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Serological tools for detection of *Trichinella* infection in animals and humans

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ABSTRACT

Trichinellosis is a serious foodborne zoonotic disease. It is an important threat to public health in both developing and developed countries. Human infections are strongly associated with consuming undercooked meat containing infective *Trichinella* larvae. The development of serological tools has enabled seroepidemiological studies and contributed to our knowledge on the importance of this parasite. Serological tests can also help the diagnosis of parasite infections in humans and the surveillance of animals. Generally speaking, serological techniques include detection methods for specific antibodies and for circulating parasite antigens in the serum or tissue fluids. Here, we present a comprehensive review of various methods used in the detection of antibodies against *Trichinella* and circulating parasite antigens in animals and humans.

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1. Introduction

Trichinellosis is one of the most important food-borne parasitic zoonoses throughout the world. Humans acquire trichinellosis by ingesting

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raw or undercooked meats containing the infective *Trichinella* larvae. In the past several decades, human outbreaks have been reported in many parts of the word [24,33]. Trichinellosis is regarded as an emerging or re-emerging disease in some parts of the world. The global importance of Trichinellosis has prompted the development of a number of serological tools for the detection of *Trichinella* infection in humans and animals. Serological techniques include detection methods for specific antibodies and for circulating parasite antigens in the serum or tissue fluids. For detection of *Trichinella* infection in humans, serological tests for detecting *Trichinella*-specific antibodies are valuable methods for human trichinellosis diagnosis. For detection of *Trichinella* infection in animals, according to the International Commission on Trichinellosis, serological methods are not recommended as substitutes for meat inspection of individual carcasses. However, serological methods for antibody detection are suitable for the surveillance of domestic animals and wildlife and contribute to the knowledge on *Trichinella* circulation [19]. This paper summarizes the progress on serological test tools for the detection of *Trichinella* infections, as well as their advantages and shortcomings.

2. Antigens used in serological tests

Trichinella antigens are divided into a fast-responding group (group I) and a slow-responding group (group II). The group I antigens are mainly composed of somatic antigens and are detected after two weeks of infection. The group II antigens are mainly composed of cuticular and excretory/secretory (ES) antigens of the muscle larvae (ML) and are detected after 4–5 weeks of infection.

2.1. Cuticular antigens

The cuticle is the most obvious point of contact between a parasite and its host. Thus, it is very useful in the indirect fluorescent-antibody test. Investigations on surface antigens indicated that cuticular antigens are stage-specific. Four major antigens are present on the new born larvae (NBL) cuticle, with molecular masses of 20, 30, 58, and 64 kDa. First-stage larvae were shown to contain four major antigens with molecular masses of 47, 55, 90, and 105 kDa. The adult cuticle contains three major antigens with molecular masses of 20, 33 and 40 kDa [8].

2.2. ES antigens

ES antigens are synthesized by *Trichinella* from different developmental stages, and the source of ES antigens is the stichosome. The antigenicity and composition of ES antigens of *Trichinella* vary according to the developmental stage. The ES antigens of the ML consist of a group of structurally related glycoproteins with molecular weights of 45–53 kDa [39]. *Trichinella* ML antigens have been classified into eight groups (TSL-1 to TSL-8) based on their recognition by different monoclonal and polyclonal antibodies. TSL-1 (45–100 kDa in the non-reduced form), TSL-2 (45 kDa in the non-reduced form), TSL-3 (45 kDa in the non-reduced form), and TSL-5 (35 kDa in the non-reduced form) are present in ES antigens of the ML. TSL-1 is the most abundant ES antigen [31]. A immunocytolocalization study showed that the antibodies are distributed in the hypertrophic nuclei and cytoplasm of parasitized nurse cells and in the lumen of the larvae oesophagus and intestine tissues. On the contrary, ES antigens of adult parasites are sometimes poorly immunogenic and lack the specific 45–53 kDa antigens [31]. The NBL cannot excrete/secrete any antigen; however, the NBL starts to form a stichosome after invading muscle cells.

