Accurate differentiation of *Trichosporon asahii* and *Trichosporon asteroides* from other human pathogenic yeasts and moulds by using highly specific monoclonal antibodies

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ABSTRACT

Members of the genus *Trichosporon* are emerging opportunistic pathogens of humans, causing the invasive fungal disease trichosporonosis in immunocompromised patients, and summer-type hypersensitivity pneumonitis (SHP) in immunocompetent individuals through inhalation of arthroconidia. Trichosporonosis is frequently misdiagnosed as candidiasis or cryptococcosis due to a lack of awareness and the inaccuracy of immunodiagnostic tests for these yeast pathogens. Delays in identification and differentiation of *Trichosporon* spp. from other yeasts and timely administration of appropriate antifungal drug treatments add to the poor prognosis and high mortality rate associated with this trichosporonosis. This thesis describes the use of hybridoma technology to produce two highly specific murine monoclonal antibodies (MAbs), CA7 and TH1, for detection and differentiation of *Trichosporon* from other yeast pathogens. The MAbs react with extracellular antigens from *T. asahii* and *T. asteroides,* the two most common pathogenic agents of trichosporonosis. CA7 and TH1 do not recognise related *Trichosporon* spp., or unrelated pathogenic yeasts and moulds including *Candida* spp., *Cryptococcus* spp., species of *Aspergillus*, *Fusarium*, *Scedosporium*, and etiologic agents of mucormycosis. Immunofluorescence and western blotting studies show that MAb CA7, an immunoglobulin G1 (IgG1), binds to a major ~60kDa glycoprotein antigen produced on the surface of hyphae, while TH1, an immunoglobulin M (IgM), binds to an antigen produced on the surface of conidia. I show how the MAbs can be used with standard mycological growth medium (Sabouraud Dextrose Agar) and an enzyme-linked immunosorbent assay (ELISA) to accurately differentiate *T. asahii* from *Candida albicans* and *Cryptococcus neoformans* in single and mixed species cultures.

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INTRODUCTION

The genus *Trichosporon* contains approximately 50 species of fungi found in a wide variety of habitats including soil and indoor environments [1-3]. A number of species colonise the skin, gastrointestinal, respiratory and urinary tracts of humans [4-7], and superficial *Trichosporon* infections of immunocompetent individuals (for example white piedra infections of the hair shaft [1, 8]) have been described for many years [9]. As well as causing superficial infections, repeated inhalation of *Trichosporon* athroconidia can cause summer-type hypersensitivity pneumonitis (SHP) [1, 3], an immunologically induced lung disease. It is the most common form of hypersensitivity pneumonitis (HP) in Japan [10-12] and the number of reports of this disease are increasing [1]. *T. asahii* is the most frequent cause of SHP due to it being the most common species of the genus found in the environment [10, 12]. However, other fungi may be responsible and correct diagnosis of the disease following identification of the causative species is necessary for appropriate treatment, which can be challenging due to the number of different techniques required [11, 12].

Certain *Trichosporon* species have emerged as rare but frequently fatal pathogens causing invasive, disseminated, infections in immunocompromised individuals [4-8, 13-15]. As with SHP, the prevalence of invasive fungal infections has increased over the past decade [1, 2, 7, 9, 13, 16, 17] and *Trichosporon* species are now recognised as agents of disseminated yeast fungemia, second only in importance to the genus *Candida* [4, 13, 16-19]. High mortality rates are associated with disseminated trichosporonosis [1, 7, 14], with reports in the literature of between 50 and 80% mortality in high-risk patient groups [9].

Invasive trichosporonosis is mainly found in patients with haematological malignancies [7, 9, 13, 16] and granulocytopenia [14]. Incidences of haematological malignancies are increasing [13] and the use of immunosuppressive therapies [5], chemotherapy [13], corticosteroids [2, 14] and organ transplantation [7, 13], are adding to the ever expanding populations of patients at high risk for contracting disseminated fungal infections. A further risk factor is the use of intravenous catheters [13, 14, 17], since *Trichosporon* spp. can form biofilms on implanted devices, allowing escape from the host immune system and antifungal drugs [9]. Other risk factors include the use of broad-spectrum antibiotics [8, 13] and previous gastrointestinal/urinary tract colonisation by *Trichosporon* spp [6, 14].

Currently, for a proven diagnosis of invasive trichosporonosis demonstration of the fungus in tissue biopsy specimens and in culture is required [9, 17], but obtaining biopsy samples from critically ill patients is problematic. Methods for identifying *Trichosporon* to species level, based on morphological characteristics in culture and biochemical profiling are time consuming, require specialist mycological training, appropriately equipped laboratory facilities, and often provide inconsistent results [2, 9, 13, 15]. Molecular methods are necessary to distinguish *Trichosporon* spp., but no such methods have been standardised for clinical use and no commercially available tests currently accommodate the revised taxonomic classification of the genus [9, 13].

Early diagnosis of invasive trichosporonosis is critical for prompt and effective treatment [4, 17] but this is difficult for *Trichosporon* infections [4, 13-15], especially in resource-limited settings with rudimentary diagnostic procedures [8], where *Trichosporon asahii* may be mistaken for *Candida* spp in

culture [2, 8, 16, 20]. Further complications are infections caused by mixed yeast or mould species [21], and cross-reactivity of commercial immunoassays with *Trichosporon,* such as the *Cryptococcus* antigen test [22], leading to mis-identification and inappropriate use of drugs [15, 23].

To facilitate the early treatment of invasive trichosporonosis, development of non-invasive diagnostic procedures that allow accurate detection of *Trichosporon* spp. need to be developed [24]. Hybridoma technology allows the generation of highly specific monoclonal antibodies (MAbs) [25] that can be used to develop rapid and highly accurate immunodiagnostic tests capable of differentiating fungal pathogens to genus-, species- or even isolate-level from patient samples such as serum [26-28]. This paper describes the use of hybridoma technology to develop two murine hybridoma cell lines (CA7 and TH1) producing MAbs specific for *Trichosporon asahii* and the closely related species *Trichosporon asteroides.* The accuracy of the MAbs in differentiating *T. asahii* from other yeast pathogens in mixed cultures is demonstrated using a highly specific ELISA.

