

A BRIEF UPDATE ON THE DIAGNOSIS OF TRICHINELLOSIS

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Abstract. The latest immunological and molecular methods for the diagnosis of swine and human trichinellosis are briefly reviewed. The following topics are discussed in more detail: isolation of specific antigens by continuous elution-isoelectric focusing methods, production of recombinant antigens, nature of immunodominant antigens, potential use of heat shock proteins (HSPs) as diagnostic antigens, roles of specific IgE and circulating antigens (CA). The immunodominant antigens were found to be highly heat resistant. The specificity and sensitivity of colorimetric sandwich ELISA, microfluorescence (ELFA), enhanced chemiluminescence (ECIA) and dissociated enhanced lanthanide fluoroimmunoassay (DELFLIA) in detecting CA were compared. The last method is the most sensitive, detecting as little as 1 ng of antigens/ml of serum. CA was detected as early as 7 days postinfection of mice. The serum from a patient suspected to have acute trichinellosis in Hong Kong was also tested positive for CA.

INTRODUCTION

The major objectives of the present paper are to provide a brief update and to stimulate further discussion on the diagnosis of trichinellosis. Three main areas will be reviewed; production of specific antigens, antibody detection and antigen detection. Some of our recent findings will also be reported.

Immunodiagnosis of trichinellosis is generally more straightforward in human than in porcine infections. This is due to the fact that the pigs in many endemic regions are commonly infected with a large variety of indigenous nematodes which produce cross-reacting antibodies against *Trichinella spiralis*. Therefore, in view of such a problem, the diagnosis of swine trichinellosis is also included in this update.

ANTIGEN PRODUCTION

Recombinant antigens

As early as 1990-1991, genes encoding the 53, 46 and 49 kDa antigenic polypeptides were cloned. Zarlenga and Gamble (1990) found that their 123 kDa B-galactosidase fusion protein did not crossreact with antiserum against *Trichuris suis* or *Ascaris suum*. The cDNA transcript was 539 bp in length. Sugane

and Matsuura (1990) reported that the gene for 46 kDa antigenic polypeptide was a single copy in the genome. Their recombinant antigen showed no cross-reactivity with antisera against *Entamoeba histolytica*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Schistosoma mansoni*, *Hymenolepis nana* and *Toxocara canis*. However, most of these heterologous antisera are from parasites not closely related to *T. spiralis*. Therefore, further studies are required to ascertain the specificity of the recombinant antigen.

Su *et al* (1991) cloned a cDNA which encoded a 49 kDa antigen. However, after comparing the sequence with the partial amino acid sequences of the 43 kDa protein published by Gold *et al* (1990), they suggested that the two antigens might be the same.

Yan *et al* (1994) produced a 37 kDa fusion protein which were recognized in ELISA by pig anti-*T. spiralis* serum and monoclonal antibodies.

Connolly *et al* (1995) cloned and characterized two small repetitive DNA sequences of 516 and 604 bp. Since similar sequences were not detected in other species by hybridization, the repetitive sequences may be exploited for diagnostic purpose.

Although all the above recombinant antigens have successfully been produced in the laboratory, it is not known how extensively they are being employed in

diagnostic kits. At present, most kits available in the market are still based on culture-derived reagents.

Production of specific antigens by isoelectric focusing

Besides molecular cloning, specific antigens can also be extracted from the worms using other cheaper and less laborious methods.

Ko and Yeung (1991) successfully isolated immunodominant antigens of 45, 47 and 53 kDa from crude somatic worm extracts using a rotating ampholine column (Rotofor, Biorad). The antigens, with pI 5.5, 4.3 and 4.4, respectively possessed the same epitopes as those of excretory/secretory (ES) antigens that were recovered by *in vitro* culture of muscle larvae. Antigens from the two sources showed similar specificity and sensitivity in ELISA. Presumably, the immunodominant antigens in the stichosome of infective larvae would retain the specific epitopes after their release as ES products.

Subsequently, Leung and Ko (this volume) succeeded in producing highly specific antigens by fractionating ES and somatic antigens from infective larvae by continuous SDS-PAGE using a Preparative Cell (Biorad). The sample was further purified by preparative isoelectric focusing using the Rotofor method (Biorad).

