(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 July 2021 (22.07.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/145757 A1

(51) International Patent Classification:
G01N 33/53 (2006.01) *C07K 16/44* (2006.01)
C07K 14/00 (2006.01)

(21) International Application Number:
PCT/MY2020/050044

(22) International Filing Date:
19 June 2020 (19.06.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PI2020000313 17 January 2020 (17.01.2020) MY

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: A BIOMARKER FOR DETECTING STRONGYLOIDES, METHODS OF DETECTING STRONGYLOIDES AND DIAGNOSING STRONGYLOIDIASIS IN A BIOLOGICAL SAMPLE, AND A DIAGNOSTIC KIT COMPRISING THEREOF

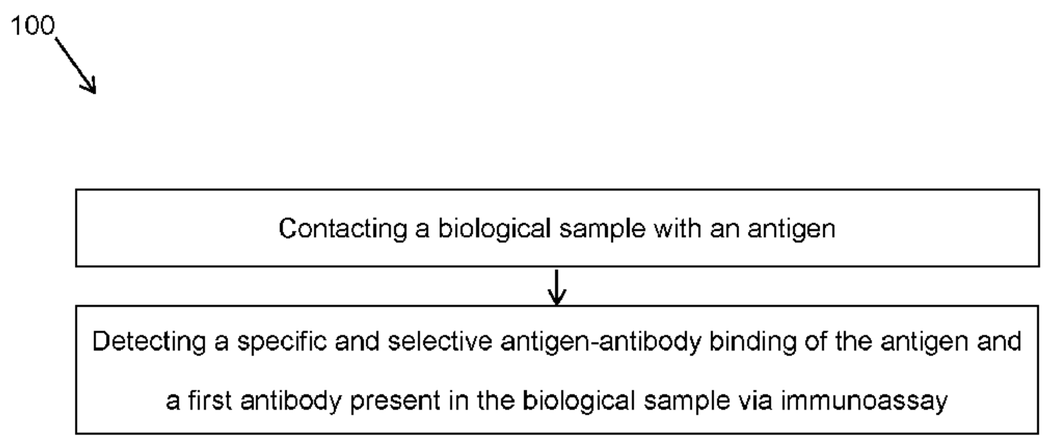

Fig. 1

(57) Abstract: The present invention relates to a biomarker for detecting *Strongyloides*, characterized by a *Strongyloides stercoralis* protein comprising an amino acid sequence set forth in SEQ ID NO: 2, in which the *Strongyloides stercoralis* protein is encoded by a nucleic acid molecule comprising a nucleic acid sequence set forth in SEQ ID NO: 1. The present invention also relates to a method (100) of detecting *Strongyloides* in a biological sample and a method (200) of diagnosing Strongyloidiasis in a subject comprising the biomarker as an antigen via an immunoglobulin E, IgE, immunoassay. More specifically, the method (200) is configured to detect an acute Strongyloidiasis in the subject. Further, the present invention provides a diagnostic kit for *Strongyloides* comprising the biomarker for detecting *Strongyloides*.

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Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

A BIOMARKER FOR DETECTING *STRONGYLOIDES*, METHODS OF DETECTING *STRONGYLOIDES* AND DIAGNOSING STRONGYLOIDIASIS IN A BIOLOGICAL SAMPLE, AND A DIAGNOSTIC KIT COMPRISING THEREOF

TECHNICAL FIELD

The present invention relates to a biomarker for detecting *Strongyloides* and uses thereof, more particularly to methods of detecting *Strongyloides* and strongyloidiasis in a biological sample from human and non-human species. Further, the present invention relates to a diagnostic kit comprising the biomarker.

BACKGROUND ART

Strongyloidiasis is one of the neglected tropical diseases. It is caused by soil-transmitted helminth, specifically *Strongyloides stercoralis*. However, in some cases, it may be caused by *Strongyloides fuelleborni* and *Strongyloides fuelleborni kellyi*. The most common route of infection is skin penetration, but it may be transmitted via oral route and transmission after solid organs transplant.

Although strongyloidiasis is prevalent in tropical, subtropics and temperate regions, other factors may facilitate its transmission in other regions such as poor sanitation, especially in poor countries. While *S. stercoralis* generally causes clinically asymptomatic infection and chronic infection in an immunocompetent patient, it may cause a life threatening hyper-infection and dissemination in an immunocompromised patient. Many patients are asymptomatic or show mild acute symptoms of cutaneous irritation at the site of skin penetration. In chronic cases, the intestinal tract and skin are clinically affected and frequently goes unnoticed. On the other hand, immunosuppression may lead to severe manifestation of the hyper-infection syndrome and disseminated strongyloidiasis.

Strongyloidiasis patient secretes different classes of antibodies such as immunoglobulin A (IgA), immunoglobulin E (IgE), immunoglobulin M (IgM), immunoglobulin G (IgG) and subclasses of IgG to combat the disease. More specifically, the presence of IgE antibody indicates an acute infection of

strongyloidiasis, whereas the presence of IgG4 indicates to chronic infection of strongyloidiasis. In addition, IgE antibodies are important as defence against parasites and have been long known to be induced during helminthic infection. Further, there are significant positive correlations between specific IgG4 and IgE, where a single-positive IgE+/IgG4- may indicates a more recent infection of strongyloidiasis, while a single-positive IgE-/IgG4+ may indicates a chronic infection of strongyloidiasis.

Diagnosis of helminthic infection including *S. stercoralis* infection remains a challenge to date. The conventional diagnosis by observing the larvae directly or through culture is laborious, has low sensitivity and provides a high index of the false-negative result due to the intermittent larvae release. Promising result can be obtained by molecular diagnosis through real-time polymerase chain reaction (RT-PCR). However, RT-PCR is not widely available, costly and requires technological support, hence does not provide the best solution for diagnosis of helminthic infection, especially in endemic areas which are mostly resource-limited. On the other hand, enzyme-linked immunosorbent assay (ELISA) significantly increases the sensitivity and negative predictive value; however, its general reliance on a crude antigen that requires larvae culture makes it difficult to use.

Apart from that, the use of native *Strongyloides* protein may cause potential cross-reactivity and standardization issues. Further, diagnosis of acute strongyloidiasis is challenging to ascertain and active and past infections are hard to differentiate due to unreliable indicator. For example, IgG-based immunoassay may detect past infection and hence resulting in inaccurate diagnosis.

Hence, there is a need to develop alternative diagnostic methods that include the development of reliable immunological assays and the use of a recombinant protein which reduces time, cost and cross-reactivity. There have been a number of solutions that provide alternative diagnostic methods for helminthic infections and few of them are discussed below:

WO 2017091059 disclosed methods of screening biological samples for the presence of *Strongyloides spp*. More particularly, the prior art relates to a sensitive and specific screening test for the presence of anti-*Strongyloides spp* antibodies, protein or nucleic acid in a subject by using a *Strongyloides spp* L3 stage antigen. In addition, the *Strongyloides spp* L3 stage antigen is an isolated or a recombinant *S. stercoralis* protein comprising an amino acid sequence and a nucleic acids sequence that encodes the *Strongyloides spp* L3 stage antigen. However, the anti-*Strongyloides spp* antibodies detected are IgG antibodies. Hence, the prior art does not provide a means to detect an acute strongyloidiasis.

CA3051466A1 relates to generation of human allergen and helminth-specific IgE monoclonal antibodies for diagnostic and therapeutic use. The prior art also relates to a method of detecting an IgE antibody with binding affinity or specificity for an antigen such as a dust mite antigen in a subject. The helminth infections diagnosed by the prior art are *Wuchereria bancrofti* or *S. stercoralis*, which serve as antigens in the prior art. The prior art comprises a test antibody or a fragment having complementarity-determining region (CDR) of the heavy chain and its corresponding nucleic acid sequence. However, the antibody or antibody fragment comprises heavy and light chain variable sequences in the prior art only have 70 %, 80 % or 90 % identity to clone paired heavy and light chain variable sequences. Hence, the prior art has lower sensitivity and specificity against helminth antigen such as *S. stercoralis* antigen as it is desirable to have sensitivity and specificity higher than 95 %. Hence, the prior art still needs to be improved to provide a more reliable diagnosis result.

US20120308599 A1 disclosed a vaccine and method of use against *S. stercoralis* infection. The prior art comprises an isolated *S. stercoralis* immunoreactive antigen (SSIR), more particularly to an antigen from *S. stercoralis* stage L3, which is mixed with an adjuvant to immunize a subject. Further, the prior art applies immunoassay, more particularly to ELISA for quantifying antibodies generated to the *S. stercoralis* immunoreactive antigen. However, the prior art relates more to a therapeutic composition against *S. stercoralis* rather than detection of acute or chronic strongyloidiasis.

Accordingly, it can be seen in the prior arts that there exists a need for a biomarker as an antigen that provides high specificity and selectivity against *Strongyloides*, specifically *S. stercoralis*. In addition, there exists a need to utilize the biomarker in both diagnostic and therapeutic applications. There also exists a need to apply the biomarker in producing a diagnostic kit for strongyloidiasis, especially acute strongyloidiasis, with a diagnostic sensitivity and specificity higher than 95 %.

SUMMARY OF THE INVENTION

The following presents a simplified summary of the invention in order to provide a basic understanding of some aspects of the invention. This summary is not an extensive overview of the invention. Its sole purpose is to present some concepts of the invention in a simplified form as a prelude to the more detailed description that is presented later.

An objective of the present invention is to provide a biomarker for detecting *Strongyloides*, preferably *Strongyloides stercoralis*. Preferably, the biomarker may be used for diagnosis, epidemiological studies and research.

It is also an objective of this invention to provide a biomarker for detecting *Strongyloides*, in which the biomarker is an isolated *S. stercoralis* protein or a recombinant *S. stercoralis* protein comprising an amino acid sequence.