METHODOLOGY

Fungal culture

Trichosporon asahii is currently the most common cause of invasive trichosporonosis [4, 18]. Consequently, a clinical isolate of the fungus (*Trichosporon asahii* var. *asahii* CBS 8972) was used to prepare immunogen for immunisation of mice. The fungus was grown in malt yeast liquid medium (MYB; bactopeptone, 0.5% w/v; malt extract (OXOID Ltd. Basingstoke, United Kingdom; LP0039) , 1.0% w/v; glucose, 0.4% w/v; yeast extract, 0.4% w/v; adjusted to pH7.3 with 1M NaOH) or on malt yeast agar (MYA; MY containing

2.0% w/v agar). Sabouraud dextrose agar (SDA; 3.0% w/v SD broth (Oxoid; CM0147) containing 2.0% w/v agar) was used in mixed species specificity screening. *Candida, Rhodotorula, Magnusiomyces, Kluyveromyces, Geotrichum, Wickerhamomyces* and *Pichia* species were all grown on Glucose-Peptone-Yeast extract agar (GPYA; 4.0% w/v glucose, 0.5% w/v bacteriological peptone, 0.5% w/v yeast extract, 1.5% w/v agar.). *Aspergillus, Penicillium, Scedosporium, Paecilomyces, Pythium, Alternaria, Verticillium* and *Pseudallescheria* species were all grown on Malt Extract Agar (MEA; 2.0% w/v malt extract containing 2.0% w/v agar). *Malassezia furfur* was grown on Leeming-Notman agar (LNA; 1.0% w/v bacteriological peptone, 0.5% w/v glucose, 0.01% w/v yeast extract, 0.8% w/v ox bile (SIGMA Chemical Company, Poole, United Kingdom; 70168), 0.0001% w/v glycerol, 0.05% w/v glycerol m-monostearate, 0.00005% w/v Tween-60 (Sigma; P1629), 1.0% w/v liquid, whole fat, cow's milk (Sainsbury's), 1.2% agar). All other fungi were grown on Potato Dextrose Agar (PDA; 2.4% w/v potato dextrose broth (Sigma; P6685) containing 2.0% w/v agar). All media were autoclaved at 121°C for 15mins before use and cultures grown at 26°C with a 16h photoperiod of fluorescent light, with the exception of *M. furfur* which was grown at 30°C in the dark.

Preparation of immunogen

For preparation of the immunogen, flasks containing 100ml of sterile MYB were inoculated with 10^3 spores of *T. asahii* per ml of medium. The flasks were incubated at 26^oC with shaking (125rpm) for 2d, after which the contents were centrifuged at 4000rpm for 5mins, the bulk of the supernatant was discarded and the pelleted cells re-suspended in the remaining liquid. The surface of 2-d-

old MYA petri-dish cultures were scraped and combined with the re-suspended cells. The combined cell preparations were snap frozen in liquid nitrogen, lyophilized for 3d and the dried material stored at -20°C prior to use. Before immunisation the immunogen was reconstituted with phosphate buffer saline (PBS; 137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, and 1.5mM KH_2PO_4 [pH 7.2]) to make a cell suspension containing 10mg per ml of buffer.

Immunisation regime

Four female BALB/c white mice were each given four injections of immunogen into the peritoneum at two-week intervals. Serum obtained from tail bleeds was used to monitor antisera titres of the mice by using a Plate-Trapped-Antigen-Enzyme-Linked-Immunosorbent-Assay (PTA-ELISA). The mouse exhibiting the strongest immune response (highest titre of anti-*Trichosporon* antibodies) was selected for hybridoma production and a single booster injection was given three days before fusion.

Production of hybridomas

The selected mouse was euthanized and the spleen removed aseptically and stored in 20% tissue culture medium (20% TCM: RPMI 1640 medium containing 20% fetal bovine serum). Spleen cells were removed by rupturing the spleen and left to stand to let the connective tissue settle. The cell suspension was transferred to a centrifuge tube and, along with a centrifuge tube of murine myeloma cells (Sp2/0-Ag14) (each containing a total of 10^7 cells), were centrifuged at 250g for 5mins. Supernatants from both tubes were discarded and the cells were re-suspended in RPMI serum-free medium, before combining the myeloma and spleen cells suspensions together. The combined

samples were centrifuged at 250g for 5mins. The supernatant was discarded to dry the cells and 0.3ml of polyethylene glycol solution (PEG: 0.2ml PEG mixed with 0.4ml RPMI1640 serum-free medium) was added. After 6mins, the cells were centrifuged for 1min at 500g. The PEG solution was removed, the cells re-suspended in 5ml of serum-free RPMI medium and added to 95ml of HAT (hypoxanthine, aminopterin, thymidine) selective medium (TCM containing sterile HAT). 100µl of the HAT-cell suspension was added to wells of sterile 96-well culture plates and the cells incubated in a CO_2 incubator at 37°C.

Screening of hybridomas

After 7 to 10d, colonies of hybridoma cells were clearly visible in the wells. Supernatants bathing hybridomas were screened for *Trichosporon* MAbs in PTA-ELISA tests, using microtitre plates coated with soluble antigens from *T. asahii* and other related and unrelated fungi of clinical significance (see Table.1. for species used).

Preparation of antigen coated microtitre plates

Hybridoma supernatants were tested by PTA-ELISA using 96 well microtitre plates (Nunc Maxisorp) coated with antigens from lyophilised cells or with antigens prepared as surface washings (see Table. 1. for species used). Lyophilised cells were prepared as described for the preparation of the *T. asahii* immunogen. For surface washings, slant cultures of fungi grown at 26°C were washed with 3ml PBS using a pipette tip to gently stroke the slant surface. The washings were added to 1.5ml eppendorf tubes and centrifuged at 14000rpm for 5mins to precipitate spores and hyphae. The resulting supernatants, containing soluble antigens, were diluted 1 in 10 in PBS. 50µl samples were

used to coat the wells of 96 well microtitre plates and the content incubated overnight at 4°C in sealed plastic bags. Plates were washed three times with PBST (PBS containing 0.05% [v/v] Tween-20 (polyoxyethylene-sorbitan monolaurate)(Sigma; P7949), once each with PBS and dH_2O (5min each wash), and then air dried in a laminar flow hood. Plates were stored in sealed plastic bags at 4°C prior to use.

Table. 1.

Organism	Isolate number	Source
Trichosporon asahii var. *asahii*	8972	CBS
Trichosporon asahii var. *asahii*	8973	CBS
Trichosporon asahii var. *asahii*	5286	CBS
Trichosporon asahii var. *asahii*	7632	CBS
Trichosporon asahii var. *asahii*	5599	CBS
Trichosporon asahii	2479	CBS
Trichosporon asteroides	6183	CBS
Trichosporon asteroides	7623	CBS
Trichosporon asteroides	2481	CBS
Trichosporon asteroides	7624	CBS
Trichosporon cutaneum	2466	CBS
Trichosporon dermatitis	2043	CBS
Trichosporon inkin	7630	CBS
Trichosporon inkin	7655	CBS
Trichosporon loubieri	7065	CBS
Trichosporon mucoides	7625	CBS
Trichosporon mycotoxinivorans	9756	CBS
Trichosporon ovoides	7556	CBS
Alternaria infectoria	137.9	CBS
Aspergillus cervinus	537.65	CBS
Aspergillus fumigatus	AF293	SK
Aspergillus nidulans	A4	FGSC
Aspergillus niger	102.4	CBS
Aspergillus oryzae	AO1	CRT
Aspergillus terreus var. *terreus*	601.65	CBS
Botrytis cinerea	R2	CRT
Candida albicans	5314	SB
Candida dubliniensis var. *dubliniensis*	8500	CBS
Candida glabrata	4962	CBS
Candida krusei	5590	CBS
Candida parapsilosis var. *parapsilosis*	8836	CBS
Candida tropicalis var. *tropicalis*	1920	CBS

