Review Articles

Recombinant Proteins as Antigens for Serological Detection of *Toxoplasma gondii* and *Neospora caninum* in Livestock

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Received: 27 January 2021; Revised: 14 June 2021; Accepted: 16 June 2021

Abstract

Toxoplasmosis and neosporosis are diseases of livestock worldwide caused by infections with closely related parasitic protozoa, *T. gondii* and *N. caninum*, respectively. Toxoplasmosis is a cause of reproductive failure in small ruminants and zoonotic, while neosporosis is a major cause of bovine abortion without zoonotic reports. The clinical signs associated with both infections are often nonspecific. Therefore, serological diagnosis is important for detection of specific antibodies induced by the infection. However, propagation of *T. gondii* and *N. caninum* tachyzoite *in vitro* or *in vivo* is required prior to crude antigen extraction, high risk in contamination of cell culture or animal facilities for parasite propagation and time-consuming process. With the use of recombinant proteins as antigens, the risk of handing viable parasites can be avoided with improving in sensitivity and specificity for the detection. Although some of *T. gondii* or *N. caninum* recombinant proteins showed a high efficacy for diagnosis, more validation and optimization are still needed to provide a high throughput performance for using in animals. This review presents advance in the application of recombinant antigens as a serological marker for the above parasites detection in livestock.

Keywords: diagnosis, *Neospora caninum, Toxoplasma gondii,* recombinant proteins, serology

รีคอมบิแนนท์โปรตีนเพื่อเป็นแอนติเจนสำหรับการตรวจวินิจฉัยทางซีรั่มวิทยาของเชื้อทอกโซพลาสมา กอนดิไอและนีโอสปอรา แคนินุ่มในปศุสัตว์

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Received: 27 January 2021; Revised: 14 June 2021; Accepted: 16 June 2021

บทคัดย่อ

โรคทอกโซพลาสโมซิสและโรคนีโอสปอโรซิสเป็นโรคที่พบได้ในปศุสัตว์ทั่วโลกเป็นโรคเกิดจากเชื้อโปรโตซัวทอกโซพลาสมา กอนดิไอและนีโอสปอรา แคนินุ่ม ซึ่งที่มีรูปร่างลักษณะทางโครงสร้าง พันธุกรรมและภูมิคุ้มกันคล้ายคลึงกันมาก โรคทอกโซพลาสโมซิสเป็นสาเหตุทำให้เกิดปัญหาในระบบสืบพันธุ์ในสัตว์เคี้ยวเอื้องขนาดเล็กและเป็นโรคติดต่อจากสัตว์สู่คน ในขณะที่โรคนีโอสปอโรซิสเป็นสาเหตุหลักของการเกิดการแท้งในวัวแต่ยังไม่มีรายงานว่าเป็นโรคสัตว์สู่คน การติดเชื้อมักไม่มีอาการแสดงที่จำเพาะ ดังนั้นการตรวจทางซีรั่มวิทยาจึงถือเป็นปัจจัยสำคัญในการวินิจฉัยโรคโดยการตรวจหาแอนติบอดีจำเพาะต่อการติดเชื้อ วิธีการตรวจหาแอนติบอดีต่อการติดเชื้อโปรโตซัวทั้งสองชนิดนี้โดยทั่วไปจะต้องเพาะเลี้ยงเชื้อระยะแทคคิซอยในหลอดทดลองหรือในหนูทดลองเพื่อนำมาเป็นแอนติเจน ซึ่งข้อเสียของการผลิตแอนติเจนสามารถเกิดการปนเปื้อน การจัดการสัตว์ทดลอง ใช้เวลานาน ดังนั้นการพัฒนาการตรวจวิเคราะห์โดยใช้รีคอมบิแนนท์โปรตีนเพื่อใช้เป็นแอนติเจนสามารถลดความเสี่ยงดังกล่าวอีกทั้งยังเพิ่มความไวและความจำเพาะในการตรวจวิเคราะห์มากยิ่งขึ้นด้วย ถึงแม้ว่ารีคอมบิแนนท์โปรตีนเชื้อทอกโซพลาสม่า กอนดิไอและนีโอสปอรา แคนินุ่มบางชนิดมีประสิทธิภาพในการตรวจสูง อย่างไรก็ตามการทดสอบความถูกต้องและหาสภาวะที่เหมาะสมของวิธีตรวจวิเคราะห์เป็นสิ่งจำเป็นอย่างยิ่งโดยเฉพาะการตรวจสัตว์ปศุสัตว์ เพื่อให้วิธีตรวจวิเคราะห์นั้นมีประสิทธิภาพ บทความปริทัศน์นี้มีเนื้อหามุ่งเน้นประโยชน์และประยุกต์ใช้รีคอมบิแนนท์โปรตีนสำหรับเป็นเครื่องหมายการตรวจทางเซรุ่มวิทยาของเชื้อทั้งสองชนิดในปศุสัตว์