It is also an objective of this invention to provide a biomarker for detecting *Strongyloides* that is encoded by a nucleic acid molecule comprising a nucleic acid sequence.

A further objective of the present invention is to provide a method of detecting *Strongyloides* in a biological sample from a human or a non-human species. More particularly, the method is using the biomarker for detecting *Strongyloides* as an antigen.

Another objective of the present invention is to provide a method of diagnosing strongyloidiasis in a subject via a biological sample taken from the subject, more particularly to a method of diagnosing acute strongyloidiasis in the subject using the biomarker for detecting *Strongyloides* as an antigen.

Further, another objective of the present invention is to provide a method of detecting *Strongyloides* in a biological sample and a method of diagnosing strongyloidiasis in a subject using an immunoassay, more particularly to an immunoassay for the measurement of immunoglobulin E (IgE) to diagnose an active infection, including an acute infection.

It is a further objective of the present invention to provide a method of detecting *Strongyloides* in a biological sample and a method of diagnosing strongyloidiasis using an immunoassay, which provide sensitivity and specificity higher than 95 % against *Strongyloides and* strongyloidiasis, respectively.

It is a further objective of the present invention to provide a diagnostic kit comprising the biomarker for detecting *Strongyloides*, more particularly to *S. stercoralis*.

Accordingly, these objectives may be achieved by following the teachings of the present invention. The present invention relates to a biomarker for detecting *Strongyloides*, characterized by a *S. stercoralis* protein comprising an amino acid sequence set forth in SEQ ID NO: 2, in which the *S. stercoralis* protein is encoded by a nucleic acid molecule comprising a nucleic acid sequence set forth in SEQ ID NO: 1. The present invention also relates to a method of detecting *Strongyloides* in a biological sample and a method of diagnosing strongyloidiasis in a subject comprising the biomarker as an antigen via an immunoglobulin E, IgE, immunoassay. More specifically, the method is configured to detect an acute strongyloidiasis in the subject. Further, the present invention provides a diagnostic kit for *Strongyloides* comprising the biomarker for detecting *Strongyloides*.

The foregoing and other objects, features, aspects and advantages of the present invention will become better understood from a careful reading of a detailed description provided herein below with appropriate reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

So that the manner in which the above recited features of the present invention can be understood in detail, a more particular description of the invention, briefly summarized above, may have been referred by embodiments, some of which are illustrated in the appended drawings. It is to be noted, however, that the appended drawing illustrates only typical embodiments of this invention and are therefore not to be considered limiting of its scope, for the invention may admit to other equally effective embodiments.

These and other features, benefits, and advantages of the present invention will become apparent by reference to the following text figure, with like reference numbers referring to like structures across the views, wherein:

Fig. 1 is a flowchart illustrating a method of detecting *Strongyloides* in a biological sample in accordance with an embodiment of the present invention.

Fig. 2 is a flowchart illustrating a method of diagnosing strongyloidiasis in a subject in accordance with an embodiment of the present invention.

Fig. 3 shows a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a western blot analysis of (A) a purified biomarker, (B) probed with anti-His horseradish peroxidase (HRP) antibody, and (C) probed with positive biological samples.

Fig. 4 is a column chart showing a mean value with a standard deviation of immunoglobulin E-enzyme-linked immunosorbent assay (IgE-ELISA) optical density (OD) readings from different biological sample groups.

Fig. 5 illustrates column charts showing comparison of a mean value with a standard deviation of IgE-ELISA OD readings from the different biological sample groups, and the T-test analysis.

Fig. 6 is a receiver operator characteristic (ROC) curve analysis of the IgE-ELISA cut-off value (COV) 0.22 for determination of strongyloidiasis with the area

under the ROC curve (AUC) 1.000 (95% confidence interval=0.999 to 1.000, P>0.0001).

Fig. 7 shows OD readings of IgE-ELISA using positive and control biological samples.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

While the present invention is described herein by way of example using embodiments and illustrative drawings, those skilled in the art will recognize that the invention is not limited to the embodiments of drawing or drawings described, and are not intended to represent the scale of the various components. Further, some components that may form a part of the invention may not be illustrated in certain figures, for ease of illustration, and such omissions do not limit the embodiments outlined in any way. It should be understood that the drawings and detailed description thereto are not intended to limit the invention to the particular form disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the scope of the present invention as defined by the appended claims. As used throughout this description, the word "may" is used in a permissive sense (i.e. meaning having the potential to), rather than the mandatory sense, (i.e. meaning must). Further, the words "a" or "an" mean "at least one" and the word “plurality” means “one or more” unless otherwise mentioned. Furthermore, the terminology and phraseology used herein is solely used for descriptive purposes and should not be construed as limiting in scope. Language such as "including," "comprising," "having," "containing," or "involving," and variations thereof, is intended to be broad and encompass the subject matter listed thereafter, equivalents, and additional subject matter not recited, and is not intended to exclude other additives, components, integers or steps. Likewise, the term "comprising" is considered synonymous with the terms "including" or "containing" for applicable legal purposes. Any discussion of documents, acts, materials, devices, articles and the like is included in the specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention.

In this disclosure, whenever a composition or an element or a group of elements is preceded with the transitional phrase “comprising”, it is understood that we also contemplate the same composition, element or group of elements with transitional phrases “consisting of”, “consisting”, “selected from the group of consisting of, “including”, or “is” preceding the recitation of the composition, element or group of elements and vice versa.

The present invention is described hereinafter by various embodiments with reference to the accompanying drawing, wherein reference numerals used in the accompanying drawing correspond to the like elements throughout the description. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiment set forth herein. Rather, the embodiment is provided so that this disclosure will be thorough and complete and will fully convey the scope of the invention to those skilled in the art. In the following detailed description, numeric values and ranges are provided for various aspects of the implementations described. These values and ranges are to be treated as examples only and are not intended to limit the scope of the claims. In addition, a number of materials are identified as suitable for various facets of the implementations. These materials are to be treated as exemplary and are not intended to limit the scope of the invention.

The present invention relates to a biomarker for detecting *Strongyloides*, the biomarker is **characterized by**: a *Strongyloides stercoralis* protein comprising an amino acid sequence set forth in SEQ ID NO: 2; wherein the *Strongyloides stercoralis* protein is encoded by a nucleic acid molecule comprising a nucleic acid sequence set forth in SEQ ID NO: 1.

The present invention also relates to a method (100) of detecting *Strongyloides* in a biological sample, the method comprising the steps of: contacting a biological sample with an antigen; and detecting a specific and selective antigen-antibody binding of the antigen and a first antibody present in the biological sample via immunoassay; **characterized in that**, the antigen is the biomarker for detecting *Strongyloides* comprising the amino acid sequence set

forth in SEQ ID NO: 2; wherein the first antibody is a *Strongyloides stercoralis*-specific antibody; and wherein presence of *Strongyloides* in the biological sample is detected via presence of the specific and selective antigen-antibody binding of the antigen and the first antibody in the biological sample.

In addition, the present invention relates to a method (200) of diagnosing strongyloidiasis in a subject, the method comprising the steps of: collecting a biological sample from a subject; contacting the biological sample with an antigen; detecting presence of *Strongyloides* protein in the biological sample via an immunoassay using a first antibody; contacting the biological sample with a second antibody; and determining presence of strongyloidiasis in the subject; **characterized in that**, the antigen is the isolated *Strongyloides stercoralis* protein comprising the amino acid sequence set forth in SEQ ID NO: 2; wherein the first antibody is a *Strongyloides stercoralis*-specific antibody; and wherein the second antibody is an antibody that specifically binds to the first antibody.

Further, the present invention relates to a diagnostic kit for *Strongyloides*, the diagnostic kit comprising: the biomarker for detecting *Strongyloides*; an immunoassay reagent; and an immunoassay device configured to detect presence of *Strongyloides*.

Referring to the drawings as shown in Fig. 1 to Fig. 6, the invention will now be described in more detail.

The biomarker for detecting *Strongyloides* comprises the amino acid sequence set forth in SEQ ID NO: 2. More particularly, the *S. stercoralis* protein is encoded by the nucleic acid molecule comprising the nucleic acid sequence set forth in SEQ ID NO: 1. Preferably, the biomarker may be used for, but not limited to, diagnosis, epidemiological studies and research.

In accordance with an embodiment of the present invention, the *S. stercoralis* protein is an isolated *S. stercoralis* protein or a recombinant *S. stercoralis* protein. Preferably, the *S. stercoralis* protein is produced from an

isolated complementary deoxyribonucleic acid (cDNA) clone from immunoscreening of *S. stercoralis* cDNA library.

In accordance with an embodiment of the present invention, the amino acid sequence set forth in SEQ ID NO: 2 preferably including a complete amino acid sequence, a fragment of the amino acid sequence or a variant of the amino acid sequence.

In accordance with an embodiment of the present invention, the nucleic acid molecule is preferably an isolated nucleic acid molecule or a recombinant nucleic acid molecule.

In accordance with an embodiment of the present invention, the biomarker including, but not limited to, a *Strongyloides*-specific antigen.

Fig. 1 is a flowchart illustrating the method (100) of detecting *Strongyloides* in the biological sample in accordance with an embodiment of the present invention. The method (100) begins with contacting the biological sample with the antigen, followed by detecting the specific and selective antigen-antibody binding of the antigen and the first antibody present in the biological sample via immunoassay. Presence of *Strongyloides* in the biological sample is detected via presence of the specific and selective antigen-antibody binding of the antigen with the first antibody in the biological sample. More particularly, the antigen is the biomarker for detecting *Strongyloides* comprising the amino acid sequence set forth in SEQ ID NO: 2, while the first antibody is a *S. stercoralis*-specific antibody.