Table. 1. continued

Cryptococcus neoformans serotype D	5728	CBS
Cryptococcus neoformans var. *neoformans*	7779	CBS
Cryptococcus saitoi	1975	CBS
Cunninghamella elegans	151.8	CBS
Filobasidiella bacillispora	10865	CBS
Filobasidiella neoformans	10490	CBS
Filobasidiella neoformans	10496	CBS
Fusarium oxysporum f.sp. lycopersici	167.3	CBS
Fusarium solani	224.34	CBS
Geotrichum candidum	115.23	CBS
Kluyveromyces marxianus	3073	CBS
Lichtheimia corymbifera	TJAFJ713070	CRT
Magnusiomyces capitatus	207.83	CBS
Malassezia furfur	9596	CBS
Paecilomyces variotii 10.1	10.1	CRT
Penicillium islandicum	338.48	CBS
Pichia norvegensis	6564	CBS
Pseudallescheria boydii	835.96	CBS
Pythium ultimum var. *ultimum*	656.68	CBS
Rhizomucor miehei	360.92	CBS
Rhizopus stolonifer var. *stolonifer*	389.95	CBS
Rhodosporidium toruloides	6016	CBS
Rhodotorula mucilaginosa var. *mucilaginosa*	326	CBS
Scedosporium apiospermum	117407	CBS
Scedosporium prolificans	467.74	CBS
Sporidiobolus salmonicolor	6781	CBS
Verticillium dahliae	178.66	CBS
Wickerhamomyces anomalus	5759	CBS

Table. 1. Fungal species used with their respective isolate numbers and source. CBS; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. SK; S. Krappman, Institute of Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August University, Gottingen, Germany. FGSC; Fungal Genetics Stock Centre, University of Missouri, Kansas City. CRT; C. R.Thornton, University of Exeter, England. SB; S. Bates, School of Biosciences, University of Exeter

Plate-Trapped-Antigen-Enzyme-Linked-Immunosorbent Assay (PTA-ELISA)

Wells were blocked for 15mins with 100µl of PBS containing 1% Bovine Serum Albumin (BSA) and, after one 5min wash with PBS, 50µl of primary antibody was added to each well and incubated for 1h. For testing of tail bleeds, the wells were incubated with a doubling dilution of serum in PBST. For screening of hybridomas, 50µl of hybridoma supernatant was added to the wells and TCM

was used as the negative control. Plates were given three 5min washes with PBST before adding 50µl of goat anti-mouse polyvalent (IgG, IgA, IgM) peroxidise conjugate (Sigma; A-0412) diluted 1/1000 in PBST, and incubated for a further hour. Plates were washed again three times with PBST and then once with PBS. 50µl of tetramethyl benzidine (TMB)(Sigma; T2885)(48.54% [v/v] Milli-Q water, 48.54% [v/v] 0.2 sodium acetate, 1.89% [v/v] sodium citric, 0.05% [v/v] H_2O_2, 0.98% [v/v] TMB (10 mg/ ml)) substrate solution was added to the wells for 30mins to visualise any bound antibody, and the reaction stopped by adding 50µl of 3M H_2SO_4 to each well. Absorbance values were measured at 450nm using an automated plate reader (iMark^{TM} microplate reader MPM6, BIORAD, Hertfordshire, UK). All incubation steps were performed in sealed plastic bags at 23°C.

Sub-cloning of positive hybridomas

Hybridoma cells were dislodged from the well surface by gently flushing with the TCM supernatant, and 50µl of the cell suspension added to 5ml warmed TCM in a sterile universal tube. This dilution was labelled ‘dilution B’ and from this, 100µl was added to 10ml of TCM in a universal tube, labelled ‘dilution C’. From dilution C, 1ml was added to a tube labelled ‘dilution D’ (containing 4ml of TCM), and 500µl added to a tube labelled ‘dilution E’ (containing 4.5ml of TCM). Before transferring volumes between dilutions, cell suspensions were mixed gently to ensure even cell distribution. Each dilution was plated out into half of a 96 well culture plate, at 100µl per well. The plates were incubated at 37°C and 5% CO_2 and fed after 5d with fresh TCM.

Expansion of cell lines and cryopreservation

Antibody positive cells lines were expanded in stages, from 96 well plates to 24 well plates, 6 well plates and then into petri dishes. As with limiting dilutions, cells were dislodged from the well surface by washing with their supernatant before removal of cell suspension from culture vessels. Prior to cryopreservation, cells lines were sub-cultured into to petri dishes approximately 24h prior to freezing to produce cultures of dividing cells. The contents of two plates each containing 25ml of cell suspension were added to sterile 50ml falcon tubes and the cells pelleted by centrifugation at 2000rpm for 5mins. The supernatant was discarded and the cells re-suspended in 600µl of freezing medium (fetal bovine serum and dimethyl sulfoxide 92:8 [v/v]). Cells were slowly frozen in cryovials at -80°C and stored long-term in liquid nitrogen.

Antibody isotyping

Immunoglobulin class was determined by using PTA-ELISA. Wells containing immobilised *T. asahii* antigen, were incubated with 50µl hybridoma supernatant for 1h. Following three 5min washes with PBST, the wells were incubated for 30mins with goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM or IgA – specific goat antiserum (Sigma; ISO-2), each diluted 1 in 3000 in PBST. Plates were washed again with PBST and incubated for a further 30min with rabbit anti-goat peroxidise conjugate (Sigma; A-5420) diluted 1 in 1000 in PBST. Plates were washed three times with PBST and once with PBS, before bound antibody was visualised with TMB substrate as described previously.

Antigen characterisation by heat treatment

6ml of immunogen was added to three 15ml falcon tubes and placed in a boiling water bath. At 10min intervals, 1ml was removed from each falcon tube and the samples centrifuged before analysing the supernatant by PTA-ELISA.

Antigen characterisation by periodate oxidation

Wells containing immobilised immunogen were incubated with 50µl of sodium *meta*-periodate solution (20mM $NaIO_4$ in 50mM sodium acetate buffer [pH4.5]) at 4°C in sealed plastic bags. Plates were given four, 3min PBS washes before processing by PTA-ELISA as described previously.

Antigen characterisation by protease digestion

Wells containing immobilised immunogen were incubated with 50µl of pronase (protease XIV (Sigma; P2143); 9mg/ml in PBS) or trypsin (T7168)(1mg/ml in Milli-Q H_2O) solution for 4h at 37°C or 4°C. Plates were given four, 3min rinses with PBS. Treated wells were assayed by PTA-ELISA as described previously.

