

RESEARCH ARTICLE

Humoral immune responses to *Candida albicans* complement receptor 3-related protein in the atopic subjects with vulvovaginal candidiasis. Novel sensitive marker for *Candida* infection

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One sentence summary: Novel *Candida*-derived antigen exerts immunobiological efficacy as a prospective sensitive diagnostic tool for serodiagnosis of *Candida* infections.

Editor: Richard Calderone

ABSTRACT

In vitro evaluation of specific anti-*Candida albicans* sera antibodies based on synthetically prepared complement receptor 3-related protein (CR3-RP) mimicking the structure of native complement receptor 3 in a cohort of 72 patients with atopy and recurrent *Candida* vulvovaginitis (RVC) revealed effective humoral response against *Candida* CR3-RP. The most significant have been IgM and IgA isotype antibodies (33 and 47% positive cases, respectively). The quantitative evaluation of anti-CR3RP isotype antibodies was confronted with results of commercial ELISA anti-*C. albicans* antibodies diagnostics based on *C. albicans* cell wall mannan and β -glucan antigens, the most significant correlation being observed with anti-CR3-RP IgM and anti- β -D-glucan IgM ($r^2 = 0.624$) followed by isotype IgA ($r^2 = 0.381$). The immunogenicity and immunoreactivity of CR3RP antigen in RVC patients' sera had been evaluated with regard to the results reached by counterimmunoelectrophoresis and heterogeneous enzyme immunoassay. Obviously, synthetically prepared CR3-RP mimicking the *Candida* cell-wall-derived structure moiety represents a promising immunological tool not only for *Candida* serodiagnostics, but also prospectively for follow-up of targeted antifungal therapy and as promising *Candida* vaccine candidate.

Key words: *Candida albicans*; CR3-related protein; recurrent vulvovaginal candidiasis; mannan; glucan

INTRODUCTION

Vulvovaginal candidiasis is one of the most frequent reasons of female genital tract infections, which affects approximately

70–75% of childbearing age women at least once throughout their lifetime. About half of them will suffer a second exacerbation and approximately 5% of cases will develop a recurrent vulvovaginal candidiasis (RVC), frequently accompanied by the

Received: 21 July 2014; Accepted: 30 October 2014

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refractory episodes (approximately three to four times per year) (Ferrer 2000). The prevalence increases with the age up to menopause. Predisposing factors for developing of vulvovaginal candidiasis include e.g. active sexual life, antibiotic and oral contraceptive usage, estrogen therapy, pregnancy, diabetes mellitus, HIV, systemic immunosuppression, atopy, deteriorated local cell immunity, etc. (Magliani et al., 2002; Cassone, De Bernardis and Santoni 2007; Xu et al., 2008; Fisher 2012). *Candida albicans* is the major etiological agent of vulvovaginal candidiasis with 95% prevalence in genital candidiasis (Ferrer 2000; Calderone and Gow 2002). Approximately 15–30% of RVC cases are due to non-*C. albicans* spp. The most frequently isolated spp. are *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* (Ferrer 2000; Sobel 2003; Diba et al., 2012; Faraji et al., 2012; Mendling and Brasch 2012; Hamad et al., 2014). *Candida glabrata* is the second most abundant species, causing approximately 5–15% of cases of vaginal candidiasis, particularly recurrent cases (Ferrer 2000; Diba et al., 2012). In rare cases, *Saccharomyces cerevisiae* also causes a vulvovaginitis, often with fewer clinical signs and symptoms (Sojakova et al., 2004; Ventolini and Baggish 2006; Mendling and Brasch 2012).

The mechanism by which commensal *Candida* can become an opportunistic pathogen i.e. a switch between saprophytic and pathogenic behavior is linked to blastospore germination, mycelium or hyphae development, adhesion to host constituents and proteinase secretion (Calderone and Fonzi 2001; Romani 2002; Mayer, Wilson and Hube 2013).