In accordance with an embodiment of the present invention, the first antibody is preferably an immunoglobulin E, IgE, antibody.

In accordance with an embodiment of the present invention, the immunoassay including, but not limited to, an enzyme-linked immunosorbent assay (ELISA), a dot-blot, a Western blot, a vertical flow test, a lateral flow assay, and a biosensor.

In accordance with an embodiment of the present invention, the biological sample is from a human or non-human species.

Fig. 2 is a flowchart illustrating the method (200) of diagnosing strongyloidiasis in the subject in accordance with an embodiment of the present invention. The method (200) begins with collecting the biological sample from the subject, contacting the biological sample with the antigen, and detecting presence of *Strongyloides* protein in the biological sample via the immunoassay using the first antibody. The method (200) is then followed by contacting the biological sample with the second antibody, and finally determining presence of strongyloidiasis in the subject.

Specifically, the antigen is the isolated *S. stercoralis* protein comprising the amino acid sequence set forth in SEQ ID NO: 2. Further, the first antibody is a *S. stercoralis*-specific antibody, whereas the second antibody is the antibody that specifically binds to the first antibody.

In accordance with an embodiment of the present invention, the second antibody is preferably conjugated to a detector molecule selected from, but not limited to, an enzyme, a fluorescence molecule or a colloidal gold.

In accordance with an embodiment of the present invention, each of the first antibody and the second antibody is an immunoglobulin E, IgE, antibody.

In accordance with an embodiment of the present invention, the method (200) is used for determining acute strongyloidiasis in the subject via detection of the first antibody and the second antibody.

Further, the diagnostic kit for *Strongyloides* is also provided, in which the diagnostic kit comprises the biomarker for detecting *Strongyloides*, the immunoassay reagent and the immunoassay device configured to detect presence of *Strongyloides*.

In accordance with an embodiment of the present invention, the diagnostic kit is configured to detect presence of *Strongyloides* antibody or *Strongyloides* protein.

In accordance with an embodiment of the present invention, the diagnostic kit including, but not limited to, an enzyme-linked immunosorbent assay, a dot blot assay, a Western blot assay, a vertical flow assay, a lateral flow assay, and a biosensor.

Hereinafter, example of the present invention will be provided for more detailed explanation. The advantages of the present invention may be more readily understood and put into practical effect from these examples. However, it is to be understood that the following examples are not intended to limit the scope of the present invention in any way.

Examples

Example 1

Biological Samples

(1) Selection of biological samples

Biological samples, namely serum samples of patients, were divided into two groups; Group 1A and Group 1B. Group 1A had biological samples from strongyloidiasis patients that were positive by both of polymerase chain reaction (PCR)/microscopy and serology (n=20), whereas Group 1B had biological samples from strongyloidiasis patients that were positive by stool-PCR but negative by serology (n=11). The initial conclusion are Group 1B is more likely to have an acute infection, while Group 1A samples is more likely to have a chronic infection.

The diagnostic specificity was evaluated using 69 serum samples from other parasitoses, specifically Group 2 that comprises patients who had amebiasis (n=4), ascariasis (n=4), echinococcosis (n=4), filariasis (n=9), giardiasis (n=1)

hookworm (n=6), schistosomiasis (n=11), taeniasis (n=2), toxocariasis (n=18), toxoplasmosis (n=5), trichuriasis (n=2) and mixed infection (n=3). In addition, Group 3 comprised 25 biological samples from healthy donors.

(2) Pre-adsorption of biological samples for immunoscreening

Prior to immunoscreening, the biological samples, namely serum samples, were pre-adsorbed against two kinds of *Escherichia coli* XL1-Blue antigen, which are *E. coli* whole-cell pellet at 100 mg per tube and *E.coli* whole cell lysate 250 µL lysate per 100ul of 0.5-µm microsphere beads. A volume of 30 µl of biological sample was added to XL1-Blue whole-cell pellet and the mixture was mixed thoroughly and incubated at 4°C on rotator overnight. Two rounds of overnight incubation of serum were performed with both pellet and beads coated with *E. coli* lysate. The final pre-adsorbed serum was then stored at -20 °C. Further, the efficiency of serum pre-adsorption was determined using immunoglobulin G-enzyme-linked immunosorbent assay (IgG-ELISA).

Example 2

Immunoscreening of *Strongyloides stercoralis*

(1) Preparation for immunoscreening of *Strongyloides stercoralis*

A complementary deoxyribonucleic acid (cDNA) library was constructed in a λTriplEx2 vector by Clontech Laboratories Inc. from a mixture of L3 and adult worms of *S. stercoralis*. Prior to immunoscreening, the titration of the phage cDNA library with lambda (λ) dilution buffer was performed to produce approximately 300 to 500 well-separated plaques per plate. 20 µL of the phage at dilution of 10^3 was added to 600 ul of diluted xl-1 blue cells of optical density (OD) of 0.5 at 600 nm. The tubes were then incubated for 15 min at 37 °C to allow attachment and transduction. 7 ml of melted soft top agarose was added to the mixture and then poured to pre-warmed Luria–Bertani/magnesium sulfate (LB/$MgSO_4$) agar plates, followed by 6 hours incubation at 37 °C for plaque formation.

A nitrocellulose membrane (NC) soaked with 10 mM of isopropyl ß-D-1-thiogalactopyranoside (IPTG) was overlaid on top of the plate containing visible plaques and incubated for 4 hours at 37 °C. The NC membrane was washed 3

times for 5 min each using Tris-buffered saline having 0.05% Tween 20 (TBS-T), namely 20 mM of Tris and 150 mM of sodium chloride (NaCl), followed by 1 hour blocking with diluted blocking buffer. The NC membrane was then washed and incubated with a serum that had been pre-adsorbed with *E. coli* antigen earlier, in a total dilution of 1:100 in TBS having pH 7.5 and incubated overnight at 4 °C. After the overnight incubation, the blot was washed and incubated with secondary antibodies, namely monoclonal mouse anti-human immunoglobulin E-horseradish peroxidase (IgE-HRP) at 1:2,000 dilution in TBS-T for 2 hours at room temperature. The signal of the reaction was detected using chemiluminescent substrate, which developed an X-ray film. Dark spots on the film represented reactive phage clones. The reactive clones were cored out from the plate and allowed to diffuse in 200 µL in λ-dilution buffer overnight at 4°C, recovered on the following day by centrifugation at 15,000 × g for 5 min.

The procedure for immunoscreening of *S. stercoralis* cDNA library was performed according to the procedure by Sambrook *et al.* (Sambrook J, Russell DW, Russell DW. 2001. Molecular cloning: a laboratory manual (3-volume set). Cold spring harbor laboratory press New York.). Primary and secondary immunoscreenings of the library were performed using the pre-absorbed pooled biological samples from *Strongyloides* patients. The selected clones was further immuno-screened using pooled positive pre-absorbed biological samples from *Strongyloides* patients, pooled negative serum samples from patients with other parasitoses, specifically soil-transmitted helminths, and healthy donor's biological samples. Finally, only the clones that react with the biological samples from pooled positive and not reactive with pooled negative biological samples were chosen for a tertiary immunoscreening. In the tertiary immunoscreening, individual pre-adsorbed biological samples were used to determine the diagnostic sensitivity and specificity of the selected clones.

(2) Results of immunoscreening of *Strongyloides stercoralis*

S. stercoralis cDNA library was primary immuno-screened with pooled positive serum in 11 series and a total of 122 IgE cDNA clones were isolated. 27 out of the 122 isolated clones from the primary immunoscreening having good

intensity were chosen for the secondary and tertiary immunoscreening. Secondary immunoscreening was performed with pooled positive and pooled negative biological sample, while tertiary immunoscreening was performed with individual positive and negative biological sample. 6 clones were found to have good diagnostic potential ranging from 70 to 100 %. The highest potential clone was identified as A133, corresponding to the biomarker, where its sensitivity and specificity was 100 % and 92.85 %, respectively.

(3) Sequence analysis of biomarker A133

The recombinant clone was identified through immunoscreening of cDNA library of *S. stercoralis*, in which a novel recombinant protein antigen A133 was produced from the cloned cDNA of *S. stercoralis* and used for detecting parasite-specific IgE antibodies in strongyloidiasis patients.

The gene insert sequence of A133 revealed 100% similarity with *S. stercoralis* genome assembly S_stercoralis_PV0001, scaffold SSTP_contig0000002 Sequence ID: LL999051.1, having Identities = 723/723 [100%], and gaps = 0/723 [0%]. Further, the translated protein analysis of A133 showed highest similarity to *Strongyloides ratti* PDZ signaling domain protein (GH21964p), having identities = 207/237 [87%%], positives = 225/237 [94%], and gaps = 0/237 [0%]) and Sequence ID: CEF67580.1. PDZ domain, also known as discs-large homologous regions (DHR) or GLGF, is one of the most important domains for protein-protein interaction and considered as a novel target of drug discovery. Further, PDZ domain is one of the important modules reserved in the parasite to mediate multiple biological processes.

Example 3

***In-vivo* Excision of Potential Clones**

The highly sensitive and most specific selected Immunoglobulin E-complementary deoxyribonucleic acid (IgE-cDNA) clones were cored out and excised *in-vivo* from the λ vector to form phagemids containing the cloned inserts, allowing conversion of cDNA clones in the form of phagemids into plasmids. 150ul of the eluted plaque was combined with a 200 ul overnight culture of *E. coli*

BM25.8 and incubated at 31 °C for 30 min. 400 uL of LB broth was added to the cell suspension and incubated for 1 hour at 31 °C with shaking at 225 rpm. 5 ul of cell suspension was spread on LB/ampicillin agar plate using a sterile glass spreader and incubated overnight at 37 °C. The isolated colony was cultured and the plasmid was then purified by using QIAprep Spin Miniprep Kit according to the manufacturer's instructions. DNA sequences were performed and sequences were examined for similarity to the already available sequences in the GenBank non-redundant nucleic acid database using BLAST analysis and other specific public databases for nematodes such as nematode.net and Wellcome Trust Sanger Institute. In addition, Open Reading Frame Finder (ORF finder) was used to analyze the presence of start and stop codons in nucleotide sequence. The translation of the nucleic acid sequence to amino acid sequence was performed using the Expasy translates tool software.