PAGE and Western blotting

SDS-PAGE was carried out using 4-20% (w/v) gradient polyacrylamide gels under denaturing conditions. Surface washings of *T. asahii* var. *asahii* CBS 8972, *T. asteroides* CBS 6183 and *T. inkin* CBS 7630 cultures on PDA slants were taken every 24h after inoculation, using 3ml of sterile Milli-Q water in place of PBS. The supernatant was added to eppendorfs and frozen for storage until use. At the time of use, samples were thawed, vortexed and centrifuged for 5mins at 14000rpm to precipitate cells and hyphae. The supernatants, containing soluble antigens were denatured by mixing with Laemmli buffer [29]

and heating at 95°C for 10mins in the presence of β-mercaptoethanol, prior to gel loading. Proteins were separated for 1.25h at 23°C (165V). Pre-stained, broad-range markers (Bio-Rad Laboratories Limited, Hemel Hempstead, UK) were used for molecular mass determination.

For western blotting, separated proteins were transferred electrophoretically on to a polyvinylidene difluoride (PVDF) membrane for 2h at 75V. Membranes were washed three times with PBS and then blocked for 16h at 4°C, with PBS containing 1% (w/v) bovine serum albumin (BSA). Blocked membranes were incubated with MAb supernatant, (diluted 1 in 2 with PBS containing 0.5% [w/v] BSA (PBSA)), for 2h at 23°C. After washing three times with PBS, membranes were incubated for 1h with goat anti-mouse alkaline phosphatase conjugate, diluted 1 in 15,000 in PBSA (either IgM µ-chain specific, Sigma; A9688 or IgG whole molecule, Sigma; A3562, depending on determined isotype). Membranes were washed three times with PBS, once with PBST and bound antibody visualised by incubation in substrate solution [26]. Reactions were stopped by immersing membranes in dH_2O and membranes were then air dried between sheets of Whatman filter paper.

Immunofluorescence

Sterilised slides were coated with a yeast cell suspension containing 1% w/v glucose and incubated at 26°C for 16h. After air drying, the slides were fixed in fixative solution (ethanol/chloroform/3% paraformaldehyde solution 6:3:1 by volume) for 3mins, followed by immersion in 95% methanol for 4mins and washed once in dH_2O before air drying. Hybridoma supernatant was added to the slides and incubated for 1h, followed by three, 5min PBS washes. Slides were then incubated with goat anti-mouse polyvalent fluorescein isothiocyanate

conjugate (diluted 1 in 40 in PBS)(Sigma; F1010) for 30mins. Slides were given 3x5min washes with PBS and mounted in PBS-glycerol mounting medium before overlaying with coverslips. Slides were examined using a fluorescence microscope (Olympus IX81), under UV. All incubation steps were performed at 23°C in a humid environment, to prevent evaporation. Slides were stored in the dark, to prevent bleaching, at 4°C.

Mixed yeast culture screen

A yeast inoculating loop was used to inoculate SDA plates with the surface washing suspension from slant cultures and grown at 26°C. After 24h, surface washings of the plates were taken using 10ml PBS and samples were processed by PTA-ELISA.

RESULTS

Production of hybridoma cell lines and isotyping of MAbs

Two fusions were performed and 1284 hybridoma cell lines were screened for MAb production by PTA-ELISA. Two of the MAbs (CA7 and TH1) were selected for further testing on the basis of their high absorbance values (A_{450} 0.400 and A_{450} 1.122 respectively). The cell line CA7 was sub-cloned twice and TH1 sub-cloned three times. Isotyping of the MAbs showed that CA7 belongs to immunoglobin class G1 (IgG1) and TH1 to immunoglobin class M (IgM).

MAb specificity tests

In secondary specificity screening tests, CA7 and TH1 reacted strongly in PTA-ELISA against surface antigens from *T. asahii* and *T. asteroides*. Even though CA7 failed to recognise *T. asahii* var. *asahii* CBS 5286, TH1 gave a positive absorbance value (Abs_{450} >0.100) for this strain. Together the MAbs gave a combined positive reaction with all strains tested of the two species (FIG. 1.).

The antibodies did not react with surface antigens from other *Trichosporon* species or a broad range of other clinically important yeasts and moulds such as *Candida, Cryptococcus* and *Aspergillus* species [30-32]. The MAbs were also tested against other emerging invasive pathogens such as *Rhodotorula* [19, 33], and the other causative agent of white piedra (folliculitis) *Malassezia furfur* [34]. Neither MAb reacted with surface antigens from these species.

FIG. 1.