The protein and glycan constituents of *Candida* cell wall are engaged in the close intercellular interactions between the fungus and the host immune cells (Calderone and Gow 2002; Chauhan et al., 2002; Netea et al., 2006; Romani 2011; Gauglitz et al., 2012; Hall et al., 2013). These structural moieties representing candidal pathogen-associated molecular patterns (PAMPs) are recognized by several unique soluble or membrane-bound pattern recognition receptors (PRR) as mannose receptor, DC-SIGN, SIGN-R1, galectin-3, Dectin-1, Dectin-2, TLR2, TLR4, TLR6, Mincle, complement receptor 3 (CR3), scavenger receptors CD5, CD36, SCARF1, etc. (Poulain and Joault 2004; Ladgé 2010; Perez-Garcia et al., 2011; Romani 2011; Cunha et al., 2012; Gauglitz et al., 2012; Hardison and Brown 2012; Ifrim et al., 2013).

Immune evasion of *Candida* is supported by a variety of cell surface molecules expressed by *Candida*, helping it to evade the host immune response by mimicry of host receptors (Bujdaková et al., 1997; Phan et al., 2007; Zipfel et al., 2011; Luo et al., 2013).

The *Candida* complement receptor 3-related protein (CR3-RP) the member of the DING protein family (Bernier 2013) has been described to be a 'mimicry' antigen, functionally comparable with the human CR3 (Mac-1, CD11b/CD18), which is expressed on the surface of neutrophils, macrophages and monocytes, with the ability to bind the human complement fragment iC3b and to mediate leukocyte diapedesis (Beller, Springer and Schreiber 1982; Todd and Petty 1997; Xia and Ross 1999). The fragment of the CR3-RP has already been purified and sequenced (DINGGGATLPG) and its DING motif corresponds to widely spread ubiquitous DING protein family (Bujdaková et al., 2008). The DING proteins have already been mentioned in prokaryotic as well as in high eukaryotic organisms (Berna et al., 2009; Bernier 2013). The CR3-RP has been reported to be a surface antigen participating in adherence to buccal epithelial cells as well as in *in vitro* biofilms (Bujdaková et al., 2010). CR3-RP-protein conjugate effectively induce antibody and cell-mediated immune responses in rabbits following prime-boost vaccination (Paulovičová et al., 2008).

The present study reveals for the first time the bioimmunological activities of novel synthetically prepared CR3-RP mimicking the structure and function of native complement receptor 3, and points out the opportunity of this structure to be exploited in the diagnostics of human candidiasis.

MATERIALS AND METHODS

Study subjects

Seventy-two female atopic subjects (34.6 ± 12.2 yr) with microbiologically proven *Candida* infection together with clinical observation and history of RVC were enrolled in the study. Primary exclusion criteria were recent or ongoing antibiotic or immunosuppressive therapy. *Candida* spp. isolated from vaginal (92.61%) or cervical (7.39%) swabs undergo typing and identification (HPL Laboratories, Bratislava, Slovakia). Skin prick test (SPT), including *C. albicans* allergen (Alyostal® Stallergenes), was done on the patients' forearm according to the international and national guidelines. SPT was evaluated after 15–20 min and rated as positive if the wheal diameter was ≥ 3 mm and the negative control was negative.

Control group

Sixty-five female blood donors (National Blood Service, Slovak Republic) aged 18–56 yr (average 35.9 ± 18.6) without allergic diseases were enrolled as healthy control subjects.

Material

Sera samples have been taken before onset of anti-mycotic and/or immunomodulative therapy, respectively. The samples for determinations of specific anti-CR3-RP, anti-mannan and anti-glucan immunoglobulins' levels were collected and immediately stored at -70°C . The specimens were analysed retrospectively and results had no influence on therapeutic decisions.

Ethics

The study protocol has been approved by the Local Ethical Committees of Oncology Institute of St. Elisabeth, Bratislava, Slovakia, and Faculty Hospital of Comenius University in Bratislava, Slovakia. Written, informed consent for blood collection and subsequent laboratory examinations, in accordance with the principles of the Helsinki Declaration, has been obtained from each patient before enrolment in the study. Patient age, disease process, drug history, family history and clinical signs and symptoms were documented at the first visit.