The immunodominant antigens of 45-53 kDa and other constituent proteins were fractionated into single band per lane. Five isoforms, with pIs varying from 4.97-5.89, did not cross-react with antisera against the common nematodes of Chinese pigs in immunoblotting. Therefore, these isoforms have good potentials to serve as diagnostic antigens. The above purification method is easy to perform and relatively inexpensive to run. The yield was as high as 85%.

NATURE OF IMMUNODOMINANT ES ANTIGENS

Glycoproteins

The ES products of the infective-stage larvae contain many types of molecules. They may play different roles in initiating the infectious process and modulating the host environment (Ko *et al*, 1994). The

composition of the ES products also varies according to the stage of worm development. Ko and Wong (1992) found that ES antigens from pre-encysted and encysted larvae showed differences in SDS-PAGE profile. The former ES products were dominated by low molecular mass proteins and the antigenic profile resembled that of adult worms (Ko and Yeung, 1989).

Using periodic acid-Schiff staining, Gamble (1985) found that the immunodominant 45, 49 and 53 kDa antigens in the ES products were glycoproteins. We also made similar observations in 1990 by staining gels with a modified diamine silver method (unpublished data, see Fan, 1993). Eleven glycoprotein bands, ranging from 20-84 kDa, were observed in the SDS-PAGE gels containing somatic worm extracts. Of these, the 45 and 47 kDa bands were most prominent. Fourteen glycoprotein bands, ranging from 18-80 kDa, were observed in the ES products. Bands of 29, 45, 47 and 53 kDa were prominent. Moreover, after sodium periodate treatment, epitopes which were previously recognized in the 45, 47, 53, 66 and 72 kDa bands by various monoclonal antibodies disappeared. This indicates that some active epitopes are in the carbohydrate moiety which is degraded as the result of cleaving the hexose ring by sodium periodate.

Using N-glycanase treatment, Gold *et al* (1990) and Denkers *et al* (1990) observed that the epitopes of 43, 45, 50 and 68 kDa antigens are probably associated with N and O-linked oligosaccharides. Wisniewski *et al* (1993) studied the monosaccharide composition of affinity purified larval glycoproteins originated from the stichosome by gas chromatography/mass spectrometry. Novel fucosyl- and tyvelosyl-containing glycoconjugates were characterized. Reason *et al* (1994) also discovered that TSL-1 glycans contained clusters of hydrophobic terminal structures which are likely to be highly immunogenic.

Several studies were carried out to evaluate the role of carbohydrate epitopes in immunity to *T. spiralis* (Denkers *et al*, 1991; Jarvis and Pritchard, 1992; Ellis *et al*, 1994). The last authors suggested that the coating of the larval surface with glycans may protect the parasite from enzymatic attack.

Heat resistant nature

The immunodominant antigens of infective larvae appear to have some unique physical properties

eg they are highly heat resistant. We found that the antigens could retain their reactivity even after being heated for 20 minutes at 43, 65 and 100 °C (Figs 1-3). After heat treatment, the antigens still reacted intensely with polyclonal and monoclonal antibodies in immunoblotting. The result may reconfirm the suggestion that some active epitopes are located on the carbohydrate moiety of the glycoprotein antigens. This would explain why they could withstand heat treatment.

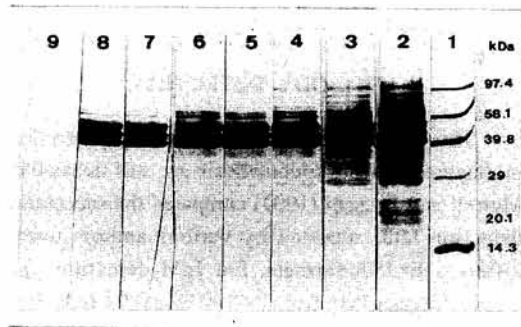


Fig 1 - Result of immunoblotting after heating ES antigens of *T. spiralis* for 20 minutes at 43 °C. Various polyclonal and monoclonal antibodies were used as developing antibodies. Lanes- 1. SDS-6 marker; 2. rabbit anti-ES serum; 3. pig anti-ES serum; 4. mab 7C2C5 (IgM); 5. mab 1D11 (IgG1); 6. mab 5D11 (IgG1); 7. mab 1H7 (IgM); 8. mab 3B2 (IgM) ; 9. myeloma NSI supernatant.