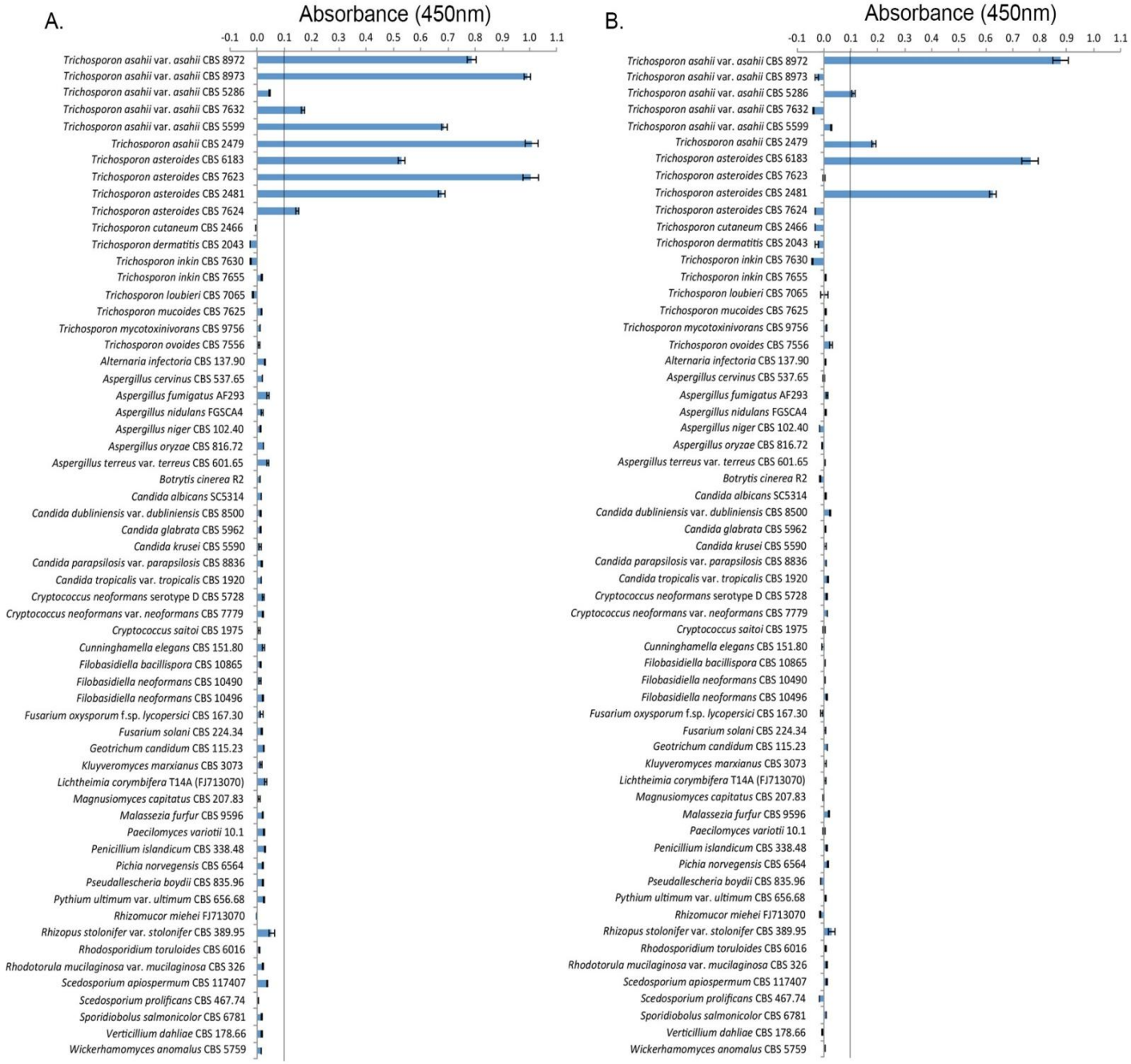


FIG. 1. Absorbance values from secondary specificity screening of MAbs against surface washing antigens. (A) CA7 (B) TH1 by PTA-ELISA, against surface washing antigens. Each bar represents the average of 8 technical replicates with the average TCM values subtracted. Threshold for positive value = +0.100 (shown by line on graph) and error bars shown = ±standard error.

Characterisation of antigen

The *T. asahii* var. *asahii* CBS 8972 freeze dried antigen was subjected to different treatments including heat treatment (FIG. 2G) or periodate, pronase and trypsin treatments (FIGS. 2A-F).

FIG. 2.

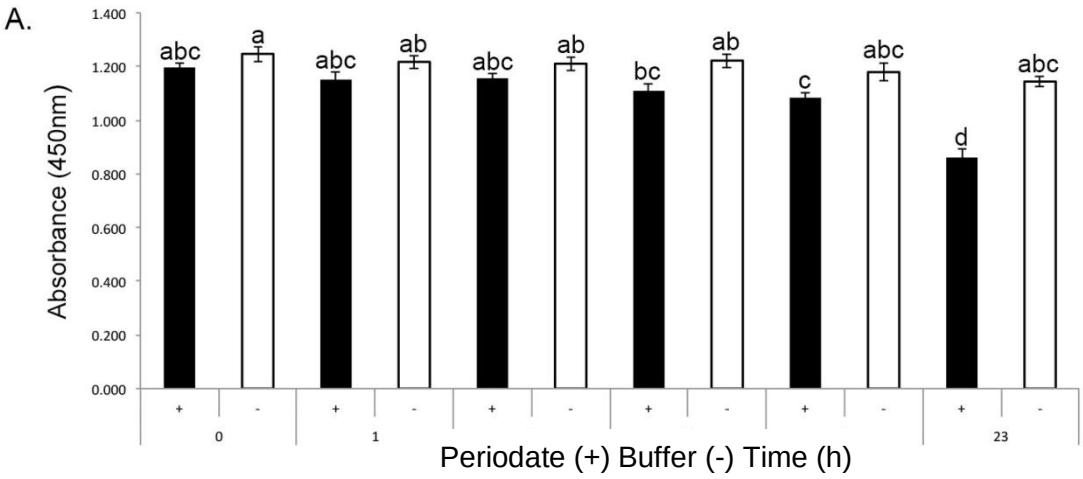
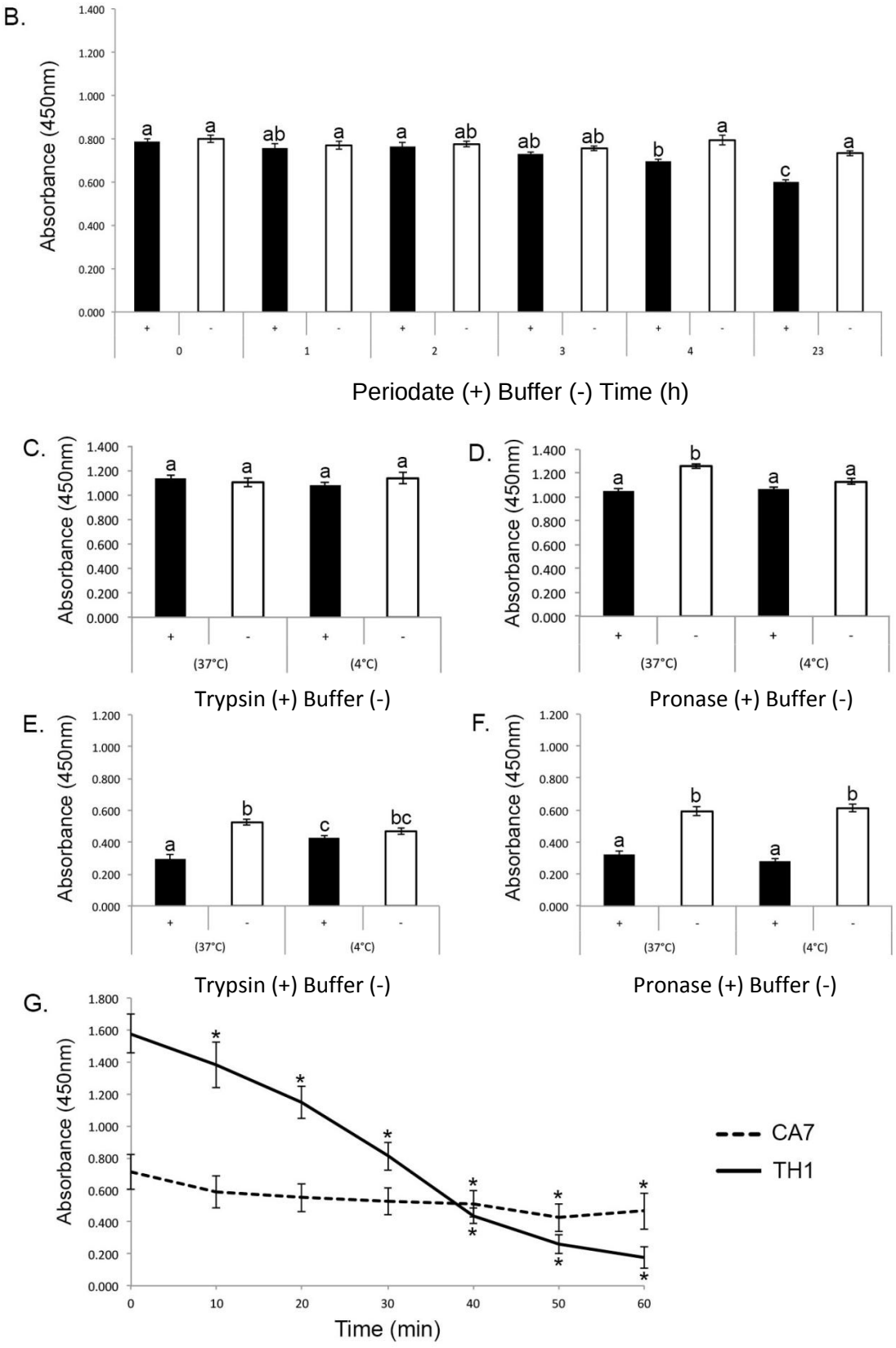