Standard strains and clinical *Candida* isolates

In this study, the standard strains *C. albicans* CCY 29-3-162 and *C. albicans* CCY 29-3-32 (serotype A) (CCY Culture Collection of Yeasts, Chemical Institute, Slovak Academy of Sciences, Slovakia) were used. Originally, both strains were recovered from patients with RVC. *Candida albicans* CCY 29-3-162 was selected because of its high CR3-RP expression (Bujdaková et al., 2008). Additionally, clinical isolates *C. glabrata* 12889/1, *C. krusei* 8989 and *S. cerevisiae* 21755 originating also from patients with vulvovaginal candidiasis were tested for the expression of the CR3-RP employing SDS-PAGE, Western blotting and ELISA.

Determination of the CR3-RP in yeast crude lysates—SDS-PAGE, Western blotting and ELISA

The crude protein extracts of tested strains (approximately 1 mL of each) were obtained from 20 mL of the overnight yeast suspension (10^7 /mL) by glass beads (diameter 2 mm, SIGMA-Aldrich, USA) breaking in breaking buffer (20% glycerol, 0.1 M Tris HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, all from Sigma-Aldrich, USA). Cells were disrupted and crude extracts were prepared as it was described by Bujdaková et al. (2008). Protein lysates of *C. albicans*, *C. glabrata*, *C. krusei* and *S. cerevisiae* were separated by SDS-PAGE in a 12.5% polyacrylamide gel (Applichem, Germany) followed by blotting to nitrocellulose membrane (Serva, Germany) as it was previously described (Bujdaková et al., 2008). The level of CR3-RP expression in different *Candida* spp. was determined by enzyme-linked immunosorbent assay (ELISA, Voller, 1978) in 96-well microtitre plates (Sarstedt, Germany). In Western blotting as well as in ELISA, as the primary antibody, polyclonal anti-CR3-RP antibody was applied in the dilution 1:100 in phosphate buffered saline (PBS) (Bujdaková et al., 2008). For ELISA, 10 µg of appropriate protein lysate was applied to well, coated with the coating buffer (0.1 M NaHCO₃, pH 9.6) to final volume 200 µL, and incubated overnight at 4°C. After one washing step, non-specific binding was blocked by 1-h incubation in 1% (w/v) gelatin (Applichem, Germany) in PBS supplemented with 0.05% (v/v) Tween 20 (Sigma-Aldrich, USA). After three washing steps with PBS-Tween 20 (0.05%, v/v), goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, USA) was added in final dilution of 1:30 000 and the plate was incubated for 1 h at room temperature. After four additional washing steps, alkaline phosphatase substrate containing p-nitrophenylphosphate (pNPP, Sigma-Aldrich, USA) was used for development. The reaction was stopped by 3 M NaOH and evaluated at 490 nm using a microplate reader (MRX II, Dynex, USA). Results were calculated as mean value $\pm$ SD from five parallel wells and two independent experiments.

Preparation of CR3-RP

The synthetic peptide DINGGGATLPQAL corresponding to the 13 amino acids (KL Ross Petersen, ApS, Chemical Research and development Laboratory, Denmark) was designed on the CR3-RP sequence already published by Bujdaková et al. (2008). Peptide was synthesized as white powder with high purity (HPLC, $\geq 95\%$). N-terminal sequence of this protein has been indexed in the UniProt Knowledgebase under accession number P85437.

Counterimmunoelectrophoresis (immunoelectrosyneresis)

The qualitative detection of anti-*Candida* antibodies and anti-CR3-RP antibodies was performed via counterimmunoelectrophoresis/immunoelectrosyneresis of patient sera using CR3-RP antigen (0.4 µg/well) and *C. albicans* metabolic and somatic antigens (*C. albicans* antigens, Bio-Rad Labs., CA, USA) as standard *Candida* antigens for comparison. The procedure was conducted under the conditions recommended by manufacturer.