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Introduction

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii* with a worldwide significant concern in human as infection with *T. gondii* can induce abortion in primary infection during or just before pregnancy and cause serious clinical symptoms in immunocompromised hosts (Dunay et al., 2018). Besides the disease in human, *T. gondii* infection can cause abortion, stillbirth or neonatal deaths in farm animals especially sheep, goats and pigs which are the sources of infection to human (Hill and Dubey 2013). Neosporosis has emerged as a serious disease in cattle and dogs worldwide (Dubey and Schares 2011). It is an important cause of reproductive failure and neonatal mortality in cattle due to abortion which leads to economic losses including infertility, reduced milk yield, and weight reduction (Wilkowsky et al., 2011; Horcajo et al., 2016). In dog, several clinical forms have been described (Silva and Machado 2016), affecting very young individuals, adults, or even old dogs, mainly characterized by nervous manifestations. Up to now, there is no evidence for *N. caninum* infection in human.

Serology is the one of the major methods which is used to establish the diagnoses of *T. gondii* and *N. caninum* an indirect way, based on the research of antibodies against the parasite (Lindsay and Dubey 2020). Several serological tests have been used for detection of these parasites in animals which are the different antigen formats such as indirect immuno-fluorescent antibody test (IFAT), agglutination test and enzyme-linked immunosorbent assay (ELISA) (Dubey et al., 1996; Canada et al., 2004; Wiengcharoen et al., 2012; Udonsom et al., 2018; Udonsom et al., 2019). However, *T. gondii* and *N. caninum* are closely related parasites and share the same class of organelles and have several shared genomic features. Therefore, the use of whole parasites obtained from mouse inoculation or cell culture may cause false positive due to cross reaction (Conrad et al., 1993; Bjorkman and Uggla 1999; Chahan et al., 2003) . Moreover, parasites lysate antigens mixed with lipids, carbohydrates and other non-specific proteins extracted from whole parasites could affect the sensitivity and specificity of the tests (Ghalmi et al., 2014; Singh et al., 2015).

With the advances in designing and prediction of protein epitopes, recombinant antigens are made to increase the sensitivity and specificity of the diagnostic tests which could be used as alternative antigens to overcome the cons of whole *T. gondii or N. caninum* tachyzoites obtained from mouse inoculation or cell culture and/or parasites lysate antigen problems (Bai et al., 2012; Dong et al., 2012; Wang et al., 2016; Márquez-Contreras 2018). Here we review the development of recombinant antigens based on various formats, and their applications for toxoplasmosis and neosporosis diagnosis in livestock.

Development of recombinant proteins for serodiagnosis of toxoplasmosis and neosporosis

Serological tests play a crucial role in the diagnosis of toxoplasmosis and neosporosis when a specific clinical sign is absent. Several serological tests have been developed using either live tachyzoites or native soluble antigens however, they are expensive, laborious, and low specificity (Velmurugan et al., 2008; Márquez-Contreras, 2018). One approach to improving these antigens is to replace the native antigens with recombinant proteins. Using recombinant proteins for the diagnosis have been shown to be useful to improve standardization of the test and reduce the costs of production. The advances in molecular biology, genomics, proteomics, and bioinformatics have been allowing and facilitating the design of new strategies for the development of more sensitive and specific diagnostic tests in the form of recombinant antigens (Laín et al., 2008; Wang et al., 2013; Shaddel et al., 2018). In recent years, The recombinant *T. gondii* and *N. caninum* protein antigens have been expressed from several genes including surface antigen (SAGs), microneme proteins (MICs), rhoptries proteins (ROPs) and dense granule proteins (GRAs) (Liao et al., 2005; Fernández-García et al., 2006; Jiang et al., 2008; Gatkowska et al., 2010; Wang et al., 2014; Bachan et al., 2018).

The recombinant proteins are widely applied as antigen marker in various serological methods including Enzyme-linked immunosorbent assay ELISA, latex agglutination test and rapid immunochromatographic test.