FIG. 2. Absorbance of MAb binding against treated antigen. (A) TH1 and (B) CA7 against periodate treated antigen = ■ , treatment time shown in hours, each bar represents the average of 8 technical replicates; (C) TH1 and (E) CA7 against trypsin treated antigen =■ , (D) TH1 and (F) CA7 against pronase treated antigen =■ , each bar represents the average of 12 technical replicates; respective buffer solutions = □ ; (G) 100°C, each point shown represents the average of 3 biological replicates (each with 4 technical replicates) with average TCM values subtracted. (A) TH1, one-way ANOVA (F(11,84) = 17.12, p= <0.001); (B) CA7, one-way ANOVA (F(11,84) = 14.02, p= <0.001); (C) TH1, one-way ANOVA (F(3,44) = 0.62, P=0.607); (D) TH1, one-way ANOVA (F(3,44) = 19.21, p<0.001); (E) CA7, one-way ANOVA (F (3,44) = 23.97, p<0.001); (F) CA7, one-way ANOVA (F (3,44) = 57.16, p<0.001); Tukey-kramer: results that do not share a letter are significantly different; (G) TH1, one-way ANOVA (F(6,77) = 163.54, p<0.000); CA7, one-way ANOVA (F(6,77) = 4.39, p=0.001); Tukey-kramer: asterisks show significant decrease compared to time point zero. Error bars shown = ±standard error

FIG. 2. Continued.



Following 40mins, or longer, of boiling treatment of the antigen, CA7 binding ability to the antigen was significantly reduced compared to no heat treatment ($p<0.001$) (FIG. 2G). CA7 binding to the antigen was also statistically significantly reduced ($p<0.001$) following periodate treatment for 4 and 23h (FIG. 2B), pronase treatment at 37°C and 4 °C (FIG. 2F) and trypsin treatment at 37°C (FIG. 2E) compared to treatment with the respective buffer solutions.

TH1 binding to the antigen was also significantly reduced following boiling treatment of the antigen after 10mins, compared to no boiling treatment (FIG. 2G), periodate treatment for 23h (FIG. 2A) and pronase treatment at 37°C (FIG. 2D), when compared to the controls ($p<0.001$). Trypsin treatment of the antigen did not significantly reduce TH1 binding (FIG. 2C).

PAGE and Western blotting

Surface washings of fungal slant cultures were taken every 24h and the samples used for SDS-PAGE and western blotting studies.

CA7 reacted to immuno-reactive glycoprotein antigen(s) with molecular weights in the region 48-74KDa (FIG. 3A). TH1 did not bind to an immuno-reactive antigen in western blotting studies.

FIG. 3.

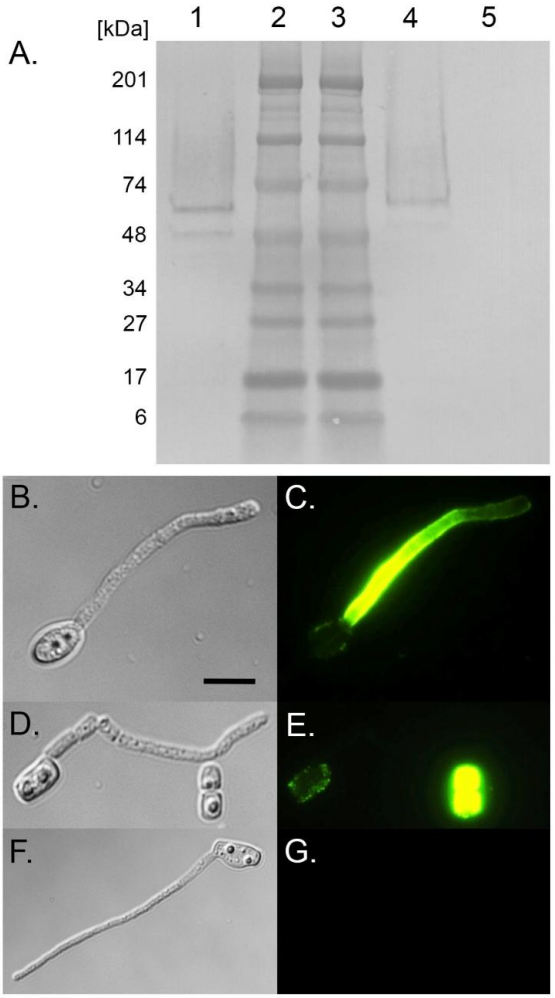


FIG. 3. (A) Analysis of antigen by PAGE and Western Blotting. Lane 1, Western immunoblot with MAb CA7 after separation of antigen from *T. asteroides* (72h of growth), by sodium dodecyl sulphate PAGE under reducing conditions; the well was loaded with 40µl of sample; Lanes 2 and 3, molecular mass marker; Lane 4, Western immunoblot with MAb CA7 after separation of antigen from *T. asahii* (96h of growth), by sodium dodecyl sulphate PAGE under reducing conditions; the well was loaded with 40µl of sample; Lane 5, Western immunoblot with MAb CA7 after separation of antigen from *T. inkin* (96h of growth), by sodium dodecyl sulphate PAGE under reducing conditions; the well was loaded with 40µl of sample. (B-G) Photomicrographs of *T. asahii* var. *asahii* CBS 8972 immunostained with MAb: (B, C) CA7, (D, E) TH1, (F, G) TCM as negative control and anti-mouse polyvalent Ig fluorescein isothiocyanate. (B) conidium with hyphae examined under a brightfield microscope. (C) Same field of view in panel B but examined under fluorescence, note the staining of hyphae and not conidia. (D) conidium with hyphae and ungerminated conidia examined under a brightfield microscope. (E) Same field of view in panel D but examined under fluorescence, note staining of conidia not hyphae. (E) conidium with hyphae examined under a brightfield microscope. (F) Same field of view in panel E but examined under fluorescence, no staining seen in negative control. Bar, 8µm.

Immunofluorescence

T. asahii var. *asahii* CBS 8972 cells were cultured for 16h on slides in sterile 1% (w/v) glucose solution. The slides were processed for immunofluorescence and viewed at x100 under bright field and immunofluorescence microscopy.

Immunofluorescence studies showed that CA7 binding was specific to the surface of hyphae (FIG. 3C), whereas TH1 binding was specific to the surface of conidia (FIG. 3E).

Mixed yeast culture screen

Yeasts were cultured on SDA plates for 24h (FIG. 4A) before surface washings were prepared and tested by PTA-ELISA using both MAbs (FIG. 4B&C). Monoclonal antibodies CA7 (FIG. 4B) and TH1 (FIG. 4C) were highly accurate in detecting *T. asahii* var. *asahii* CBS 8972 both when grown individually, and when grown on plates containing mixed populations of *C. albicans* SC 5314 and *C. neoformans* var. *neoformans* CBS 7779. The MAbs did not cross react with *C. albicans* or *C. neoformans*, when grown either as single species or as mixed populations.