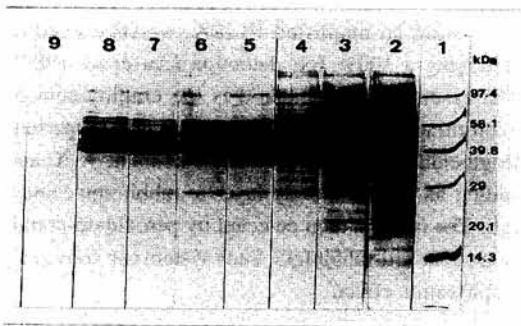


Fig 2 - Result of immunoblotting after heating ES antigens of *T. spiralis* for 20 minutes at 65 °C. Various polyclonal and monoclonal antibodies were used as developing antibodies. Lanes- 1. SDS-6 marker; 2. rabbit anti-ES serum; 3. pig anti-ES serum; 4. mab 7C2C5 (IgM); 5. mab 1D11 (IgG1); 6. mab 5D11 (IgG1); 7. mab 1H7 (IgM); 8. mab 3B2 (IgM); 9. myeloma NSI supernatant.

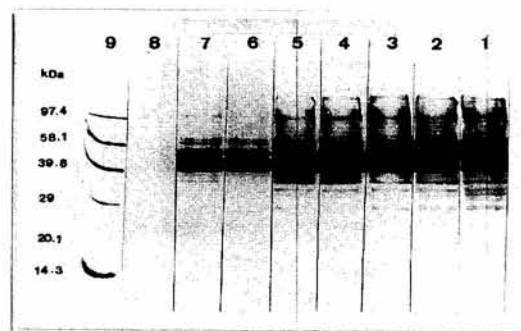


Fig 3 - Result of immunoblotting after heating ES antigens of *T. spiralis* for 20 minutes at 100 °C. Various polyclonal and monoclonal antibodies were used as developing antibodies. Lanes- 1. rabbit anti-ES serum; 2. pig anti-ES serum; 3. mab 7C2C5 (IgM); 4. mab 1D11 (IgG1); 5. mab 5D11 (IgG1); 6. mab 1H7 (IgM); 7. mab 3B2 (IgM); 8. myeloma NSI supernatant; 9. SDS-6 marker.

Heat shock proteins

At present we still cannot answer the following questions: What are the immunodominant glycoproteins? What are their specific functions as related to the survival of *T. spiralis* in host muscles?

A possible conjecture is that some immunodominant antigens in the somatic and ES products of the infective larvae may actually be heat shock proteins (HSPs). Using metabolic labeling with L-[³⁵S] methionine and *in vitro* translation experiments, Ko and Fan (1995) documented the presence of HSPs in both somatic extracts and ES products of *T. spiralis* and *T. pseudospiralis*. The optimal heat shock temperature is 43°C. The major HSPs in somatic extracts of *T. spiralis* are: 20, 47, 50, 70, 80 and 86 kDa. The major HSPs in ES products are: 11, 45, 53 and 64 kDa (Figs 4-5).

The conjecture is based on two major observations. Firstly, some HSPs have the same molecular masses and isoelectric points as the major immunodominant antigens commonly used in diagnosis. Secondly, several HSPs of other parasites are known to be immunogenic (Hedstrom *et al*, 1988; Rothstein *et al*, 1989; Selkirk *et al*, 1989). In *Schistosoma mansoni*, the reading frame of a 86 kDa antigen is highly homologous to those of large HSPs of *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Johnson *et al*, 1989).

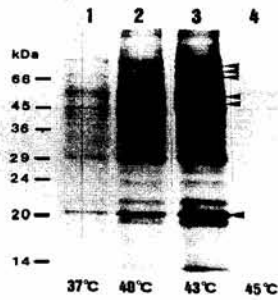


Fig 4 - Fluorogram showing SDS-PAGE profile of [³⁵S] methionine-labeled crude somatic extracts from infective larvae of *T. spiralis*. Worms were given heat shock and radiolabeling simultaneously for 2 hours at different temperatures. Lane- 1. control sample, radiolabeled at 37°C. Lanes 2-4. samples labeled at 37, 40, 43, and 45°C respectively. Arrows indicate heat shock proteins of 86, 80, 70, 50 and 20 kDa.