Example 4

Preparation of Recombinant Proteins

(1) Custom synthesis into a pET32a expression vector

The IgE reactive clone with the best potential diagnostic value obtained from the IgE-phage immunoblots was selected for production of recombinant protein. The nucleic acid sequence of the clone was sent to a EPOCH Life Science Inc. for codon optimization, DNA synthesis, and cloning into a pET32a expression system.

(2) Expression and purification of recombinant proteins

The custom-cloned recombinant plasmid was transformed into *E. coli* host cells C41 (DE3) and protein expression was performed in 2 L of terrific broth supplemented with 100 µg/mL of ampicillin at 37 °C with shaking at 200 rpm until the OD600 reached to 0.6. The culture was then induced by adding 1 mM of IPTG and further incubated for 4 hours at 30 °C. The cells were harvested by centrifugation, specifically 10,000 × g for 10 min at 4 °C, and the cell pellet was then resuspended in cold lysis buffer having 50 mM of sodium dihydrogen phosphate (NaH_2PO_4), 300 mM of NaCl, 10 mM of imidazole and pH 8.0, with lysozymes at a final concentration of 0.5 mg/mL and protease inhibitors. After 30 min of incubation on ice, the cell lysate was disrupted by a French press and

centrifuged. The obtained supernatant was treated with 0.5 µg/mL of deoxyribonuclease I (DNase1), incubated on ice for 30 min and then centrifuged at 10,000 × g for 30 min. The lysate obtained was filtered and incubated for 1 hour with nickel nitrile tri acetic acid resin slurry on a rotator.

Further, a gradient washing was performed using phosphate buffers having 50 mM of NaH2PO4, 300 mM of NaCl and pH 7.4, with imidazole concentration of 20, 30 and 40 mM, and the target protein was eluted with phosphate buffer containing 250 mM of imidazole. Eluted fractions (having 500 µL each were collected and then purity of protein in each fraction was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions with high-purity proteins were pooled and concentrated using a protein concentrator spin column. The protein concentration was determined using Bio-Rad Protein assay reagent and then stored at -20 °C.

Example 5

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

(1) Preparation of SDS-PAGE and Western Blot

10 ug/well of purified protein electrophoresed in 10 % of SDS-PAGE and transferred to NC membranes. The blot was washed 3 times for 5 minutes each using TBS-T having 0.05 % Tween 20, and was subsequently blocked for 1 hour with 5 % of skimmed milk, followed by washing with TBS-T and incubated overnight at 4 °C with biological sample. After the overnight incubation, the membrane was washed and incubated with monoclonal mouse anti-human IgE-HRP at 1:1000 for 2 hours at room temperature. The signal of the reaction was detected using chemiluminescent substrate and developing an X-ray film. The potential diagnostic value was evaluated using several biological samples from *Strongyloides* patients with other parasitoses and also healthy donors.

(2) SDS-PAGE and Western Blot Analysis

Fig. 3 shows SDS-PAGE and western blot analysis of (a) a purified biomarker, (b) probed with anti-His horseradish peroxidase (HRP) antibody, and

(c) a sandwich format with an aptamer as a capturing element. More specifically, Fig. 3 (C) represents purified biomarker probed with positive biological samples from Group 1A on lane 1 and 2, probed with positive biological samples from Group 1B on lane 3 and 4, and control biological samples from Group 2 on lane 5 to 19, Group 3 of healthy individuals on lane 20 to 22, and an M lane that represents the Precision Plus Protein Unstained Standard Marker. Further, Group 2 comprising control biological samples of ascariasis on lane 5 to 8, filariasis on lane 9 to 14, mixed hookworm infection and trichuriasis on lane 15 and 16, amoebiasis on lane 17, toxocariasis on lane 18, and giardiasis on lane 19.

SDS-PAGE analysis showed that only the thickest band of approximately 40 kDa showed reactivity with anti-His-HRP western blot. The A133 IgE-western blot using biological sample showed 100 % sensitivity for Group 1A, 100 % sensitivity for Group 1B. The specificity for Group 3 and Group 2 was 100% and 93.7%, respectively, as one of the filariasis serum was cross-reactive.

Example 6

Immunoglobulin E-Enzyme-Linked Immunosorbent Assay (IgE-ELISA) of the Recombinant Protein

(1) Determination of the diagnostic sensitivity of the recombinant protein

Diagnostic sensitivity was determined using the biological samples from Group 1A and Group 1B, whereas the diagnostic specificity was evaluated using Group 2 and Group 3. The ELISA was performed as described earlier, except 10ug/ml of target recombinant protein A133 was used for coating the plate well and the IgE-HRP at 1:750 was used as the secondary antibodies with 2 hours incubation. The cut-off value (COV) of the test was determined by the receiver operator characteristic curve analysis of the ELISA data obtained from testing of all serum samples (n =125).

(2) Evaluation of the diagnostic value of the recombinant protein by IgE-ELISA

IgE-ELISA of the recombinant protein, namely A133 IgE-ELISA, was performed with biological sample groups described earlier and statistical analysis was performed. P-value less than 0.05 was considered significant and all the statistical analysis was performed by using Graph Pad Prism version 8.0.2. Referring to Fig. 4, the analysis of variance (ANOVA) results of all the four-group showed that it is statically significant with P-value < 0.0001. The t-test performed for Group 1A and Group 1B, Group 1B and Group 2, Group 1B and Group 3 have P-values < 0.0001. In addition, receiver operator characteristic (ROC) curve analysis of the IgE-ELISA (n=125) shown COV 0.22 produced the highest sensitivity of 100 % and specificity of 98 %, with the area under the ROC curve (AUC) is 1.000, more particularly to 95 % confidence interval of 0.999 to 1.000 and P-value > 0.0001. Antigenicity evaluation produced the diagnostic sensitivity of 100 % (n=31) and diagnostic specificity of 98 % (n=94) as shown in Fig. 5. Further, the distribution of OD of positive and negative biological sample is shown in Fig. 6.

The purified A133 was used in IgE western blot and IgE-ELISA and showed good diagnostic potential with high sensitivity and specificity. A significant difference was observed between biological samples of Group 1A and Group 1B, and also between the negative control biological samples of Group 2 and 3 with Group 1B. These results implied that A133 has a good potential to detect acute *Strongyloides* infection.

The above-mentioned biomarker overcomes the problems and shortcomings of the existing biomarker for *Strongyloides*, specifically *S. stercoralis*. The biomarker showed high potential for diagnosis, epidemiological studies and research, especially for diagnosing acute infection of strongyloidiasis. In addition, IgE-ELISA is applied in the methods of detecting *Strongyloides* (100) and diagnosing strongyloidiasis (200) in a subject. In addition, the present invention detects IgE in order to detect presence of active or acute infection of *Strongyloides*. The biomarker as antigen also showed high diagnostic sensitivity and specificity of higher than 95 % due to the biomarker being an isolated or

recombinant *S. stercoralis* protein. Due to its high sensitivity and specificity, the biomarker is utilized in preparing an improved diagnostic kit for *Strongyloides*.

The exemplary implementation described above is illustrated with specific shapes, dimensions, and other characteristics, but the scope of the invention also includes various other shapes, dimensions, and characteristics. Also, the components as described above could be manufactured in various other ways and could include various other materials.

Various modifications to these embodiments are apparent to those skilled in the art from the description and the accompanying drawings. The principles associated with the various embodiments described herein may be applied to other embodiments. Therefore, the description is not intended to be limited to the embodiments shown along with the accompanying drawings but is to be providing broadest scope of consistent with the principles and the novel and inventive features disclosed or suggested herein. Accordingly, the invention is anticipated to hold on to all other such alternatives, modifications, and variations that fall within the scope of the present invention and appended claims.

Although the present invention has been described with reference to specific embodiments, also shown in the appended figures, it will be apparent for those skilled in the art that many variations and modifications can be done within the scope of the invention as described in the specification and defined in the following claims.

SEQUENCE LISTING

<110> UNIVERSITI SAINS MALAYSIA

<120> A BIOMARKER FOR DETECTING *Strongyloides*, METHODS OF DETECTING *Strongyloides* AND DIAGNOSING STRONGYLOIDIASIS IN A BIOLOGICAL SAMPLE, AND A DIAGNOSTIC KIT COMPRISING THEREOF

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taa 723

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Thr	Lys	Thr	Ser	Asp	Ala	Leu	Gly	Ile	Thr	Ile	Thr	Asp	Asn	Gly	Ala
		35					40					45			
Gly	Tyr	Cys	Phe	Val	Lys	Lys	Ile	Lys	Pro	Gly	Ser	Thr	Ser	Ala	Leu
	50					55					60				
Ala	Cys	Pro	Ala	Ile	Leu	Ile	Gly	Asp	His	Ile	Glu	Lys	Val	Asn	Gly
65					70					75					80
Asn	Ser	Met	Val	Gly	Met	Thr	His	Cys	Ser	Val	Ala	Arg	Val	Leu	Arg
				85					90					95	
Asn	Ile	Ser	Val	Gly	Glu	Thr	Phe	Ile	Ile	Arg	Leu	Val	Glu	Pro	Tyr
			100					105					110		
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Arg Lys Ser Arg Leu Ser Gly Gln Ser Thr Lys Asn Asp Ser Asn Lys
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Claims:

1. A biomarker for detecting *Strongyloides*, said biomarker is characterized by:

a *Strongyloides stercoralis* protein comprising an amino acid sequence set forth in SEQ ID NO: 2;

wherein said *Strongyloides stercoralis* protein is encoded by a nucleic acid molecule comprising a nucleic acid sequence set forth in SEQ ID NO: 1.