FIG.4.

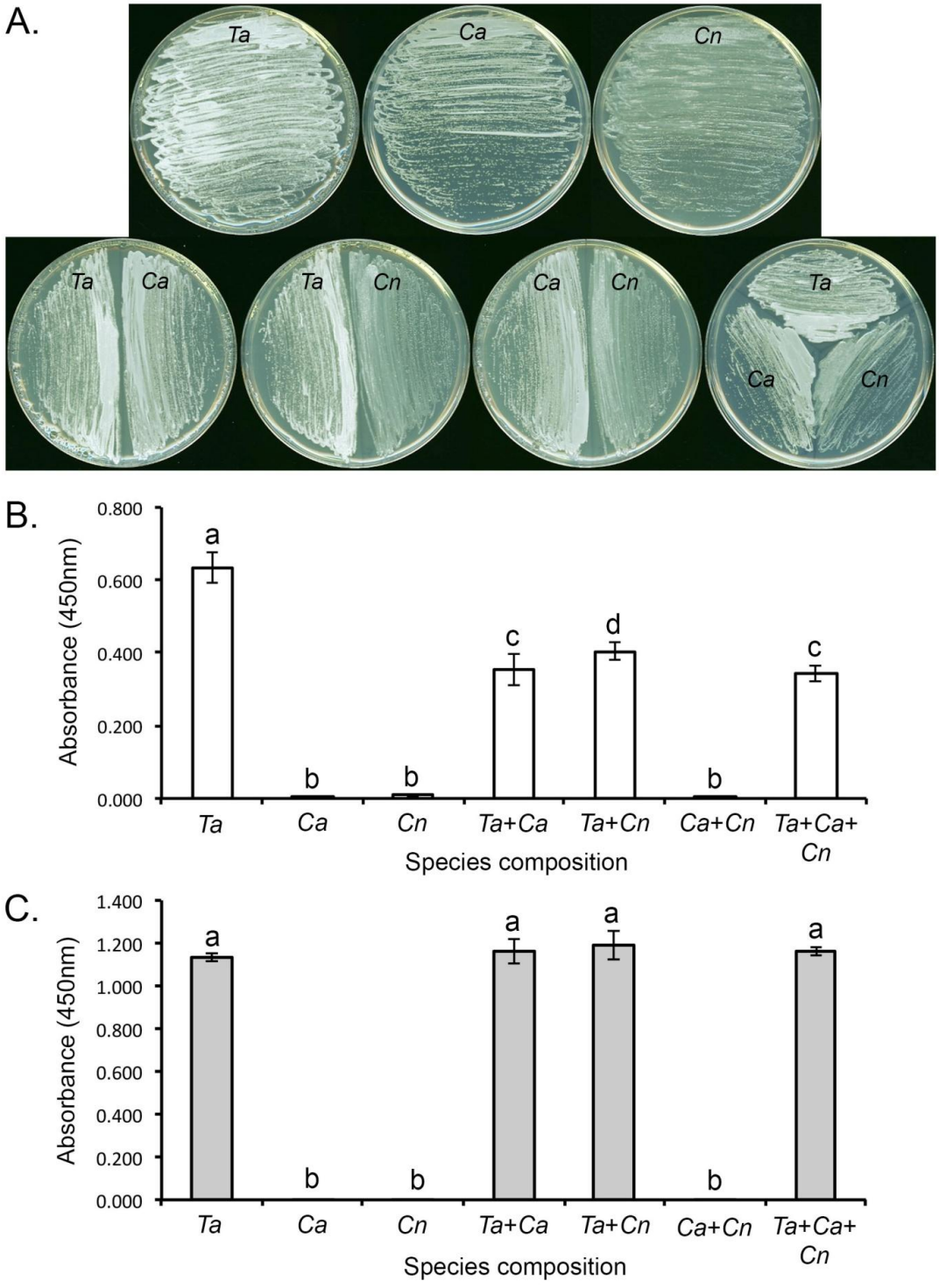


FIG. 4 (A) SDA plates inoculated with species in sections; Ta indicates *T. asahii* var. *asahii* CBS 8972, Ca indicates *C. albicans* SC 5314 and Cn indicates *C. neoformans* var. *neoformans* CBS 7779. (B) CA7 and (C) TH1 absorbance values after surface washings of SDA plates processed by ELISA, each bar shown represents the average of 3 biological replicates (each with 6 technical replicates) with average TCM values subtracted. CA7, one-way ANOVA (F (6,119) = 504.90, $p<0.001$). TH1, one-way ANOVA (F (6,119) = 1078.57, $p<0.001$). Tukey-kramer: results that do not share a letter are significantly different and error bars shown =± standard error.

DISCUSSION

This thesis describes the production of two murine MAbs, CA7 and TH1, raised against surface antigens from *Trichosporon asahii* var. *asahii* CBS 8972.

Specificity screening

Specificity screening showed that both MAbs reacted only with antigens from the species *Trichosporon asahii* and *Trichosporon asteroides.* CA7 gave a positive absorbance value (higher than the detection threshold of Abs_{450} 0.100 following correction for TCM negative controls) by PTA-ELISA for all *T. asahii* and *T. asteroides* strains tested, with the exception of *T. asahii* var. *asahii* CBS 5286. TH1 reacted positively for 5 of the 10 strains tested, including *T. asahii* var. *asahii* CBS 5286. When combined in ELISA tests, the MAbs were able to detect all of the isolates of *T. asahii* and *T. asteroides* tested.

Trichosporon asahii is the principal etiologic agent of trichosporonosis in humans [4]. While *T. asteroides* was originally described as a pathogenic agent of superficial infections [35], it's prevalence as an agent of disseminated infection is increasing, since the first recorded case of *T. asteroides* trichosporonosis in 2002 [20]. Currently, the species is reported to be second only to *T. asahii* as the cause of trichosporonosis in immunocompromised patients [13]. Consequently, MAbs CA7 and TH1 represent useful diagnostic reagents for the immunodetection of the most important agents of this disease.

Recognition of these two species, but lack of recognition of the other *Trichosporon* species tested, may be explained by their close phylogenetic relatedness. Phylogenetic studies of the *Trichosporon* genus have shown *Trichosporon asahii* and *Trichosporon asteroides* belong to a distinct clade, clade ovoides [1, 13], and are phylogenetically very similar [36]. Comparisons of

their ITS (internal transcribed spacer) regions show 98.7% similarity and for the intergenic spacer 1 (IGS1), 75.1% similarity [13]. When the mitochondrial cytochrome *b* genes of the species are compared, DNA sequences are synonymous and differ by only 4.0%, meaning that the two species contain proteins with identical amino acid sequences [35].