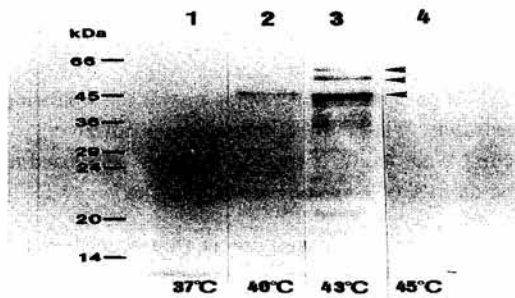


Fig 5 - Autoradiogram showing SDS-PAGE profile of [³⁵S] methionine-labeled ES products from infective larvae of *T. spiralis*. Worms were given heat shock and radiolabeling simultaneously for 2 hours at different temperatures. Lane-1. control sample, radiolabeled at 37°C. Lanes 2-4. samples labeled at 37, 40, 43 and 45°C respectively. Arrows indicate heat shock proteins of 64, 53, 45 kDa.

Proteases

Besides HSPs, Lai and Ko (1994) also identified a 47 kDa neutral metallo-protease from ES products of infective larvae of *T. spiralis* which were cultured for 24 hours in RPMI 1640 medium. This protease may be involved in the penetration of the intestine by the infective larvae. However, two serine proteases were found in the ES products from infective larvae of *T. pseudospiralis* (unpublished data).

Several discrete proteinases, dominated by the serine class, were also observed by Todorova *et al* (1995) in the ES products of adult worms. Antibodies harvested from immune mice would inhibit proteinase activity.

Therefore, it appears that the ES products of *Trichinella* consist of a wide range of molecules having different adaptive functions. A better understanding of the biological activities of the so called immunodominant antigens may produce more specific probes which can be used in diagnosis.

ANTIBODY DETECTION

The roles of specific IgM, IgA and IgE detection in diagnosing acute trichinellosis are still debatable. Murrell and Bruschi (1994) compared the successful detection rates reported by various authors using different ELISA formats. For IgM detection, the successful rates vary from 78-100 % and for IgA, they vary from 13-69%. But IgM can be detected as long as one year postinfection (Bruschi *et al*, 1990). Although IgA detection has a good potential, it is not commonly used because the traditional assays are not too sensitive.

However, the detection of IgE remains the most controversial. The detection rates reported vary from 7-100%. This is probably due to the differences in the type of assay used and the acuteness of the infection in patients.

Using an amplified ELISA, we succeeded in achieving a 100% IgE detection (Au *et al*, 1983). The essence of the method is the employment of microtitration plates with high adsorption properties, lengthening the incubation of test serum at 37 °C and adding an extra antibody layer *ie* rabbit anti-human IgE. The latter is then detected by peroxidase-conjugated goat anti-rabbit IgG. Such system can convey an amplification effect.

Bruschi *et al* (1990), using the above method, reported detection rates of 80% after two months and 20% after one year. Tassi *et al*. (1990) also concluded that the amplification method is more sensitive than the conventional indirect ELISA.

Therefore, the diagnostic value of IgE detection in acute trichinellosis should further be explored.

ANTIGEN DETECTION

Circulating antigens (CA) were detected by several authors with conventional assays. Ivanoska *et al* (1989) used an immunoradiometric assay (IRMA) with a monoclonal antibody specific for the 53 and 59 kDa antigens. CA was detected in 47% of patients showing clinical signs and in 13% of patients suspected of exposure to infection.

Candolfi *et al* (1989), who used the conventional ELISA, detected CA in only 30% of patients at 10 and 20 days after onset of clinical symptoms. The positive rate increased to 56% between 21 and 30 days. Rodriguez-Osorio *et al* (1990) detected CA with a sandwich ELISA in patients at 28 days of infection. However, at 78 days, CA seemed to have disappeared in most patients.

Nishyama *et al* (1992) used the sandwich ELISA to test 347 individuals with suspected trichinellosis. Among individuals showing clinical symptoms, CA were detected in 29.9% and the prevalence of antibodies was 18.9%. The dot-immunobinding technique can detect CA in the sera of experimentally infected rabbits as early as 4 days postinfection. CA is detected in more than one third of patients by the end of the third week of infection.