Determination of *C. albicans*-specific IgE

Sera levels of *C. albicans*-specific IgE were determined by enzyme allergosorbent test (EAST) using commercial diagnostic kit (HyCor Biomedical, USA). The results were expressed according to international EAST classification.

Determination of total IgE

Sera levels of total IgE were determined by ELISA assay using commercial diagnostic kit for Total IgE (Biogema, Slovakia). The absorbance values have been detected at dual wavelengths 450 and 630 nm and evaluated using calibration curve based on WHO 75/502 human IgE reference serum. The results are expressed as IU mL⁻¹.

Determination of the specific anti-CR3-RP IgE

Sera levels of specific anti-CR3-RP IgE were determined by ELISA assay, based on modification of commercial diagnostic kit for Total IgE (Biogema, Slovakia). In the coating step, the synthetically prepared CR3-RP in sodium bicarbonate–sodium carbonate buffer pH 9.6 has been applied onto Immulon 4HBX microplates (Dynex, USA) (5 µg/mL, 200 µL/well) for 18 h at 4°C. After that, the plates were overcoated with 2.5 M carbonate–bicarbonate buffer with 0.025% Tween 20. The plates were blocked with 1% BSA in 0.05 M carbonate–bicarbonate buffer. Then, the sera specimens (1:100 in blocking buffer) were examined for the CR3-RP-specific IgE antibodies using peroxidase-labeled goat anti-human IgE and developed with 3,3',5,5'-tetramethylbenzidine. The absorbance values have been detected at dual wavelengths 450 and 630 nm and evaluated using heterologous interpolation against WHO 75/502 human IgE reference serum (Total IgE kit, Biogema, Slovakia).

ELISA assay for the CR3-RP IgG, IgA and IgM determination

Enzyme-linked immunosorbent assay for the determination of CR3-RP-specific IgG, IgA and IgM antibodies has been developed by modification of ELISA anti-*Candida* II based on *C. albicans* cell wall mannan and β -glucan antigens (Biogema, Slovakia). Synthetically prepared CR3-RP in sodium bicarbonate–sodium carbonate buffer pH 9.6 was applied onto Immulon 4HBX microplates (Dynex, USA) (5 µg/mL, 200 µL/well) for 18 h at 4°C. After that, the plates were overcoated with 2.5 M carbonate–bicarbonate buffer with 0.025% Tween 20. The plates were blocked with 1% BSA in 0.05 M carbonate–bicarbonate buffer. Sera samples have been examined for the CR3-RP-specific IgG, IgA and IgM antibodies with peroxidase labeled anti-human IgA, IgG and IgM antibodies (KPL, USA). The plates were developed with 3,3',5,5'-tetramethylbenzidine chromogenic substrate (Kem-En-Tec Diagnostics) and scanned at 450/630 nm (Microplate reader MRXII, Dynex, USA). Due to the absence of international standards, the appropriate concentrations of different Ig isotypic antibodies were evaluated based on calibration curve using internal standard, i.e. pool of positive sera with established value of 100 arbitrary units (U). The cut-off values were calculated according to blood donors' IgG/IgM/IgA anti-CR3-RP sera values (average + 3 SD). The patients' results are expressed as calculated mean $\pm$ standard error of means (SEM) of two independent measurements.

ELISA assay for the β -D-glucan and mannan IgG, IgA and IgM determination

Anti-*C. albicans* β -D-glucan and mannan antibodies were determined by modification of ELISA anti-*Candida* II based on *C. albicans* cell wall mannan and β -glucan antigens (Biogema, Slovakia). Both, β -D-glucan and mannan isolated from clinical isolate *C. albicans* CCY 29-3-32 were used as antigens for coating of Immulon 4HBX microplates (Dynex, USA) (5 µg/mL,

200 $\mu\text{L}/\text{well}$). The application of sera and ELISA development was the same as with CR3-RP ELISA. Due to the absence of international standards, the appropriate concentrations of different Ig isotypic antibodies were evaluated based on calibration curve using internal standard, i.e. pool of positive sera with established value of 100 arbitrary units (U). The cut-off values were calculated according to blood donors' IgG/IgM/IgA anti-CR3-RP and anti-CR3-RP sera values (average $\pm$ 3 SD). The patients' results are expressed as calculated mean $\pm$ SEM of two independent measurements.