2.3. Somatic antigens

Somatic antigens are less specific and can cross with antibodies against other parasites. This cross-reaction is due to the presence of phosphorylcholine within somatic antigens. They are distributed in many internal structures in both the ML and adult worms. Phosphorycholine has been found in many parasites, including *Ascaris suum*, *Nippostrongylus brasiliensis*, *Toxocara canis*, and *Trichuris suis*. Additionally, phosphorycholine is an immunodominant bacterial and fungal cell wall component. Moreover, Boireau et al. classified eleven groups of antigens with monoclonal antibodies (mAbs) against somatic *Trichinella* ML extracts. An indirect fluorescent assay indicated that most groups belong to the surface and ES antigens, and only those of group 11 are restricted to the gut [5].

2.4. Antigen purification and cloning

TSL-1 antigens share an immunodominant carbohydrate epitope (tyvelose), which is unique for *Trichinella* and elicits the major antibody response in the late stage of *Trichinella* infection. They can be purified by affinity chromatography with mAbs. TSL-1 antigens are specific to the ML stage and are lost during the accelerated larval moulting [6]. Immunocytolocalization studies showed that TSL-1 antigens are primarily at the cuticle and in alpha- and beta stichocytes. mAbs against TSL-1 recognized the 40–70 kDa antigens from ML homogenates under reducing conditions, and they recognized the 45–55 kDa antigens in ES products under non-reducing conditions [31]. TSL-1 antigen epitopes are highly conserved and can been recognized by antibodies that are induced by different *Trichinella* species. Thus, enzyme-linked immunosorbent assays (ELISAs) that are based on TSL-1 antigens can detect any *Trichinella* species infection [19]. The 45-, 49-, and 53 kDa glycoproteins are the major ES antigens and they were purified by affinity chromatography with mAbs [13].

A TsA-12 clone encoding a 53 kDa glycoprotein was identified by immunoscreening of a *Trichinella spiralis* ML cDNA expression library. The 53 kDa glycoprotein of *T. spiralis* is expressed in the postcapsule larvae (15-day-old ML) and adult worms but not in the precaspule (35-day-old ML) or NBL. The glycoprotein showed high sequence similarities (90.7% and 89.5%, respectively) to the *Trichinella britovi* and *Trichinella native* 53 kDa proteins (encapsulated species); however, they showed low sequence similarities (66.6% and 68.8%, respectively) to the *Trichinella pseudospiralis* and *Trichinella papuae* 53 kDa proteins (non-encapsulated species) [27]. The 53-KDa glycoprotein of *T. spiralis* contains species-specific epitopes. The antibody response induced by the 53 kDa glycoprotein is mainly due to protein epitopes, and the antibody response against glycan epitopes is less important [34]. Western blot analysis with the different *Trichinella* species 53 kDa recombinant proteins indicated that the 53-kDa antigens induced an early and species-specific antibody response in mice that were infected with *Trichinella* [27].

In recent years, a number of antigens have been identified by immunoscreening of cDNA expression libraries from different *T. spiralis* developmental stages, including serine protease inhibitors, serine proteases and some early antigens [44,47]. Some of these antigens showed promising potential in early detection of *Trichinella* infection in pigs. Furthermore, epitope mapping was performed on specific immunodominant antigens using various approaches, including overlapping synthetic peptides or cDNA fragments expressed in *Escherichia coli* [4,32] and a phage display strategy combined with a monoclonal antibody [42]. However, the immunodominance of linear epitopes that were identified by screening overlapping synthetic peptides could not replace the ES antigens in the indirect ELISA. Recently, proteomic or transcriptomic (subtractive cDNA libraries) approaches were successfully used to select immunodominant targets or to identify new antigenic components [41,25].

Longitudinal studies in various host species underline the possibility of variation in the antigenic stimulation. After several weeks post infection, encapsulated *Trichinella* does not stimulate the immune system in several hosts and most ML antigens seem hidden. Table 1 illustrates the antigenic variation of the expression of some antigens that differ before and after cyst formation [43].

Table 1
Variability in the expression of *Trichinella* antigen.