Recently, we compared the sensitivity and specificity of different enzyme based assays for CA detection, *ie* the traditional colorimetric ELISA, microfluorescence (ELFA), dissociated enhanced lanthanide fluoroimmunoassay (DELFLIA, Pharmacia) and enhanced chemiluminescence immunoassay (ECIA). A sandwich format was used to capture CA. Polyclonal IgG antibodies against ES antigens served as the primary antibody. The secondary antibody was a biotinylated monoclonal *eg* 7C₂C₅, an IgM (Fig 6). The assay design was selected after testing different combinations of antibody pairs in ELISA. The highest OD value was obtained in the combination of rabbit anti-ES polyclonals and 7C₂C₅ (Fig 7).

The following readers were used to record data: for DELFLIA, a time-resolved fluorometer (model 1234, Pharmacia); for ECIA, a luminometer with injectors (ML3000, Dynatech); for ELFA, a microfluorometer (Fluoroskan II, Labsystem); for ELISA, a microplate reader (Anthoslabtec).

Among the above assays, ECIA is theoretically the most sensitive because its detection limit is 10⁻¹⁹

moles. The detection limits for ELISA, ELFA and DELFLIA are 10⁻¹⁶, 10⁻¹⁸ and 10⁻¹⁸, respectively. However, in our experiments, DELFLIA was found to be the most sensitive and provided a stable measurement of CA (Li and Ko, 1994).

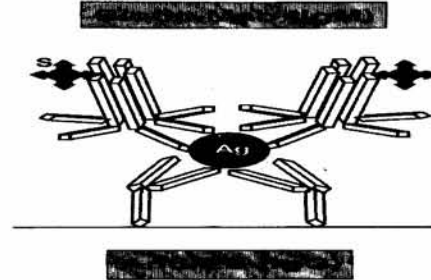


Fig 6 - Design of antibody system for capturing circulating antigens of *T. spiralis*. The primary antibody was rabbit anti-ES IgG and the secondary antibody was a biotinylated monoclonal antibody, 7C₂C₅(IgM). S= streptavidin.

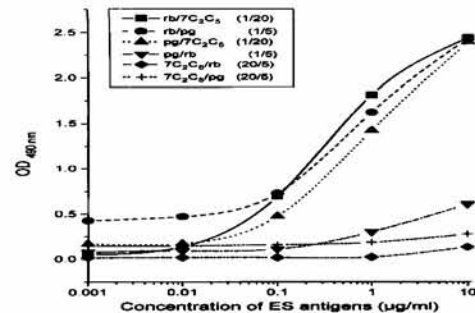


Fig 7 - Results of using different antibody pairs in sandwich ELISA to capture ES antigens of infective larvae of *Trichinella spiralis*. The numbers in parenthesis in the box are the antibody concentrations used (mg/ml). rb= rabbit anti-ES serum. pg= pig anti-ES serum. 7C₂C₅= IgM monoclonal.

DELFLIA uses a lanthanide metal chelate label (*eg* Europium) which has a long fluorescence decay time (1 ms), a sharp emission peak and high fluorescence intensity. In addition, its Stokes' Shift *ie* the difference between excitation (at 340 nm) and emission (613 nm) wavelengths, is exceptionally

large (>230 nm). The DELFIA reader measures fluorescent signal at a given time frame after onset of the reaction. The delay in measurement (400 μ s) ensures a high signal/noise ratios. The specific fluorescence is measured for 400 μ s. The excitation pulse and the measurement cycle are repeated 1000 times in 1 second.

DELFIA can give a distinct signal (counts per second) even when the concentration of *Trichinella* ES antigens in the test serum is as low as 0.01 mg/ml. There are little cross-reactions with most heterologous antigens except those of *T. pseudospiralis*. However, in ECIA using both luminol and CSPD (1,2,-dioxetane) as substrates, the lowest detection limit of antigens in the test serum is 0.1 μ g/ml. Similar results were also observed in both ELFA and ELISA experiments.

In mice experimentally infected either with 100 or 3,000 *T. spiralis* larvae, CA can be detected as early as 7 days postinfection by DELFIA (Li, 1996). CA seems to increase markedly around 10 days postinfection *ie* after the invasion of muscles by newborn larvae and around 20-30 days *ie* during the maturation and encystment of infective larvae.