Computational statistical analyses

Experimental results were calculated as mean values with $\pm$ SD. Normality of distribution was evaluated according to Shapiro-Wilk's test at the 0.05 level of significance. Statistical comparison between experimental groups was performed using one-way ANOVA and post hoc Bonferroni's and Tukey's tests. The results were significant if the difference between the analysed groups equaled or exceeded the 95% confidence level ($P < 0.05$).

Statistics were performed with ORIGIN 7.5 PRO software (OriginLab Corporation, Northampton, MA). Pearson's correlation coefficient (r^2) was used to compare the strength of the relationship between immunological parameters.

RESULTS AND DISCUSSION

Atopy and *Candida* vulvovaginitis

The RVC caused by *Candida* spp. is a general health problem affecting a large number of otherwise healthy women with a strong impact on a quality of their life. Moreover, clinical manifestations become worse in patients with atopy, suggesting an association between allergy and RVC (Moraes, De Lima Goiaba and Taketomi 2000; Moraes and Takenomi 2000; Hrubisko, Paulovicová E, Vargová 2003).

In our study, allergy to inhaled allergens has been confirmed by SPTs in 93% of patients, positive *C. albicans* SPT was established in 74% of study group and negative *C. albicans* SPT has been observed in the rest 26%. These *in vivo* results have been supported by *in vitro* quantitative determination of serum anti-*C. albicans* IgE, evident in 83.5% of patient cohort. The classification of sera levels of anti-*C. albicans* IgE revealed the distribution between EAST class 1 (0.35–0.7 kU/L), EAST class 2 (0.7–3.5 kU/L) and EAST class 3 (3.5–17.5 kU/L). EAST class 2 was the most abundant (45.3% of patients), followed by class 3 (33.4%) and class 1 (21.3%). Based on the tests of immediate hypersensitivity to aeroallergens, there has been observed a strong association between atopy and RVC. Recently, RVC has been linked to atopy (Moraes, De Lima Goiaba and Taketomi 2000; Moraes and Takenomi 2000; Neves et al., 2005) and few studies have associated RVC to allergy to *C. albicans* (Witkin, Jeremias and Ledger 1989; Rigg, Miller and Metzger 1990; Clancy et al., 1999). The research of Ferrer and Fisher implies that some allergic sensitization to candidal antigens may play a role in RVC (Ferrer 2000; Fisher 2012). Previous studies of Rigg and Moraes suggested that local hypersensitivity response to *Candida* in certain women with chronic vaginal candidiasis may be improved by specific immunotherapy using *C. albicans* extract (Rigg, Miller and Metzger 1990; Moraes, De Lima Goiaba and Taketomi 2000; Moraes and Takenomi 2000). In addition, our earlier results pointed out the close relation between *Candida* RVC and *Candida* sensitization based on anti-*C. albicans*-specific IgE and SPTs with *C. albicans* allergen (Hrubisko, Paulovicová E, Vargová 2003). In some cases, high levels of IgE in serum and vaginal secretions have been observed, particularly in patients with RVC. The sera

levels of anti-*Candida*-specific IgE and total IgE in recurring vulvovaginal candidosis have been evaluated (Weissenbacher et al., 2009).

In our RVC patients, the sera levels of total IgE ranged from 89.63 IU mL⁻¹ to 384.2 IU mL⁻¹ (average 127.3 $\pm$ 98.1 IU mL⁻¹), the RVC patients have 3.73 times higher sera levels of total IgE in comparison with non-allergic healthy blood donors (range: 9.97 to 77.72 IU mL⁻¹, average 34.121 $\pm$ 29.128 IU mL⁻¹) ($P < 0.05$).