2. The biomarker as claimed in claim 1, wherein said nucleic acid sequence set forth in SEQ ID NO: 1 is introduced into a plasmid to produce an expression construct configured to produce said *Strongyloides stercoralis* protein encoded by said nucleic acid sequence set forth in SEQ ID NO: 1.

3. The biomarker as claimed in claim 1, wherein said *Strongyloides stercoralis* protein is an isolated *Strongyloides stercoralis* protein or a recombinant *Strongyloides stercoralis* protein.

4. The biomarker as claimed in claim 1, wherein said amino acid sequence set forth in SEQ ID NO: 2 including a complete amino acid sequence, a fragment of said amino acid sequence or a variant of said amino acid sequence.

5. The biomarker as claimed in claim 1, wherein said nucleic acid molecule is an isolated nucleic acid molecule or a recombinant nucleic acid molecule.

6. The biomarker as claimed in claim 1, wherein said biomarker including a *Strongyloides* specific antigen.

7. A method (100) of detecting *Strongyloides* in a biological sample, said method comprising the steps of:

contacting a biological sample with an antigen; and

detecting a specific and selective antigen-antibody binding of said antigen and a first antibody present in said biological sample via an immunoassay;

characterized in that,

said antigen is said biomarker for detecting *Strongyloides* of Claim 1 comprising said amino acid sequence set forth in SEQ ID NO: 2;

wherein said first antibody is a *Strongyloides stercoralis*-specific antibody; and

wherein presence of *Strongyloides* in said biological sample is detected via presence of said specific and selective antigen-antibody binding of said antigen and said first antibody in said biological sample.

8. The method (100) as claimed in claim 7, wherein said first antibody is an immunoglobulin E, IgE, antibody.

9. The method (100) as claimed in claim 7, wherein said immunoassay including an enzyme-linked immunosorbent assay, a dot-blot, a Western blot, a vertical flow test, a lateral flow assay, and a biosensor.

10. The method (100) as claimed in claim 7, wherein said biological sample is from a human or non-human species.

11. A method (200) of diagnosing Strongyloidiasis in a subject, said method comprising the steps of:

collecting a biological sample from a subject;

contacting said biological sample with an antigen;

detecting presence of *Strongyloides* protein in said biological sample via an immunoassay using a first antibody;

contacting said biological sample with a second antibody; and

determining presence of Strongyloidiasis in said subject;

characterized in that,

said antigen is said isolated *Strongyloides stercoralis* protein of Claim 1 comprising said amino acid sequence set forth in SEQ ID NO: 2;

wherein said first antibody is a *Strongyloides stercoralis*-specific antibody; and

wherein said second antibody is an antibody that specifically binds to said first antibody.

12. The method (200) as claimed in claim 11, wherein each of said first antibody and said second antibody is an immunoglobulin E, IgE, antibody.

13. The method (200) as claimed in claims 11 and 12, wherein said second antibody is conjugated with a detector molecule selected from an enzyme, a fluorescence molecule or a colloidal gold.

14. The method (200) as claimed in claims 11, wherein said method is used for determining acute strongyloidiasis in said subject via detection of said first antibody and said second antibody.

15. A diagnostic kit for *Strongyloides*, said diagnostic kit comprising:

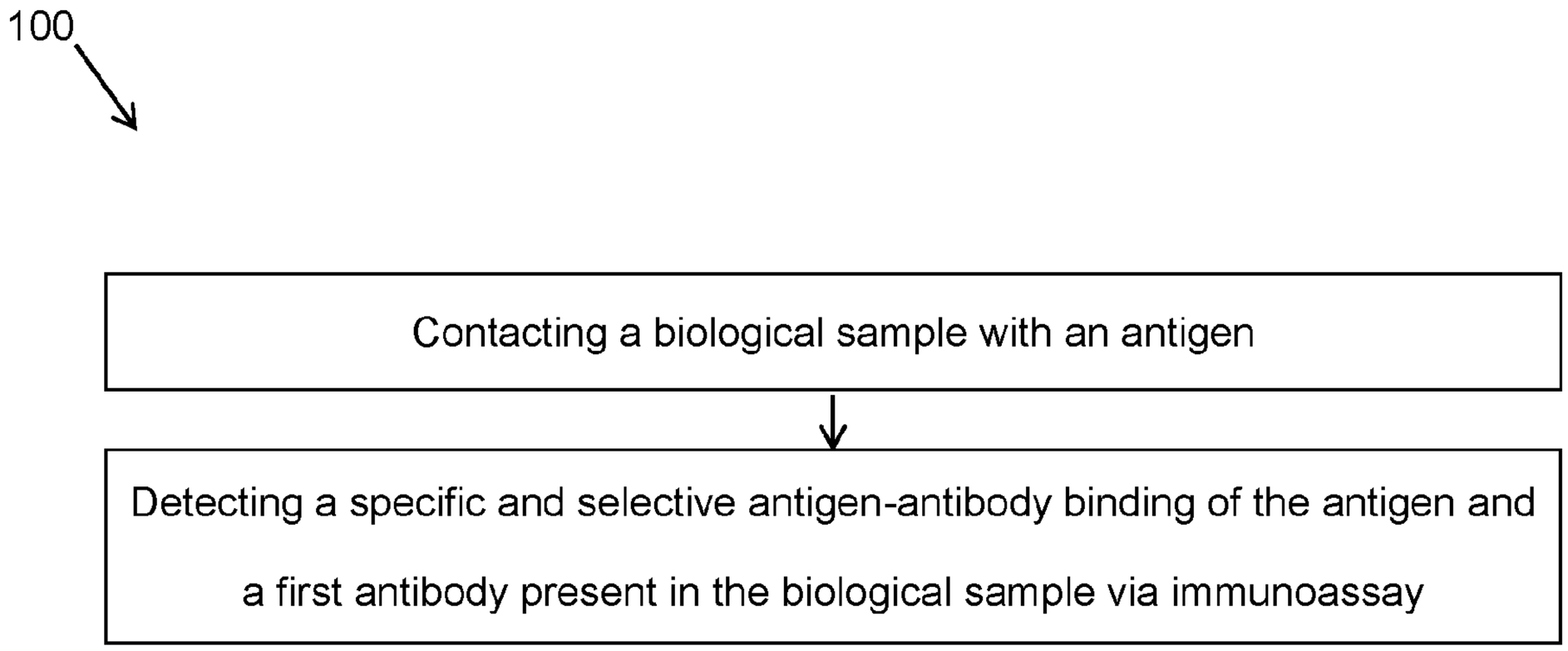
said biomarker for detecting *Strongyloides* of claim 1;

an immunoassay reagent; and

an immunoassay device configured to detect presence of *Strongyloides*.

16. The diagnostic kit as claimed in claim 15, wherein said diagnostic kit is configured to detect presence of *Strongyloides* antibody or *Strongyloides* protein.

17. The diagnostic kit as claimed in claim 16, wherein said diagnostic kit including an enzyme-linked immunosorbent assay, a dot blot assay, a Western blot assay, a vertical flow assay, a lateral flow assay, and a biosensor.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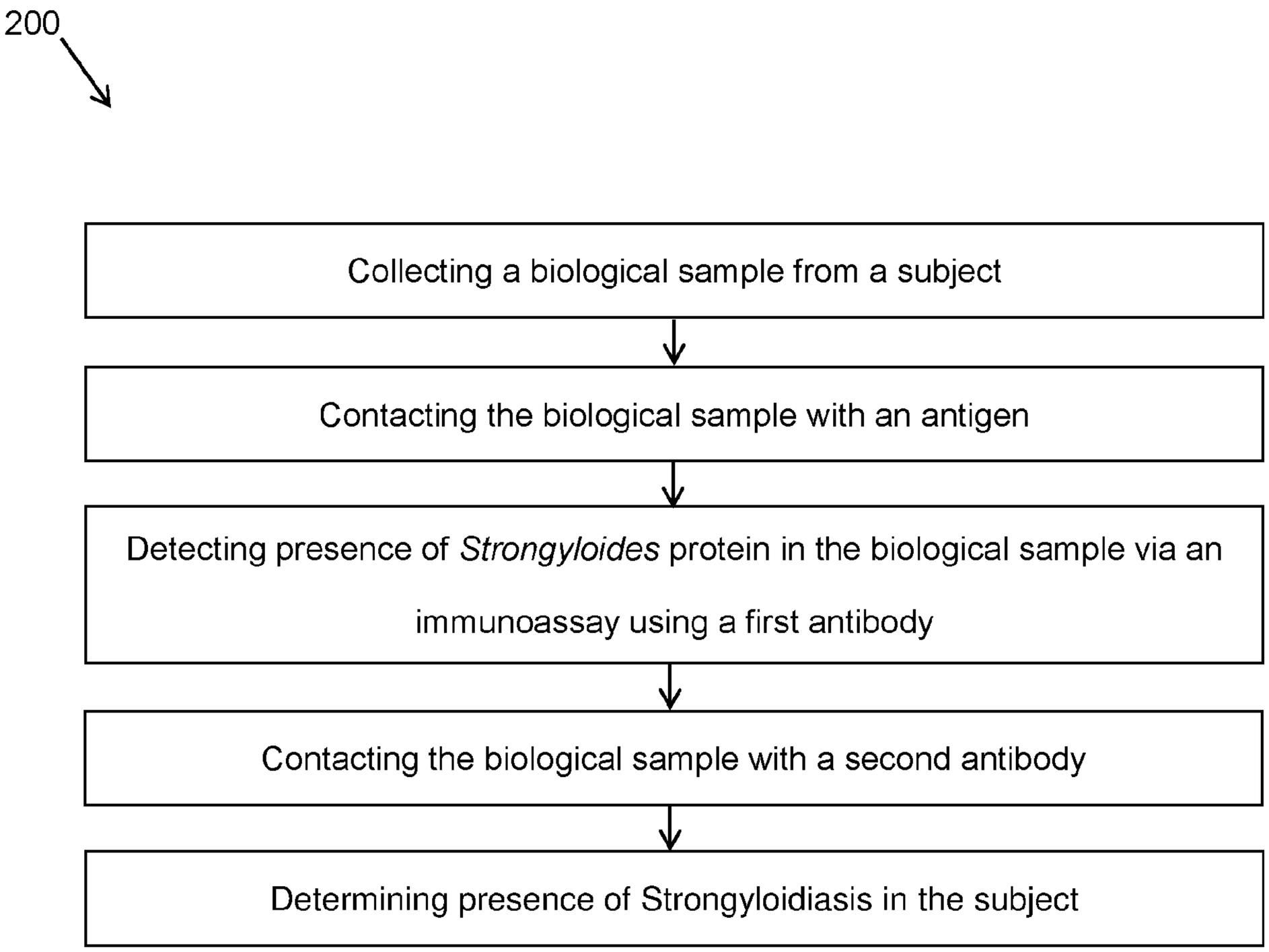
Contacting a biological sample with an antigen
Detecting a specific and selective antigen-antibody binding of the antigen and a first antibody present in the biological sample via immunoassay

