture [34,35]. Different research findings have shownno access to pasture resulted in low antibody concentrations.In contrast, in animals kept outdoors, antibody levels increased with the level of access to fully grassed pasture and herbage. The proportional bulk milk antibodylevel (measured as ODR) was positively correlated with percent of time spent grazing daily. Different research findings have indicatedherds that were managed by summer grazing and winter housing demonstrated a seasonal pattern of high ODR in late summer and early autumn and low ODR in winter [29] reflecting the build-up of parasite larvae on pasture in mid-summer [36]. Furthermore, bulk milk ELISA scores increased the earlier the date of turnout and the later the month of housing [33, 37]. Different studies have shown extensive production systems and organic herds with smaller herd sizes and lower stocking densities tend to have higher bulk milk antibody levels as compared to those animals in intensively managed Systems [38, 37]. Bennema and colleagues found that in addition to climatic and environmental factors, herd management practices had a major impact for infection of *F. hepatica* [33, 25].

[6] reported the prevalence of bovine cysticercosis is low in Western European countries which range between 0.007% and 2.4% [6]. As reported by Sanchez *et al*. [39] in Northern Spain, the prevalences of the disease were 0.54% in animals kept outdoors at pastures. Allepuz*et al*. [40] reported the bovine cysticercosis prevalence in Catalonia (North-Eastern Spain) was 0.018%. Allepuz*et al*. [40] explained the lower prevalence is that in Catalonia most of the animals are kept indoors [40]. Grazing on pastures has high potential for contamination with*T. saginta* eggs derived from human faeces directly or via sewage sediment distributed in pastures [41]. Moreover, free access of cattle to surface water (rivers, lakes, canals) and flooding of pastures have been described as important environmental risk factors for the detection of bovine cysticercosis in a herd [42]. The reported prevalences of bovine cysticercosis are based on routine meat inspection or serum and that it would be easier to get spatial distribution and follow up data when milk samples(individual and bulk milk) can be used for milk Ab ELISA testing which could be useful for monitoring and surveillance of the prevalence of bovine cysticercosis at herd level as well as at individual animal level.

Different studies have indicated bulk milk ELISA is useful tool for the veterinary practitioner as a component of a herd health monitoring or investigation programme. It is useful in regional or national surveillance programmes. Bulk milk ELISA results can provide timely information about parasite exposure status within the larger picture of a herd health monitoring programme. Furthermore, the trends of parasite-specific antibody levels and seasonal variations in disease status can determine using regular monitoring basis (4times/year).Different studies have indicated bulk milk ELISAs can also be useful tools for measuring the relative intensity or prevalence of parasite infection in the herd [20, 35, 25].

[43] indicated both individual and bulk milk samples can be tested by ELISA; however, there are significant differences in the interpretation of the results. There are many factors that can affect the titre of parasite-specific antibodies in the bulk milk. These factors are: the number and relative sero positivity of contributors, stage of infection, stage of lactation, infection and milk yield [43]. Different research findings have showna bulk milk ELISA test negative result is not mean that the herd is definitively free of a particular parasitic infection. The bulk milk ELISA assay tests can detect positive result when all ELISAshave achieved a threshold antibody concentration level. Bulk milk score is very challenging to correlate the lower the OD value for the bulk milk withthe percentage of infected animals [35, 25].

Undiluted cow milk samples were used in all tests performed for the development and optimization of milk antibody protocol for *T. saginata*. Different comparative studies explained on concerning the use of diluted and undiluted milk samples in ELISAs have displayed greater sensitivities for undiluted milk [44, 45]. Different studies have explained a greater concentration of antibodies in undiluted milk was found as compared to diluted milk. In addition, the dilution effect can result in false negative ELISA results when the antibody titre is decreasing [44, 45]. For that reason and due to faster handling the milk ELISA is evaluated for undiluted milk samples for detection of antibodies against *Dictyocaulus viviparus* in dairy cows [23, 25]. Due to the impact of the milk fat content on measured optical density values [46] , milk samples are centrifuged and after the fat layer is removed and the sediment is used for the ELISA [29].

In final optimization step, goat anti bovine IgGversus sheep anti bovine IgG1 were evaluated for the reference samples spiked in skim milk as well in cow milk.Better results were obtained using sheep anti bovine IgG1both in spiked skim milk and cow milk. All reference positive and negative serum samples spiked in skim and cow milk samples weredetected bythe developed protocol.

From this study it can be concluded that the protocol was developed and optimized using sheep anti bovine IgG1 as conjugate, 2 % casein as blocking buffer,OPD as substrate and excretory and secretory(ES) of *T. saginata* as working antigen. As no reference positive and negative milk samples were available, the protocol was developed and optimized on reference serum samples spiked in skim milk followed by cow milk samples. The preliminary results were promising for the reference samples spiked in skim milk and cow milk. In a next step, serum and milk samples should be collected from infected and non-infected dairy cows to confirm the use of the test

Recommendations: The results of this study showed that a developed and optimized milk antibody ELISA protocol using sheep anti bovine IgG1 and 2% casein is promising test for diagnosis of *T. saginata*cysticercosisin dairy cattle. Therefore, based on the above results the following recommendations are suggested:

- Testing of serum and milk samples from infected and non-infected dairy cows (individual)
- Further studies should be carried on antibodies levels in serum and milk throughout a lactation period
- The impact of mastitis on antibodies levels should be studied
- Further studies should be carried out on antibody detection in bulk milk samples versus individual samples
- We recommend also studies on cross reactions with other pathogens

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