Diagnosis of Bovine Cysticercosis in Cattle by Milk ELISA

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Abstract: Bovine cysticercosis is cosmopolitan 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. While ELISA protocols were developed for detection of circulating antigens or antibodies against *T. saginata* cysticerci in 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* metacestode excretory 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 the metacestode 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) expelled 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 by Kandil, 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 contaminated with eggs via defeacation 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 industrialized countries [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 inspection methods 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...
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 100 euro 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, detection of *C. bovis* made during post-mortem carcass examination. During post-mortem carcass examination, inspection predilection sites, namely: external and internal masseter muscles, tongue, heart, oesophagus and diaphragm is made 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 studies have 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 have described 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, the possible development and optimization of an antibody milk ELISA format for the detection of circulating antibodies in milk against *T. Saginata*cysticerci in 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 cysticercosis in 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.

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
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 plates were incubated for 30 min at 37°C. The plates 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 plates were washed five times with PBS-T20. 100 µl of goat anti bovine IgG conjugate labeled 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 phosphate buffered nitrophenyl phosphate (PNPP) prepared in RO-DI water was added. The plates were incubated in 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 655 nm. 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].

Protocol Optimization Test: Series of tests were performed by modifying the initial protocol described under initial reference 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, T20 were 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] indicated IgG1 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 diamine (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%) were obtained using 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 serum samples (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 samples.

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 compared for 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)

<table>
<thead>
<tr>
<th>Controls</th>
<th>Serum samples</th>
<th>OD values</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>B1, R196, R78</td>
<td>R9 -19</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.046</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.087</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.751</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.664</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.861</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td>0.144</td>
</tr>
<tr>
<td>CC</td>
<td>B3, R79</td>
<td>1528</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>4 -15</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.054</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.084</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.918</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.377</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td>0.138</td>
</tr>
<tr>
<td>R61</td>
<td>B5, R41</td>
<td>2501</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>7 -17</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>2.04</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>0.092</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>0.858</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>0.802</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>0.929</td>
</tr>
<tr>
<td>R61</td>
<td></td>
<td>0.108</td>
</tr>
<tr>
<td>R81</td>
<td>B7, R25</td>
<td>7701</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>8 -13</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>1.415</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>0.098</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>0.827</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>0.423</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>2.093</td>
</tr>
<tr>
<td>R81</td>
<td></td>
<td>0.104</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Substrate used</th>
<th>Negative serum</th>
<th>Positive serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>0.025</td>
<td>0.076</td>
</tr>
<tr>
<td>OPD</td>
<td>0.026</td>
<td>0.283</td>
</tr>
</tbody>
</table>

Mean OD of serum samples diluted in PBS (1/200)

<table>
<thead>
<tr>
<th>Substrate used</th>
<th>Negative serum</th>
<th>Positive serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>0.019</td>
<td>0.08</td>
</tr>
<tr>
<td>OPD</td>
<td>0.028</td>
<td>0.264</td>
</tr>
</tbody>
</table>

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% FCS versus 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) were evaluated 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 skim milk and spiked cow 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 testing, 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

<table>
<thead>
<tr>
<th>Controls*</th>
<th>Controls**</th>
<th>Serum***</th>
<th>Milk****</th>
<th>OD value</th>
<th>Od Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC Bovine 1</td>
<td>R25 47</td>
<td>0.006</td>
<td>0.012</td>
<td>0.152</td>
<td>0.023</td>
</tr>
<tr>
<td>SC Bovine 2</td>
<td>R25 9</td>
<td>0.008</td>
<td>0.014</td>
<td>0.152</td>
<td>0.064</td>
</tr>
<tr>
<td>CC Bovine 3</td>
<td>R196 37</td>
<td>0.016</td>
<td>0.015</td>
<td>0.179</td>
<td>0.044</td>
</tr>
<tr>
<td>CC Bovine 4</td>
<td>R196 19</td>
<td>0.018</td>
<td>0.023</td>
<td>0.185</td>
<td>0.082</td>
</tr>
<tr>
<td>R61 Bovine 5</td>
<td>R78 53</td>
<td>0.687</td>
<td>0.025</td>
<td>0.356</td>
<td>0.052</td>
</tr>
<tr>
<td>R61 Bovine 6</td>
<td>R78 45</td>
<td>0.699</td>
<td>0.013</td>
<td>0.383</td>
<td>0.055</td>
</tr>
<tr>
<td>R81 Bovine 7</td>
<td>R79 44</td>
<td>0.952</td>
<td>0.056</td>
<td>0.501</td>
<td>0.043</td>
</tr>
<tr>
<td>R81 Bovine 8</td>
<td>R79 47</td>
<td>0.918</td>
<td>0.056</td>
<td>0.501</td>
<td>0.043</td>
</tr>
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</table>

Cut off serum = 0.106; cutoff milk = 0.151

Goat anti bovine IgG

<table>
<thead>
<tr>
<th>Controls*</th>
<th>Controls**</th>
<th>Serum***</th>
<th>Milk****</th>
<th>OD value</th>
<th>Od Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC Bovine 1</td>
<td>R25 47</td>
<td>0.035</td>
<td>0.068</td>
<td>0.701</td>
<td>0.113</td>
</tr>
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<td>SC Bovine 2</td>
<td>R25 9</td>
<td>0.031</td>
<td>0.097</td>
<td>0.702</td>
<td>0.241</td>
</tr>
<tr>
<td>CC Bovine 3</td>
<td>R196 37</td>
<td>0.055</td>
<td>0.069</td>
<td>0.698</td>
<td>0.231</td>
</tr>
<tr>
<td>CC Bovine 4</td>
<td>R196 19</td>
<td>0.063</td>
<td>0.111</td>
<td>0.657</td>
<td>0.372</td>
</tr>
<tr>
<td>R61 Bovine 5</td>
<td>R78 53</td>
<td>1.894</td>
<td>0.081</td>
<td>0.535</td>
<td>0.252</td>
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<tr>
<td>R61 Bovine 6</td>
<td>R78 45</td>
<td>1.878</td>
<td>0.106</td>
<td>0.958</td>
<td>0.143</td>
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<tr>
<td>R81 Bovine 7</td>
<td>R79 44</td>
<td>1.434</td>
<td>0.077</td>
<td>0.893</td>
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<tr>
<td>R81 Bovine 8</td>
<td>R79 67</td>
<td>1.434</td>
<td>0.106</td>
<td>0.958</td>
<td>0.143</td>
</tr>
</tbody>
</table>

**** 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 ratios are calculated based on 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

<table>
<thead>
<tr>
<th>OD value</th>
<th>Sheep anti bovine IgG1</th>
<th>OD Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td><strong>Negative serum controls spiked in cow milk (1/200)</strong></td>
<td><strong>Positive serum controls (R61 and R81) spiked in cow milk (1/200)</strong></td>
</tr>
<tr>
<td><strong>Od Ratios</strong></td>
<td><strong>Cut off serum=0.147; cutoff milk=0.331</strong></td>
<td><strong>Cut off serum=0.147; cutoff milk=0.331</strong></td>
</tr>
</tbody>
</table>

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, slightly 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,
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 milk was slow as compared to those animals in intensively managed herds 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. saginata* 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 (4 times/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 shown a 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 ELISAs have achieved a threshold antibody concentration level. Bulk milk score is very challenging to correlate the lower the OD value for the bulk milk with the 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 IgG1 versus 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 IgG1 both in spiked skim milk and cow milk. All reference positive and negative serum samples spiced in skim and cow milk samples were detected by the 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* cysticercosis in 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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