Fig. 1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Collecting a biological sample from a subject
Contacting the biological sample with an antigen
Detecting presence of *Strongyloides* protein in the biological sample via an immunoassay using a first antibody
Contacting the biological sample with a second antibody
Determining presence of Strongyloidiasis in the subject

Fig. 2

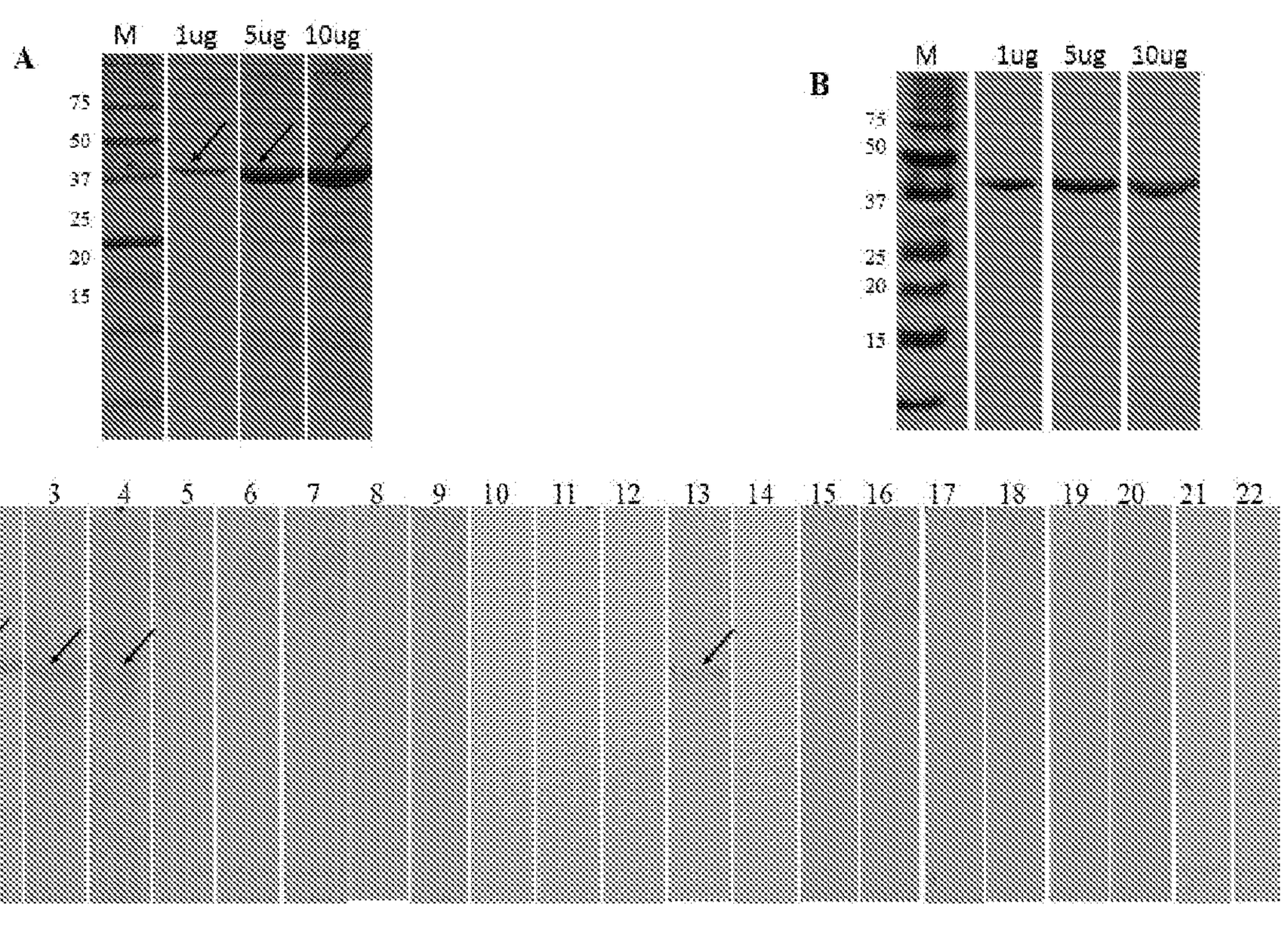
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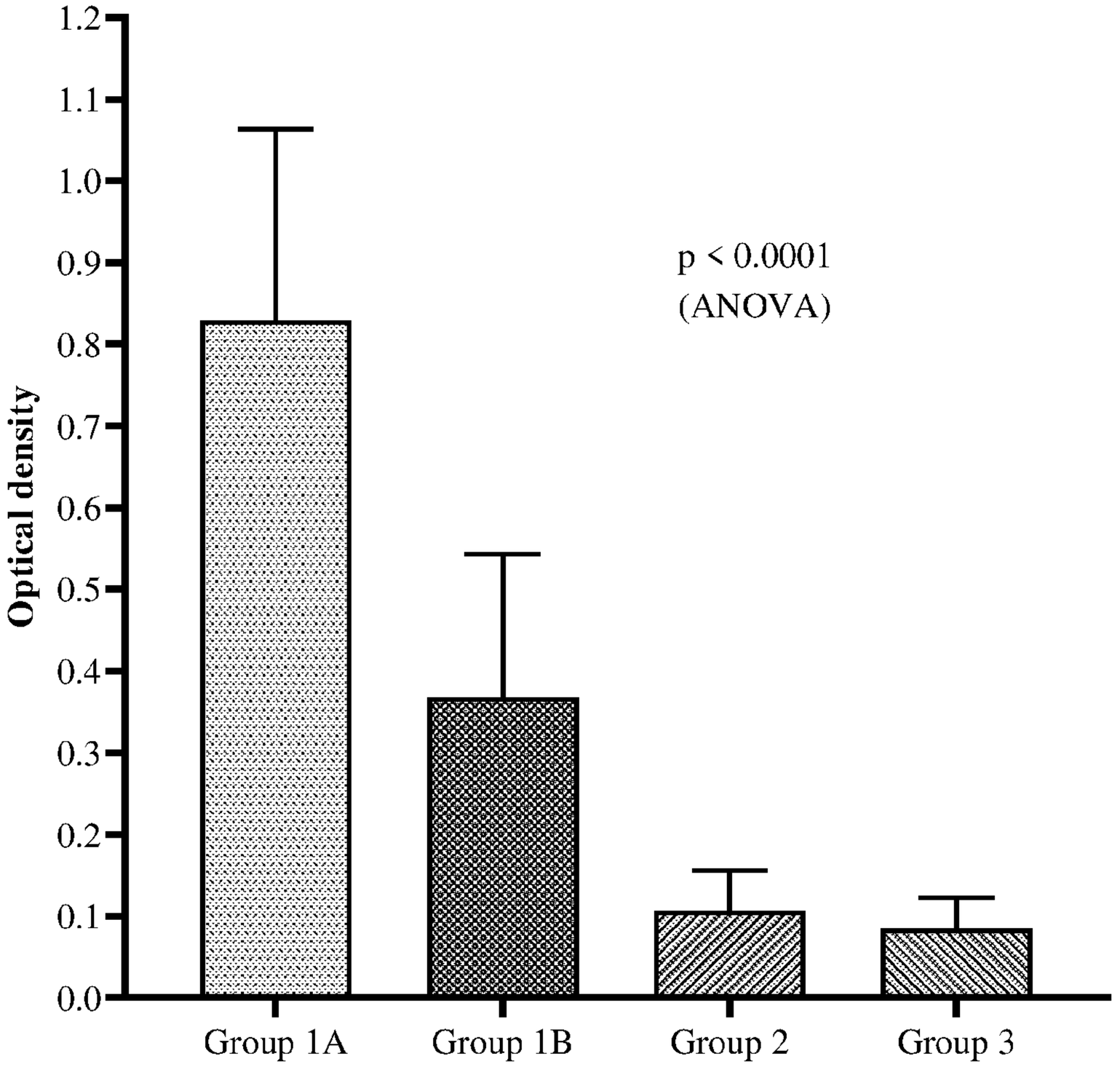
Fig. 3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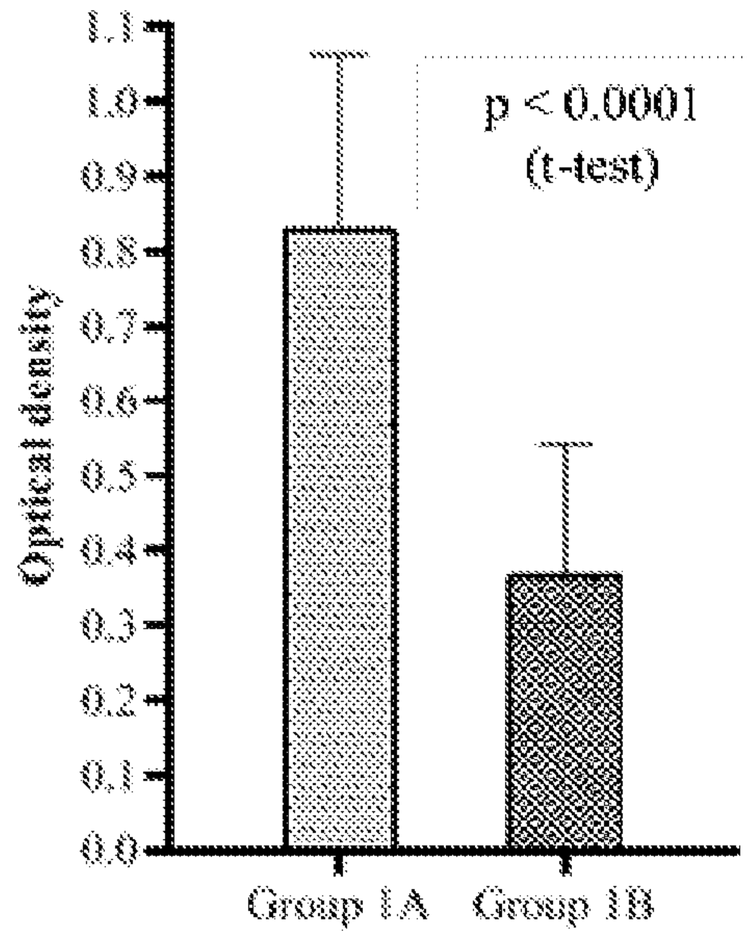
Group 1A
Group 1B
Group 2
Group 3