Species	TsORF	P43	GP53	TsJ5	Tsmyd	TbHSP
T. spiralis	Ad/ML35dpi	ML	ML35dpi/Ad	All stages	ML/Ad	All stages
T. britovi	NT	ML	ML35dpi/Ad	All stages	ML/Ad[3]	All stages
T. nativa	NT	ML	ML35dpi/Ad	NT	NT	All stages
T. pseudospiralis	Ad/ML35dpi	ML	ML35dpi/Ad	Low expression in ML	ML/Ad	All stages

dpi: days post infection; Ad: adult stage; ML muscle larvae stage; NT: not test.

3. Antibody detection methods

Trichinella infection results in a specific antibody response. The time of seroconversion is dependent upon the infection dose, *Trichinella* species, and the host species. The persistence of antibodies in different host species also varies. After primary infection with *Trichinella* larvae, seroconversion in human patients usually occurs between the second and fifth weeks, and specific antibodies may persist for years. Antibody levels do not correlate with the disease severity or the clinical course in the acute trichinosis stage [9,20]. Detectable antibody levels in animals that were infected with *Trichinella* are not usually present until 2–3 weeks or more following exposure ([14,22,23,26,28]). Antibodies against *Trichinella* can persist for at least 6 months after infection without a decline in the ES ELISA. However, in horses, antibody levels decline in a few months following infection despite the presence of infective larvae in the muscles [21]. Therefore, serological methods cannot be recommended in detecting *Trichinella* infection in horses [19]. In humans, antibody detection tests are useful adjuncts to diagnoses. The immunoglobulin G (IgG), immunoglobulin E (IgE), and immunoglobulin A (IgA)-specific antibody classes do not appear until 2–3 weeks after trichinosis infection. The IgG-specific antibodies peak around the third month post-infection and may persist in a patient's blood for years after an infection. However, the IgG antitrichinella-specific antibody titres do not correlate with the disease severity or the clinical course in the acute trichinosis stage. The seroconversion time point is dependent upon several factors, such as the number of ingested larvae, the *Trichinella* species involved, and the individual immune response (Table 2). Different techniques have been described for detecting antibodies against *Trichinella* infection in humans and animals, such as the indirect fluorescent antibody test (IFAT), Western blot analysis, and the indirect ELISA [19]. Here, the serological tools for detecting *Trichinella* specific antibodies are summarized.

Table 2
Relationship between time of seroconversion and infection does (*T. spiralis*) in pig, horse, wild boar, sliver fox, and red fox and human.

Animal species	Infection dose (No. of larvae/animal)	Time of seroconversion p.i. (week)	Reference
Swine	100	5–7	[15,28]
	500	4–5	
	1000	4–6	
	2500	4	
	8000	3	
	20,000	3–4	
	64,000	2.5–3	
Horse	1000	3–4	[14,38]
	4000	3–7	
	5000	2–4.5	
	10,000	3–4	
	20,000	2–3	
	50,000		
Wild boar	10,000	3–4	[22]
Sliver fox	500	4–6	[29]
	2000	2	
Red fox	10,000	3	[26]
Human	Unknown	2–3	[19]

3.1. IFAT

IFAT uses the whole parasite body as a series of antigens. Cryostat and paraffin sections of *T. spiralis* larvae or muscle tissue of experimentally infected mice are employed in this test. Sections are incubated with diluted serum and probed with fluorescein-labelled antibodies against immunoglobulin of the animal species. The reaction of *Trichinella* larvae with test serum is evaluated under a fluorescence microscope. Due to cross-reactions that may occur with antibodies against *filariae* (*Onchocera* spp.), *Schistoma mansoni*, and some plant parasitic nematodes, although the technique is sensitive, its application is limited. Further, investigation of cross-reactive antibodies showed that epitopes of the heat shock protein from *Trichienlla* is also recognized by antibodies from patients with autoimmune disease. Another one of its limitations is the fact that this test requires a high level of skill, experience, and expensive laboratory facilities, and serial dilutions of serum must be made, which makes the test laborious and impractical for the screening of large number of samples.