CA was also detected by DELFIA in a patient with suspected trichinellosis in Hong Kong. Unfortunately, more cases were not available for further evaluation.

DIAGNOSIS USING MOLECULAR METHODS

Several potential new diagnostic methods based on a combination of immunological and molecular biological technic have been developed. These are briefly described below.

Immuno-PCR

Immuno-PCR was developed by Takeshi *et al* (1992) using a streptavidin-biotin system (Fig 8). A linker and a marker molecules are involved. The linker molecule, which has bispecific binding affinity for a DNA molecule and antibodies, is used to attach a DNA molecule (marker) specifically to an antigen-antibody complex. The attached marker DNA can be amplified by PCR with appropriate primers. The PCR products are analysed by agarose gel electrophoresis. This method allows detection as few as 580 antigen molecules (9.6×10^{-22} moles).

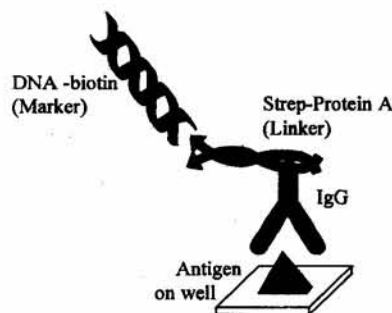


Fig 8 - Illustration of basic principle of Immuno-PCR. Streptavidin-protein A serves as the linker molecule (which has bispecific binding affinity for DNA and antibodies). Attached DNA marker is amplified by PCR.

A streptavidin-protein A chimera serves as the linker. The chimera has two specific binding abilities: one to biotin and the other to the Fc portion of an IgG molecule, derived from the protein A moiety. The bifunctional specificity, both for biotin and antibody, allows the specific conjugation of any biotinylated DNA molecule to antigen-antibody complexes.

However, high background signals and problems of fixing antigens from the test sample onto a surface may discourage routine employment of this technic. The assay was originally tested with a known concentration of bovine serum albumin. But in diagnosis, the concentration of antigens in the test sample is invariably unknown. Therefore, problems may arise in the standardization of results.

PCR-ELISA

In the present conference, Uparanukraw and Morakote (1995) reported that using PRA primers, a specific 0.6 kb fragment of the 1.6 kb repetitive DNA sequence of *T. spiralis* was amplified from blood of infected mice as early as 5 days postinfection. The presence of migratory larvae could be detected by PCR 14 days postinfection.

Recently, a PCR-ELISA kit is being marketed by Boehringer Mannheim. It is based on a non-radioactive digoxigenin (DIG)-label (Fig 9). The principle of the method involves hybridization of DIG-labeled and denatured PCR products to a biotin-labeled captured

probe. The hybrid is then bound to streptavidin-coated plate. The DIG label is detected with anti-DIG peroxidase conjugate. As little as 1 femto (f) g (10^{-15} moles) of DNA will yield a positive signal.

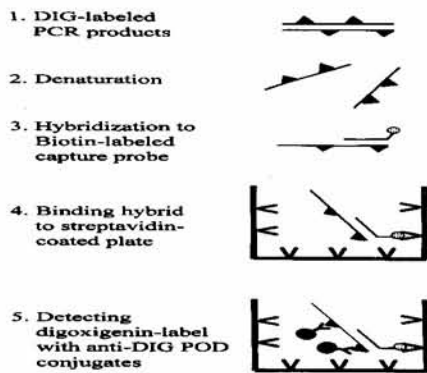


Fig 9- Illustration of basic principle of PCR-ELISA. Digoxigenin (DIG) labeled and denatured PCR products are hybridized to biotin-labeled capture probe. After binding hybrid to streptavidin-coated plate, anti-DIG peroxidase (POD) conjugate is used for detection.

This method, after modification, may be applied to detect newborn larvae in muscles. Since the assay is already available in kit form, it can be performed routinely and easily.

However, the major disadvantages of using molecular methods to diagnose trichinellosis are the high cost, high background noises and lengthy procedures. But these problems may be resolved in the near future.

CONCLUSION

To conclude, the future focus on the diagnosis of trichinellosis should be on detecting CA or DNA. Identification of key adaptive molecules in the ES products of *T. spiralis* may help to yield more specific probes. Simple, sensitive, specific and cost effective methods can be developed by exploiting the latest advancement in both molecular/immunological technology.

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