Recombinant protein-based ELISA

Numerous of the recombinant proteins have been evaluated in an ELISA test to detect antibodies against *T. gondii* or *N. caninum* infection in domestic animals (Table 1). The detection of *T. gondii* infection in goats when using *T. gondii* major surface antigen (SAG1) based ELISA demonstrated the high sensitivity and specificity when compared with immunofluorescent antibody test IFAT or commercial ELISA kit (Bachan et al., 2018; Velmurugan et al., 2008). In cattle samples, SAG1 recombinant antigen-based ELISA had sensitivity of 84.4% and specificity of 87.9% with a substantial kappa value of 0.7 when compared to IFAT (Sudan et al., 2019). To determine the activity of SAG2 antigen in cattle, the sensitivity and specificity were 80.0% and 88.57%, respectively, compared to IFAT (Sudan et al., 2019). For another study, the recombinant SAG2 showed sensitivity ranging from 81.3% to 87.1% while the specificity was 85.7% to 91.4% for *T. gondii* detection in ruminant samples compared to IFAT (Singh et al., 2015). With the pig sera, one study also evaluated SAG2 antigen, and the results showed 75.0% of sensitivity and 96.3% of specificity for *T. gondii* detection compared to LAT (Terkawi et al., 2013). The dense granule antigen, in particular of *T. gondii* GRA7 has shown to be less of sensitivity of 80% but it showed a good specificity of 88.4% in goat sera compared to IFAT (Velmurugan et al., 2008). On the other hand, GRA7 revealed a good sensitivity of 90.6% while it was less specificity of 85.2% in pig sera compared to LAT (Terkawi et al., 2013).

A study carried out by Wang Z et al., 2014, GRA7 had a high sensitivity of 96.4% and specificity of 98.6% for the detection in cattle samples collected from slaughtered in abattoirs compared to *Toxoplasma*-lysate antigen based ELISA.

Most studies of *N. caninum* antigens using NcSRS2 antigen which is a surface antigen of *N. caninum* expressed in both bradyzoite and tachyzoite stages (Hosseininejad et al., 2010; Uzeda et al., 2013). NcSRS2 showed a high sensitivity of 90.6% and specificity of 94.4% in cattle samples with kappa value of 0.8 compared to two commercial ELISA kits (Liu et al., 2007). Another study, NcSRS2 revealed a high sensitivity of 98.7% and specificity of 88.7% with kappa value of 0.8803 in cattle samples compared to IFAT (Sinnott et al., 2015) In addition, NcSRS2 showed a high sensitivity of 100% and specificity of 94.5% in field sheep samples compared to IFAT (Pinheiro et al., 2015). One study of subtilisin-like serine protease 1 proteins (NcSUB1t and NcSUB1tr) showed a good in sensitivity of 94.6-96 % and specificity of 80-100% in filed cattle samples compared to in-house SAG1 antigen based ELISA (Ybañez et al., 2013). A study of mixture the 3 antigens including NcSAG1, NcSRS2 and NcGRA2 to evaluate of known cattle samples found sensitivity of 91.7% and specificity of 100% compared to commercial ELISA kit (Dong et al., 2012).

Recombinant protein-based latex agglutination test

The advantages of latex agglutination test are the absent of secondary antibody, no special equipment needed and easy to perform (Ghalmi et al., 2014; Hill and Dubey, 2013). One study was developed recombinant antigen of *T. gondii* MIC3-sensitized with latex particle for *T. gondii* detection in pigs, the results showed a high sensitivity and specificity when tested with 50 positive and 30 negative known pig sera, and indicate TgMIC3-LAT is suitable for detecting in early stage of infection in piglets experiment infected with *T. gondii* (Jiang et al., 2008). A study of NcSAG1 based on LAT for *N. caninum* detection in cattle, NcSAG1 showed a substantial agreement of 0.8 kappa value compared to commercial ELISA kit (Moraveji et al., 2012).

Recombinant protein-based rapid immunochromatographic test (ICT)

The rapid immunochromatographic test is a qualitative test used for detection of antigen or antibody of *T. gondii*/ *N. caninum* infections. The high sensitivity (71.8-96.3%) and specificity (80-100%) when using the GRA7 were observed in field pig sera (Terkawi et al., 2013). *N. caninum* SAG1 based on ICT showed a sensitivity of 94.7% and specificity of 93.5% in cattle samples compared to in-house SAG1-ELISA (Liao et al., 2005).