Misdiagnosing invasive trichosporonosis as candidiasis is an important limitation to timely treatment of patients with appropriate antifungal drugs [8, 20]. Further confusion to species identification exists with the cryptococcal latex agglutination test, used for the diagnosis of *Cryptococcus* infections, whereby the cryptococcus test has been documented to cross react with *Trichosporon* [17, 22]. *Trichosporon* and *Cryptococcus* both secrete glucuronoxylomannans (GXM) and common domains are shared between the polysaccharides of the two species, forming antigens detected by the test [18]. Cryptococcal meningitis is described as an AIDS defining illness [33, 37] and the most common cause of adult meningitis [38, 39]. Although *Cryptococcus neoformans* is the species most frequently detected in meningitis patients, other species are isolated, including emergent human pathogens such as *Trichosporon* and *Rhodotorula* [33].

The activity of fungicides varies against different pathogenic fungi [39-42] highlighting the importance of being able to distinguish the causal agent of invasive infections (for example *Trichosporon, Candida* and *Cryptococcus*) so that the most appropriate therapeutic agent can be selected. It was therefore important in this study to ensure that both CA7 and TH1 did not cross react with surface antigens from *Cryptococcus* and *Candida* species, which was confirmed by secondary specificity screening tests.

The MAbs were screened against other clinically important species and emerging human pathogens that may be found in the same environment, all of which were not recognised by either MAb, demonstrating the high specificity of CA7 and TH1.

Antigen characterisation

Antigen characterisation indicated that CA7 and TH1 bound to antigen(s) containing both carbohydrate and protein components.

Treatment of the antigens with periodate prior to processing by PTA-ELISA significantly reduced both CA7 and TH1 binding after 4 and 23h respectively, indicating the presence of vicinal hydroxyl groups on carbohydrate moieties [27].

Reduction in the binding of both MAbs to the antigen(s) after treatment with pronase demonstrated the presence of protein components in both of the antigen(s) recognised. More specifically, the reduction in CA7 binding following trypsin treatment at 37°C indicated that the antigen bound by CA7 contains positively charged lysine and arginine side chains [24]. Reduced binding ability of a MAb to an antigen following heat treatment indicates the presence of heat-labile protein components [43]. Antigen recognition by CA7 and TH1 was significantly reduced after 40 and 10mins of boiling respectively, further indicating the presence of protein components in the antigen(s).

Western Blotting studies showed that CA7 bound to a major glycoprotein antigen with a molecular weight of approximately 60KDa. The carbohydrate components of the glycoprotein may be providing heat stability to the antigen(s) structure and explain why, despite being significantly reduced, the MAb produced an ELISA absorbance value above the positive threshold value, even

after 1h of boiling the antigen. The antigen recognised by TH1 was shown to be heat labile, significantly reducing TH1 binding after only 10 minutes of boiling of the antigen. Heating the antigen at 95°C during the denaturing step for SDS-PAGE may have caused the same reduction in binding ability and explain why TH1 did not bind to an immuno-reactive antigen during western blotting studies.

Immunofluorescence studies showed that the MAbs recognise antigens present on different morphological structures of *Trichosporon asahii*. CA7 binding was specific to antigen(s) present on the surface of hyphae, whereas TH1 binding was specific to the surface of conidia. Importantly, in combination, the MAbs are able to recognise both yeast and filamentous forms, as these different growth stages of the pathogen are implicated in tissue invasion of disseminated infections [14]. The different morphologies have also been seen in the formation of plaques [44] which, as previously mentioned, are an important risk factor associated with invasive trichosporonosis and, when present on devices such as catheters, allows the fungus to evade treatment with fungicides [9].

Mixed yeast culture screen

The increasing problem of patients with fungemia caused by mixed species infections and diagnosis of causative agents from mixed yeast cultures gave motivation to test the MAbs in mixed culture conditions. *Trichosporon asahii* var. *asahii* CBS 8972, *Candida albicans* SC 5314 and *Cryptococcus neoformans* var. *neoformans* CBS 7779 were cultured on the standard mycological growth medium SDA. Some mycological laboratories favour the use of selective media such as CHROMagar Candida, designed to aid identification and differentiation of yeasts by the presence of chomogenic substrates in the media that react with

species specific enzymes producing coloured colonies [45]. However limitations of CHROMagar media exist, for example the media struggles to detect mixed yeast cultures if one species is significantly more prevalent than others showing only the colour of the dominant phenotype [23] and, although a useful tool, the medium is recommended for use in combination with other culture media for complete isolation of species [21]. Consequently, it remains a presumptive test requiring further mycological analysis for definite species identification [23, 45, 46].

To avoid species competition, the yeasts were plated in defined sectors in SDA plate cultures. The results show that both MAbs correctly recognised the presence of *T. asahii* when grown individually and in combination with *C. albicans* and *C. neoformans.* CA7 absorbance values were statistically significantly reduced when grown in combination with the other yeasts, however these values were still well above the threshold positive value for detection. Identification of *T. asahii* was completed 3 days after inoculation of the SDA plates, following 24 h of growth and 2 days of processing the samples by PTA-ELISA. This provided a short detection time and definitive differentiation of *T. asahii* from both *C. albicans* and *C. neoformans*.

Building on this study, it will be important to test the MAbs against samples of yeasts grown with cells mixed together, rather than separated on the plate, and at varying ratios to one another. This would more accurately replicate cultures that would be obtained from patient samples suffering from co-infections.

It is not known at present if antigenic markers from *T. asahii* and *T. asteroides* could be detected during human fungemia by using serum samples or for SHP detection using bronchoalveolar lavage fluid (BALf). If they are

present in such samples then there is potential for development of the MAbs into point-of-care tests that can be used directly with human samples, rather than after culture of the pathogen. For example, lateral flow devices (LFD's), which have already been shown to have application in the detection of disseminated invasive fungemia, for instance, using serum and BALf samples for the detection of invasive aspergillosis [24, 47]. Use of BALf has also been demonstrated in the diagnosis of SHP [48] through the detection of fungal DNA [12], and might also be used for the LFD detection of *T. asahii* diagnostic antigens in SHP patients. A major advantage of using LFD's as immunoassays is the speed at which a result can be obtained [47, 49, 50], furthermore the portable assays can be performed at the site of patient care, further reducing sample analysis time. In addition, because of the simplicity of the assay format, they require less training in comparison to many laboratory based assays and using samples such as serum removes the need for invasive sampling procedures [24, 49, 51].

It is important to note that while the combination of the MAbs here detect the two most common causes of invasive trichosporonosis (*T. asahii* and *T. asteroides*) and the most common agent of SHP (*T. asahii*), the other species implicated in superficial and invasive infections (a continually expanding list including *T. inkin* and *T. cutaneum* [4, 52]) and SHP (currently the only other member of the genus noted as a causative agent is *T. mucoides* [11, 12]) would require differentiation with PCR sequencing of DNA sequences that have sufficient species differences such as the IGS1 region.

CONCLUSION

Hybridoma technology was used to produce two murine MAbs which react with antigens from *T. asahii* and *T. asteroides*, the principal agents of superficial and invasive infections in humans and of SHP. The MAbs, CA7 and TH1, bind to antigens present on the surface of hyphae and conidia respectively and can distinguish *T. asahii* from other yeasts when grown in mixed species cultures. There is potential for further development of the MAbs into diagnostic immunoassays of trichosporonosis and SHP.

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