Anti-*Candida* immunity

There were several attempts to characterize the predisposing factors for RVC including various gene polymorphisms as single nucleotide polymorphism in genes coding for mannose-binding lectin, IL-4, Dectin-1 receptor, CARD 9. More recently, gene variants of IL-22 have reported with RVC (Cassone 2014).

However, our previously performed study of serological profile in RVC did not reveal the mannose-binding lectin deficiency trend in sera levels of in-patient cohort (Hrubisko, Paulovicová E, Vargová 2003).

Generally, various *Candida*-derived glycan and protein PAMPs have been noticed as promising *Candida* invasion tools (Lopez-Ribot et al., 2004; Torosantucci et al., 2005, 2009; Mochon et al., 2010; Romani 2011; Casadevall and Pirofski 2012). Among them, the *Candida* PAMPs as β -glucan, mannoproteins, members of the secretory aspartyl proteinase (Sap) gene family, hyphal wall protein 1 (Hwp), hyphally regulated protein 1 (Hyr1) or agglutinin-like sequence 3 (Als3), representing *Candida* virulence factors, seem to be most relevant ones (Lain et al., 2008). Moreover, the Als3, Sap2, Hyr1, β -glucan, β -mannan, and/or β -1,2-mannotriose are the mostly studied candidates for future *Candida* vaccines against RVC (Fidel and Cutler 2011; Cassone 2014). The studies of vaginal-associated innate and acquired immunity in RVC are directed towards the role of vaginal T-cells, Th polarization, neutralizing antibodies, neutrophils, cervicovaginal epithelial cells and vaginal microbiota (Fidel 2007; Ledger and Witkin 2007; Weissenbacher 2009; Cassone 2014). However, no study revealed the exact mechanism involved in protection or susceptibility to RVC and dominant immune response against *Candida* invader.

Synthetically prepared PRR, PAMPs or PAMP-derived structures represent the new valuable, exactly defined models for detailed study of the host-*Candida* interactions, the evaluation of the cell and antibody-mediated immunities and cell signaling (Xin et al., 2008, 2012; Paulovicová et al., 2010, 2012, 2013a,b). Recently, the follow-up of anti-CR3-RP-specific polyclonal antibodies raised against synthetically prepared CR3-RP throughout the active rabbit vaccination has been revealed (Bujdaková et al., 2008). Additionally, the effective induction of cellular and humoral immunity in rabbits following immunization with CR3-RP-*Candida* mannan conjugate has been observed (Paulovicová et al., 2008). Next, the molecular-biological studies on CR3-RP mainly SDS-PAGE and Western blotting revealed the expression of this antigen foremost by *C. albicans* (Bujdaková et al., 1997). The recent results based on the tests with non-*albicans* spp. as *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lambica*, *C. parapsilosis*, *C. tropicalis*, *C. inconspicua* and non-*Candida* sp. *S. cerevisiae* also confirmed the expression of CR3-RP [Fig. 1A and B and Bujdakova, Chlupacova (unpublished results)].

Mentioned results, and previously published observations (Bujdaková et al., 1997), suggested a ubiquitous spread of the CR3-RP with the highest appearance in *C. albicans* confirmed by the results from ELISA (A_{490}) as follows: 0.78 $\pm$ 0.13 for *C. albicans* CCY 29-3-162, 0.34 $\pm$ 0.05 for *C. glabrata* 12889/1, 0.60 $\pm$