Fig. 4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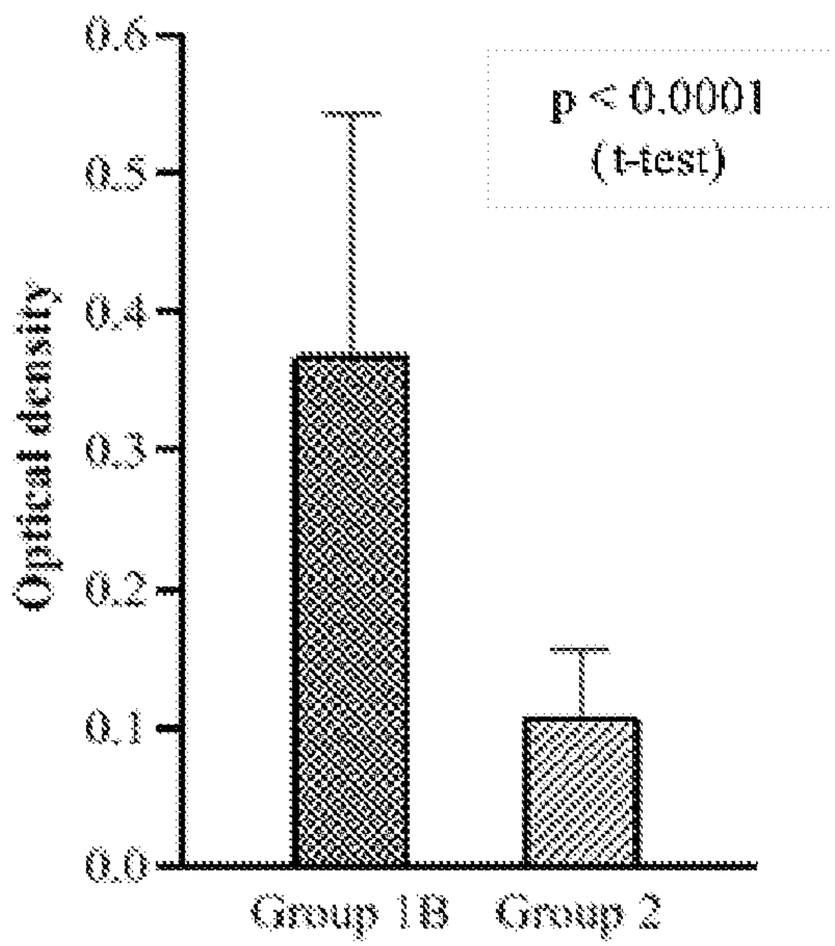
p < 0.0001
(t-test)
Group 1A
Group 1B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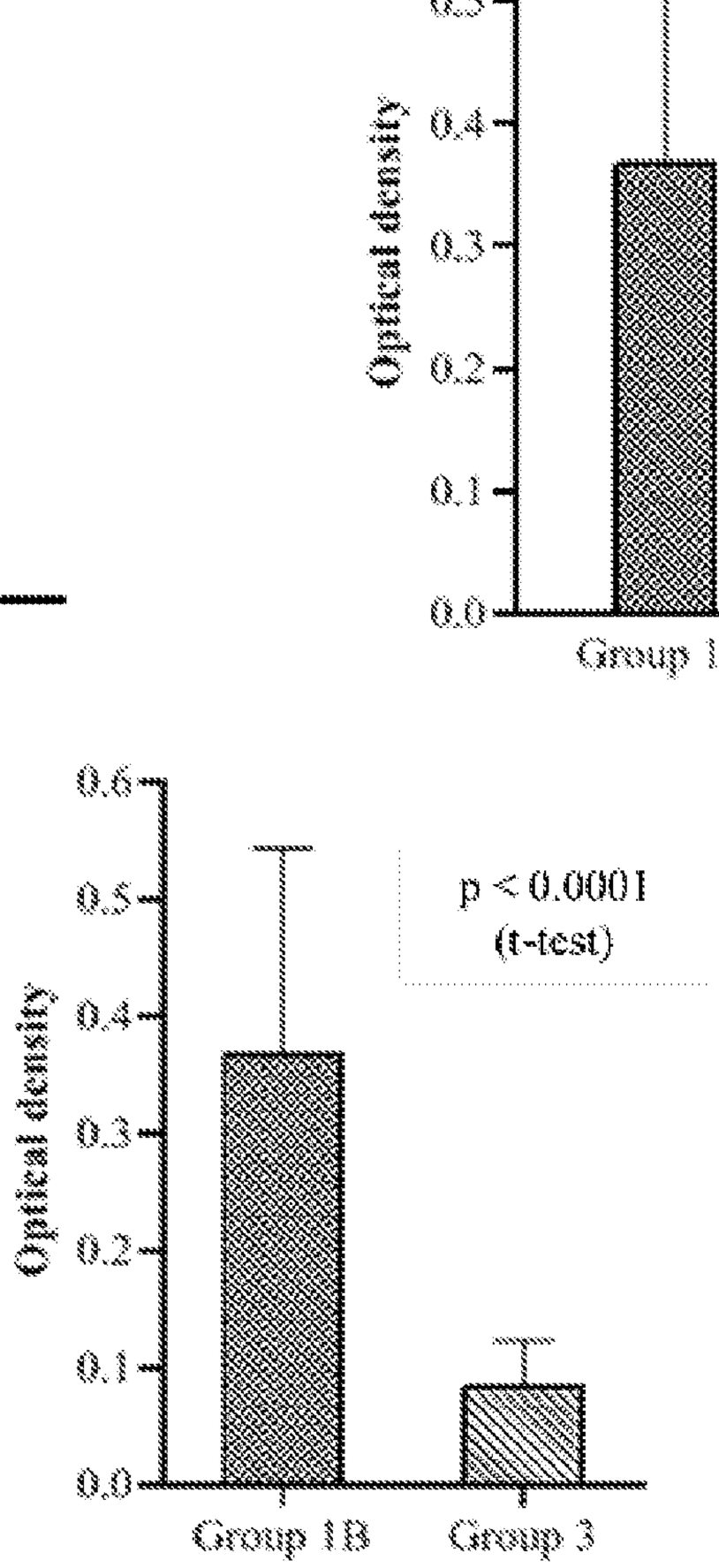
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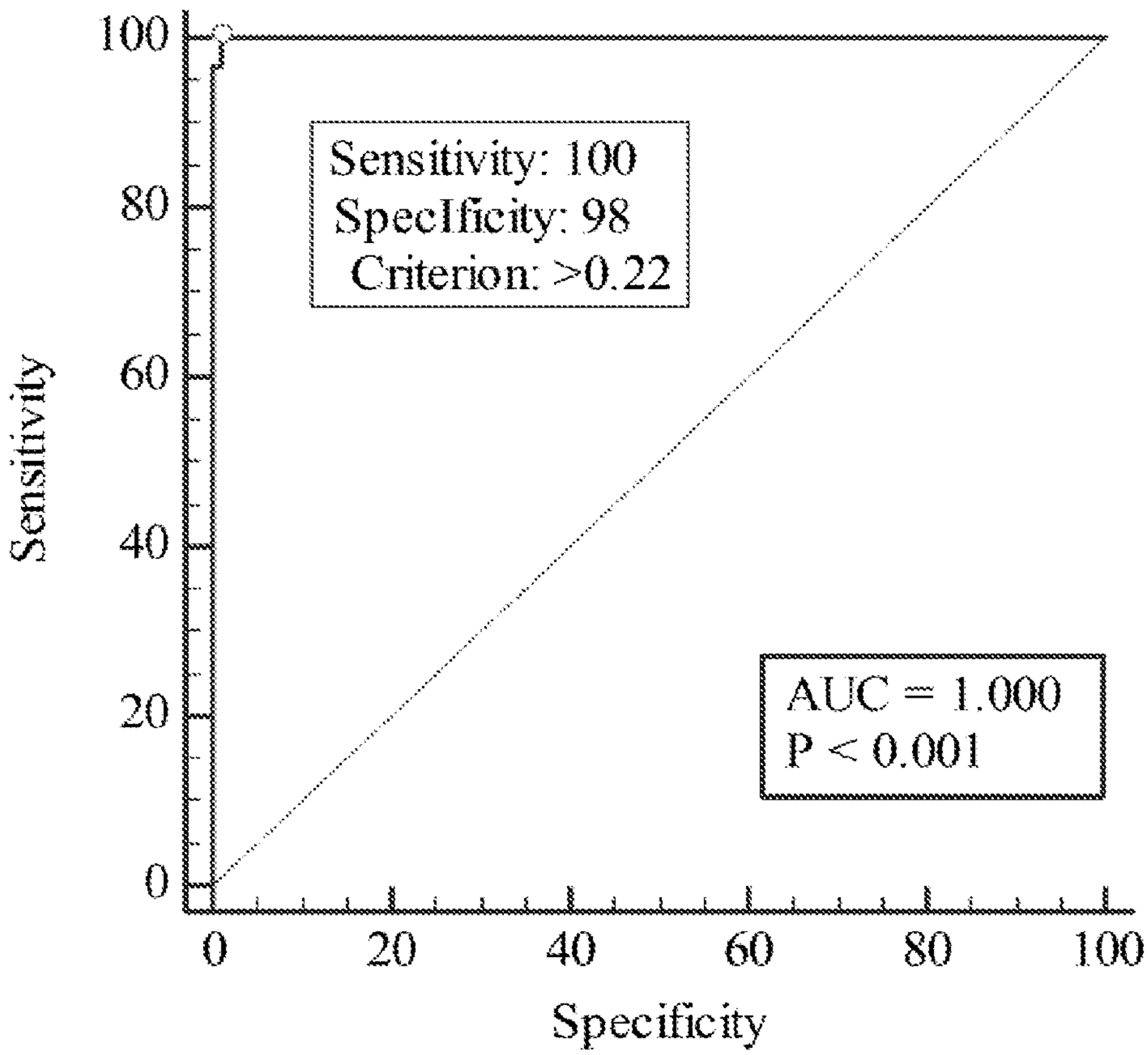
Fig. 5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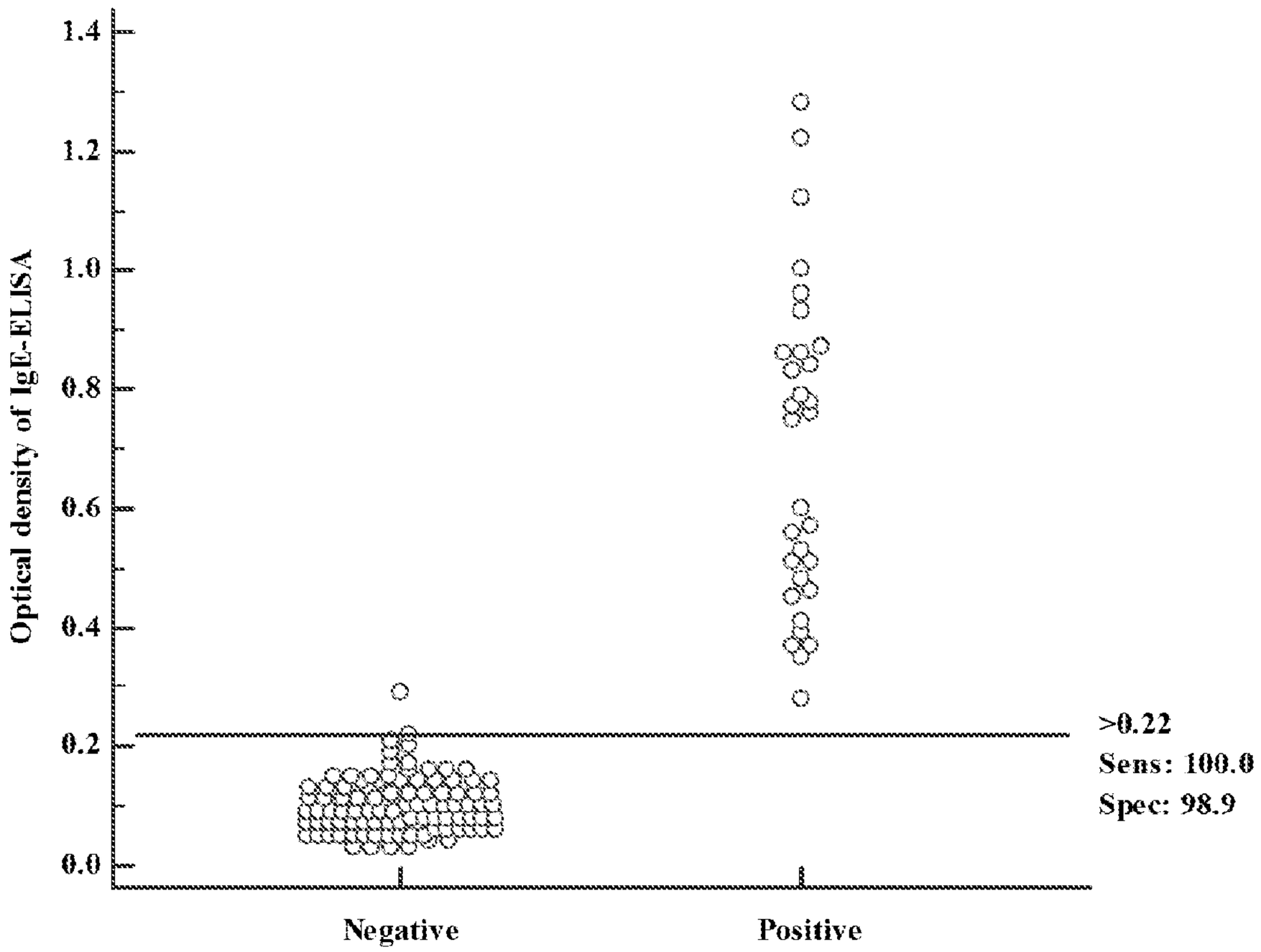
Specificity