3.2. Indirect ELISA

ELISA is the most commonly serological method to detect *Trichinella* infection in humans and animals. In comparison with other serological methods, it is easy to conduct, relatively inexpensive, readily standardized and can be automated for large-scale testing. Moreover, it is the only serological method recommended by the Office International de Epizooties to detect *Trichinella* infection in domestic pigs. ELISA for detecting *Trichinella* infection has a higher sensitivity than digestion methods in lightly infected animals. Infections as low as one larva/100 g of tissue have been detected with ELISAs. The sensitivity and specificity of ELISAs are largely dependent upon the antigen that is chosen; however, it is also relative to the host species (Table 3). At present, different antigens, such as crude antigens, ES antigens, purified antigens and synthetic carbohydrate antigens have been used in ELISA for detection of *Trichinella* infection [19,20]. Additionally, the sample quality and the individual immune response also influence the reliability of the test. Poor sample quality (e.g., samples with extensive haemolysis or bacterial growth) may decrease the specificity and sensitivity of the tests.

Initially, somatic ML antigens were used in ELISA tests. The practical advantage of somatic antigens is that they are easier to prepare with a low cost and a high yield. However, a problem with the ELISA based on somatic antigens is that cross-reactions with antibodies that are elicited by other parasites, such as *A. suum*, *T. suis*, filariae, and *Schistosoma*, occur [35], which is why somatic ML antigens are not recommended for serological testing.

Table 3
ELISA tests for detection of *Trichinella* antibodies [2].

Antigen	Sensitivity	Specificity
Crude antigens	99% (human, pigs)	60% (human)
ES antigens	99% (human)	91–96% (human)
	93.1–99.2% (pigs)	90.6–99.6% (pigs)
	98% (horses)	98% (horses)
Beta tyvelose	<98% (pigs)	>99% (pigs)
	<98% (horses)	

The specificity of ELISA in detecting *Trichinella* infection in pigs was greatly improved by ES antigens of the ML. Various field studies showed that the specificity and sensitivity of ES ELISA in pigs ranged from 90.6–99.4% and 93.1–99.2% [19]. Moreover, ES antigens from different *Trichinella* species showed high cross-reactivity between various *Trichinella species* as serology tests cannot be used in differentiating *Trichinella* species and genotypes at present.

However, ES ELISA has some disadvantages. First, the recovery is laborious. Micro-environmental factors during larvae culture may affect antigen quality, resulting in standardization problems. The second disadvantage of the ES ELISA is the occurrence of false negative results during the early stage of infection [19]. In particular, a "blind window" exists, during which larvae become infective to a new host but specific antibodies cannot be detected. In this case, the infection would already be detectable by direct detection but not by serological detection.

Some antigens that are purified with affinity chromatography have been applied in ELISA and display potential for serological detection of *Trichinella* infection in humans, pigs, and other animals. TSL-1 that is immobilized on ELISA plates using mAb against tyvelose showed 100% specificity and sensitivity in serodiagnosis of human trichinellosis [10]. The 45, 49, and 53 kDa glycoproteins have been isolated by mAb affinity chromatography and used in serological tests to detect *Trichinella* infection in pigs. These purified antigens also improved the ELISA specificity without decreasing the antibody detection sensitivity. The carbohydrate, β-tyvelose, which is the major immunodominant epitope of the TSL-1 antigens, has been synthesized and used for the detection of antibodies against *Trichinella* [19]. The synthetic β-tyvelose antigen offers stability and standardization advantages. Equal specificity was observed between E/S antigens and β-tyvelose in detecting *Trichinella*-specific antibodies in humans and pigs [7,11]. Unfortunately, synthetic β-tyvelose was less sensitive than ES antigens for *Trichinella* antibody detection in wild and domestic animals [26]. Thus, the authors suggested that the β-tyvelose antigen might not be suitable for monitoring infections in pig herds. Recently, GalNAcb1–4(Fuca1–3)GlcNAc-R (LDNF glycan) was identified as a potential antigen for diagnosis of human trichinellosis through a glycan microarray technique. ELISAs based on five LDNF glycan molecules showed a high sensitivity [1].