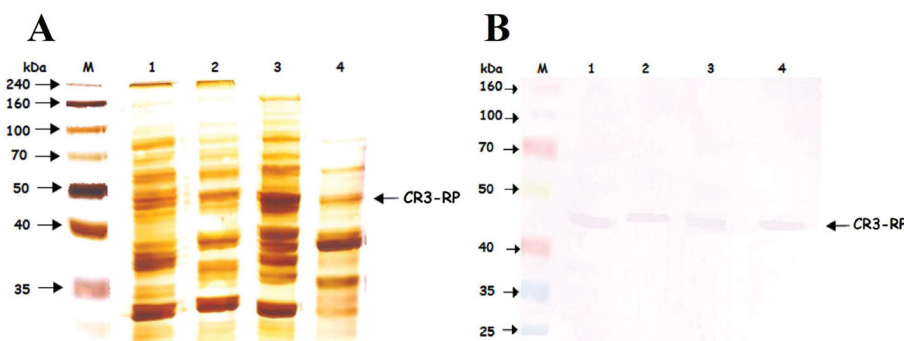


Figure 1. SDS-PAGE of the crude protein lysates (A) and Western Blotting with polyclonal anti-CR3-RP Ab (B) of different *Candida* spp. and *S. cerevisiae*. Ladder: Spectra TM multicolor broad range protein ladder; (1) *C. albicans* CCY 29–3–162; (2) *C. glabrata* 12889/1; (3) *C. krusei* 8989; (4) *S. cerevisiae* 21755.

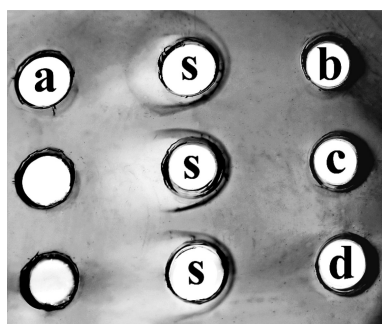


Figure 2. Detection of serum anti-*C. albicans* antibodies directed against different *Candida* antigens by counterimmunoelectrophoresis. Middle holes (s)—patients' serum (W.K. 1952), (a)—metabolic and (b)—somatic antigens (Bio-Rad), (c)—CR3-RP (0.4 µg/µL) and (d)—CR3-RP (dilution 1/100 i.e. 0.004 µg/µL).

0.05 for *C. krusei* 8989 and 0.24 ± 0.03 for *S. cerevisiae* 21 755. The epidemiological survey on causative yeasts in symptomatic women with RVC (2013, HPL Mycology Laboratory, Slovakia) revealed the 81.23% prevalence of *C. albicans*, followed by different non-*albicans* spp., namely *C. glabrata* (7.95%), *C. krusei* (2.13%) and non-*Candida* spp. such as *S. cerevisiae* (5.47%). Consequently, with regard to this survey and the ubiquitous spread of the CR3-RP, the importance of CR3-RP as promising candidosis biomarker could be assumed.

The immunobiological effectiveness of the CR3-RP in patient's sera has been evaluated using commercial *Candida* metabolic and somatic antigens as the comparative *Candida* antigens in the assay based on counterimmunoelectrophoresis (Fig. 2).

According to the manufacturer's recommendations, one precipitation arc is sufficient indication of a *C. albicans* infection. The number of precipitation arcs increases with disease spread to various organ groups. The pattern of counterimmunoelectrophoretogramme performed with serum from patient with RVC (Fig. 2) revealed the positive antibodies against metabolic and somatic (cellular) antigens, indicating the *C. albicans* infection. Using the CR3-RP as antigen under the same conditions, we have detected the precipitation arc, even if 1/100 serum dilution has been applied. Thus, it can be concluded that the serum of patient with RVC is reactive with synthetically prepared CR3-RP and thus could be used as serologic marker of *Candida* infection.