Fig. 6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Sens: 100.0
Spec: 98.9
Negative
Positive

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2020/050044

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/53 (2006.01) C07K 14/00 (2006.01) C07K 16/44 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GenomeQuest search for SEQ ID 2. PATENW, MEDLINE, BIOSIS, EMBASE, CAPLUS: Strongyloides, strongyloidiasis, stercoralis, fuelleborni, S. stercoralis, S. fuelleborni, S. Ratti, immunoassay, antigen, epitope, immunogen, marker and like terms; IPC/CPC: searched within C07K14/4354, GO1N33/53, G01N33/6854, G01N33/5308, C07K16/44, G01N2333/4353 and similar marks; ESPACENET, GOOGLE SCHOLAR, Internal databases provided by IP Australia: Applicant and Inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2020	06 August 2020
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	**Authorised officer** Cara Mortimer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832661

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/MY2020/050044**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Varatharajalu et al. "Strongyloides stercoralis excretory/secretory protein stronglyastacin specifically recognized by IgE antibodies in infected human sera" Microbiology and Immunology (2011), 55(2), 115-122. title and abstract	
A	Ramachandran et al. "Recombinant cDNA clones for immunodiagnosis of strongyloidiasis" Journal of Infectious Diseases (1998), 177(1), 196-203. abstract	
A	CA 3051466 A1 (VANDERBILT UNIVERSITY) 09 August 2018 summary page 7 line 5-19	
A	WO 2017/091059 A1 (UNIVERSITI SAINS MALAYSIA) 01 June 2017 summary page 3 and example 1 on page 12	
A	WO 2011/097216 A1 (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERVICES et al.) 11 August 2011 abstract and example 9 on page 19	
A	Anderson et al. "Comparison of three immunoassays for detection of antibodies to Strongyloides stercoralis" Clinical and Vaccine Immunology (2014), 21(5), 732-736. abstract and "InBios Strongy Detect IgG ELISA" on page 733	
A	Masoori et al. "Fatty acid and retinol-binding protein: A novel antigen for immunodiagnosis of human strongyloidiasis" PloS one (2019), 14(7), 1-15. (online journal) [retrieved from internet on 22 July 2019] <URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0218895> abstract and "Optimization of SsFAR-specific ELISA" on page 4.	

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/MY2020/050044**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
CA 3051466 A1	09 August 2018	CA 3051466 A1	09 Aug 2018
		AU 2018214946 A1	08 Aug 2019
		EP 3576758 A1	11 Dec 2019
		US 2019353661 A1	21 Nov 2019
		WO 2018144425 A1	09 Aug 2018
WO 2017/091059 A1	01 June 2017	WO 2017091059 A1	01 Jun 2017
		US 2018356409 A1	13 Dec 2018
		US 10627394 B2	21 Apr 2020
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		EP 2531210 A1	12 Dec 2012
		US 2012308599 A1	06 Dec 2012
		US 9056068 B2	16 Jun 2015

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.