Recently, some antigens were identified by immunoscreening of cDNA libraries of different *T. spiralis* developmental stages. The applications of recombinant antigens for antibody detection have been described. Two early stage immunodominant antigens of *T. spiralis*, 411 and NBL1, were evaluated to detect *Trichinella* infection in pigs. The results showed that ELISAs based on recombinant NBL1 or 411 had a high specificity level. More than 150 sera from *Trichinella*-free pigs did not react with the ELISA based on either NBL1 or 411. Additionally, ELISAs based on either NBL1 or 411 showed the same sensitivity as an ES ELISA for *Trichinella* detection in highly (20,000 ML) or moderately (1000 ML) infected animals; however, the sensitivity of the NBL1 or 411 ELISAs was lower than that of the ES ELISA in testing lightly (200 ML) infected pigs. Significantly, the NBL1- and 411-based ELISAs allowed for earlier detection (5 to 45 days and 5 to 20 days, respectively) compared with the ES ELISA. Thus, the two antigens are good candidates for improving the serological tools for early *Trichinella* infection detection in pigs [4]. The antigenic potential of the 53 kDa *T. spiralis* glycoprotein was also evaluated with an ELISA. An antibody against the recombinant 53 kDa glycoprotein could be detected in experimentally-infected mice as early as 14 days post infection [27].

3.3. Western blot assay

Western blot assay based on either ES antigens or crude worm extracts (CWE) of ML can also be applied to detect antibodies against *Trichinella.* This method allows for the detection of specific *Trichinella* antigens and can discriminate cross-reacting antibodies. However, this test is not applicable for routine diagnosis as it requires technical expertise and is time consuming, cumbersome, and expensive; therefore, the technique is often used as an adjunct to confirm other serological tests with positive results rather than as a routine screening tool for herd sera [19]. As *Trichinella* antigens from CWE present a more complex banding pattern and are easier to prepare than ES antigens, they are more favoured for use in Western blot analysis [3]. The specific patterns of these proteins that are recognized by sera from different hosts have been investigated with either ES antigens or CWE. Human sera from trichinellosis-infected patient-recognized specific protein bands with molecular weights of 47, 55, and 90 kDa or 43–44 and 64 kDa in CWE of *T. spiralis* [45]. Western blotting with ES antigens showed that the 55, 36, 29, and 14 kDa proteins were specifically recognized by sera from trichinellosis patients; however, only the 55 kDa protein was recognized by all of the trichinellosis infected patient sera [30]. Western blots are able to discriminate trichinellosis patients from patients with other helminth infections, although cross-reaction was observed from patients with toxocariasis, filariosis, anisakiasis, and other parasitic infections [17].

Patterns recognized for *Trichinella* infections in humans and pigs are significantly different. Forty-seven, 49, 52, 60, and 63 kDa proteins or 43, 47, 61, 66, and 102 kDa proteins in the CWE were recognized in the pig sera; however, the 43 kDa protein was the predominant antigen [3,12]. Variations in the molecular weights for specific protein fractions may be due to differences in the protocols that are used to prepare the antigens, quality of the matrices (serum and muscle juice), and reference ladders [3]. A recent study showed that there are specific triple-band patterns for the 53–72 kDa proteins in human sera and 48–72 kDa proteins in pig sera [16]. The sensitivities and specificities for anti-*Trichinella* antibody detection in pigs ranged from 95.8% to 98.1% and from 99.5% to 99.6%, respectively. The pattern profile frequency was irrespective of the dose and the period of infection as well as the *Trichinella* species [3,12].