Table 1. Performance of different recombinant antigens for serodiagnosis of toxoplasmosis and neosporosis in livestock

Antigen	Diagnostic test	Animals	Sensitivity (%)	Specificity (%)	References
T. gondii					
SAG1	ELISA	Goat	92.6	90.7	Bachan M et al., 2018
SAG1	ELISA	Cattle	84.4	87.9	Sudan V et al., 2015
SAG2	ELISA	Cattle	80.0	88.6	Sudan V et al., 2019
SAG2	ELISA	Goat	82.1	91.4	Singh H et al., 2015
		Sheep	81.3	85.7	
		Cattle	87.1	85.7	
GRA7	ELISA	Cattle	96.4	98.6	Wang Z et al., 2014
SAG2	ELISA	Pigs	75.0	96.3	Terkawi MA et al., 2013
GRA7			90.6	85.2	
GRA14			81.3	92.6	
SAG1	ELISA	Goat	83.3	88.4	Velmurugan GV et al.,
GRA7			80.0	88.4	2008
N. caninum					
SRS2	b-ELISA	Cattle	98.7	88.7	Sinnott FA et al., 2015
SRS2	ELISA	Sheep	100	94.5	Pinheiro FA et al., 2015
SUB1	ELISA	Cattle	94.6-96	80-100	Ybanez RH et al., 2013
SRS2	ELISA	Cattle	90.6	94.4	Liu J et al., 2007
SAG1	ICT	Cattle	94.7	93.5	Liao M et al., 2005

Recombinant protein for immunoblot

The development of immunoblot method using recombinant NcSAG4 for the detection of *N. caninum* in 52 field cattle samples was performed and showed more sensitive than that of commercial ELISA kit with high specificity (100%) (Hu et al., 2011). The NcSAG4 protein is an antigen that was expressed in the bradyzoite stage of *N. caninum* which correlated to chronic infection (Fernández-García et al., 2006). Therefore, this could be used this recombinant NcSAG4 as a serological marker of chronic stage in the infected animals.

Recombinant antigens used in serological detection for toxoplasmosis and neosporosis in livestock

Several studies have evaluated and improved the sensitivity and specificity of recombinant antigens for toxoplasmosis and neosporosis serodiagnosis in livestock based on ELISA methods. Most studies of *T. gondii* recombinant proteins used to identify the reactivity for serodiagnosis were the specific surface antigens of tachyzoites stage consisting of SAG1 and SAG2 while *N. caninum* recombinant protein used were of NcSRS2, a surface antigen expressed in both bradyzoite and tachyzoite stages. For *T. gondii* detection, SAG1 showed a high sensitivity and specificity for the detection in field goat samples (Bachan et al., 2018), followed by TgGRA7 which have shown to be a good antigen for antibody detection in cattle samples (Wang et al., 2014). For *N. caninum* detection, NcSRS2 revealed a highest sensitivity of 100% for the detection in field sheep samples (Pinheiro et al., 2015). The high specificity of 100% in cattle samples was obtained when the bradyzoite stage-specific, NcSAG4, were used for antibody detection using immunoblot showed (Hu et al., 2011). Furthermore, NcSAG1 was used to develop the immunochromatographic test and the high sensitivity and specificity were demonstrated for the rapid detection of *N. caninum* in cattle samples (Liao et al., 2005).

Conclusions and future perspectives

Toxoplasmosis and neosporosis could affect morbidity and reduce the productivity of the livestock. Conventional serological tests have limitations due to a variety of factors such as antigen production and preparation, expensive, laborious, and low sensitivity for the diagnosis. There is an increasing interest in the development of the diagnostic tests for toxoplasmosis and neosporosis diagnosis in which the test is more reliable, simple, rapid and being further developed for local veterinary fields. Performance of the immunodiagnostic tests based on recombinant proteins is beneficial as they increase the sensitivity and specificity of detection as well as overcome the limitations of whole cell derived antigens. Several recombinant proteins have been shown to be useful for the diagnosis of toxoplasmosis and neosporosis in infected animals. The performance of different recombinant antigens in the same assays needs to be further optimized to increase the diagnostic test value, since the kinetics of antibodies against *T. gondii* and *N. caninum* infection are different among

animal species. Identification of the genes and immunogenic determinants of each recombinant antigen warrants further research to study their function. Finally, the development of a rapid immunochromatoghaphic test using recombinant antigens will provide an alternative test for routine screening of *T. gondii* and *N. caninum* in farm animals.

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