3.4. Other antibody detection methods

Bentonite flocculation and latex agglutination are used in human trichinellosis diagnoses. However, they are not as sensitive and specific as ELISA. Thus, they are used only when a rapid infection confirmation is required (the result is obtained in less than 1 h) [9]. The competitive inhibition assay (CIA), which has a high level of specificity for antibody detection against *Trichinella*, is a valuable test. Due to its higher specificity, CIA can help in the interpretation of pathological symptoms at late and distant periods following invasion [19]. A novel immunoenzymatic test, which was named the thin-layer immunoassay-enzyme-linked immunosorbent assay (TIA-ELISA), was developed. A TIA-ELISA that was utilized for ES antigens showed similar sensitivity and specificity to that of the conventional ELISA [18]. Because the TIA-ELISA is easy to perform, cheap, sensitive, and specific, the test could be an acceptable alternative for use in clinical laboratories without specialized equipment.

The Dot-ELISA is easier to perform than ELISAs and Western blotting, and the results can be read without specialized equipment. ES antigens and purified antigens have been applied in the Dot-ELISA as a serological test for trichinellosis in pigs and humans [36]. Recently, an immunochromatographic strip for rapid detection of *Trichinella* infections in pigs was developed. This serological tool is used as an alternative to ELISAs in clinical laboratories lacking specialized equipment [46].

A bead-based suspension array method was recently used to develop novel serological tools. *T. spiralis* ES antigens were covalently coupled to paramagnetic beads and used to bind serum antibodies. Additionally, the use of protein A/G instead of an anti-swine antibody showed a high sensitivity with a similar specificity. The result suggested that this serological tool could potentially be used in various species [37].

4. Antigen and detection methods

Antigen detection may provide a tool for the serological monitoring of anti-parasitic therapies and for the study of the interactions

between *Trichinella* and its hosts [40,48]. Various methods have been developed to detect circulating antigens (CAg) of *Trichinella*, including counter-immunoelectrophoresis, immunoradiometric assays, Dot blots, and ELISAs [40,48]. Because the *T. spiralis* CAg levels in the serum are low, the sensitivity of antigen detection tests is lower than that of antibody detection tests. The typical successful CAg detection rate in serum samples was 19%–47% in human patients and 56% in pigs that were infected with *Trichinella*. Hence, the serological methods available for detecting *Trichinella* antigens cannot be applied for trichinellosis serological test in humans and animals [9].

5. Conclusion and perspective

It is clear that the development of serological tools for trichinellosis has greatly contributed to a better understanding of the prevalence and epidemiology of *Trichinella* infection. They may contribute to the diagnosis of human Trichinellosis and to treatment follow-ups. Serological tests also provide a valuable tool in monitoring *Trichinella* infections in domestic animals and wildlife.

Trichinella diagnoses are still challenging despite the advances made in the development of serological techniques. An equal importance should also be given to the efficacy of the laboratory test. A standard technique should have a high sensitivity and specificity, and it must be reproducible, easy to perform, and adaptable for use in local laboratories without specialized equipment or in the field. Furthermore, costs should be taken into account to choose which diagnostic tests can be applied. More research is required to achieve all of these objectives.

The antigens used in serological tests for antibody detection have evolved from crude extracts to highly purified specific antigens, synthetic peptides, and recombinant antigens. The application of ELISAs for the detection of circulating parasite antigens may present some diagnostic advantages because it demonstrates not only exposure but also active infections. The problem with the detection of antibodies that recognized *Trichinella* is the occurrence of false negative results that occur during the early stage of infection. Thus, efforts should be made to develop new or improved serological tools for early detection of antibodies that recognize *Trichinella*. Efforts in isolating and characterizing the antigens at different parasitic stages will most likely lead to the identification of specific proteins with promising antigenic potential. This information, together with genomic and proteomic data, will provide a solid base for further development of serological tools. Finally, effective work should also be conducted to develop practical, simple, effective, and economical serological tools so that they can be used in field conditions for surveys. Once these data have been assembled, the hope is that immunodiagnostic tests will be further developed for commercial use.

Contributions of authors

Y. Yang, Y N Cai, and M W Tong wrote the manuscript. I. Vallée, P. Boireau and MY Liu and S P Cheng read and reviewed the manuscript. N Song, Y H Xuan and Y J Kang provide some suggestion about the manuscript.

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