To ascertain this suggestion, we applied the anti-CR3-RP ELISA method to determine the sera levels of anti-CR3-RP IgG, IgM and IgA antibodies in the cohort of patients with RVC. Evidently, the highest concentrations of immunoglobulin isotypes have been observed with IgG, followed by IgM and IgA, respec-

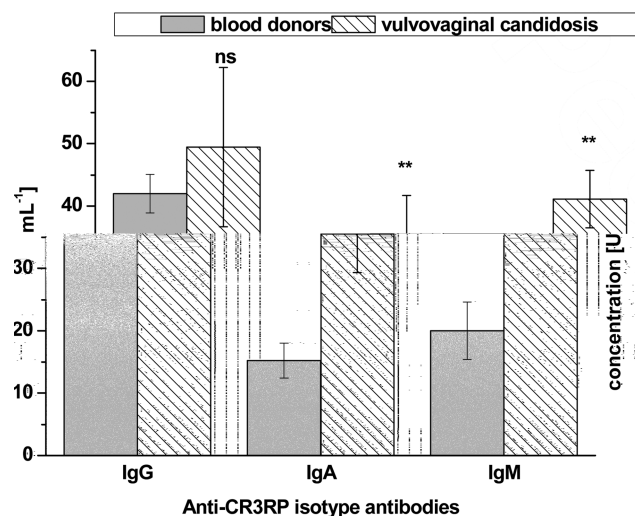


Figure 3. Distribution of sera levels of anti-CR3RP-specific antibodies in control group comprised healthy blood donors and in subjects with RVC. The experimental data are expressed as geometric means of double measurements $\pm$ SD. Levels of significance: **, $0.001 < P < 0.01$. Differences were considered significant where $0.01 < P < 0.05$.

tively. Statistically, the most significant have been the anti-CR3-RP IgM ($P < 0.01$) and IgA ($P < 0.01$) sera levels, respectively, compared to the values obtained with the healthy blood donors' anti-CR3-RP sera levels (Fig. 3).

Regarding the reference values and reference range (healthy blood donors) of anti-CR3-RP antibodies, the highest frequency of positive results in RVC patients was observed with IgA anti-CR3-RP isotype antibodies (47%), followed by IgM anti-CR3-RP (33%) antibodies. The number of anti-CR3-RP IgG positive patients was the lowest one (23%). To follow IgE response directed against CR3-RP, sera levels of specific IgE anti-CR3-RP have been determined in RVC patients and compared to those of blood donors. Obviously, the RVC patients exhibit 3.636 times higher values of specific IgE anti-CR3-RP ($P < 0.05$) (Fig. 4). Neves et al. (2005) declared the elevated specific IgE against *Candida* in recurrent vaginal candidiasis with personal history of atopy.

To evaluate the serological anti-*Candida* glycan responses in RVC patients, we have analysed the anti-mannan and anti- β -glucan sera levels as well (Fig. 5).

The results on mannan- and β -glucan-specific antibody responses revealed that the most abundant isotype has been IgM, followed by IgA and IgG. Obviously, the highest and statistically significant sera levels of antibodies were directed

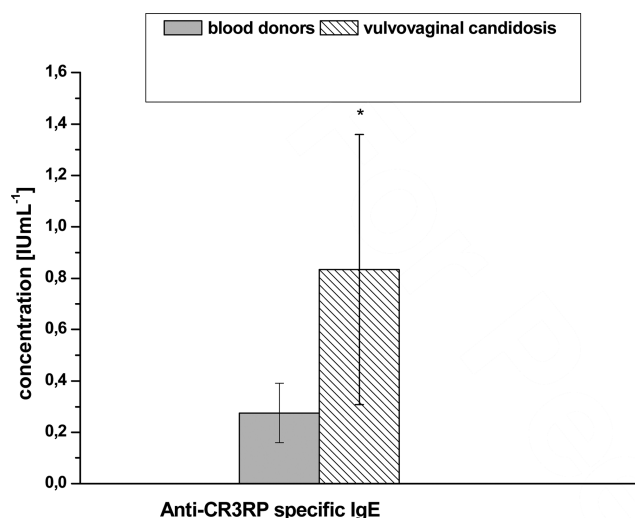


Figure 4. Sera levels of anti-CR3-RP-specific IgE antibodies in patients with RVC vs healthy blood donors. The experimental data are expressed as geometric means of double measurements $\pm$ SD. Levels of significance: *, $0.05 < P < 0.01$. Differences were considered significant where $0.01 < P < 0.05$.

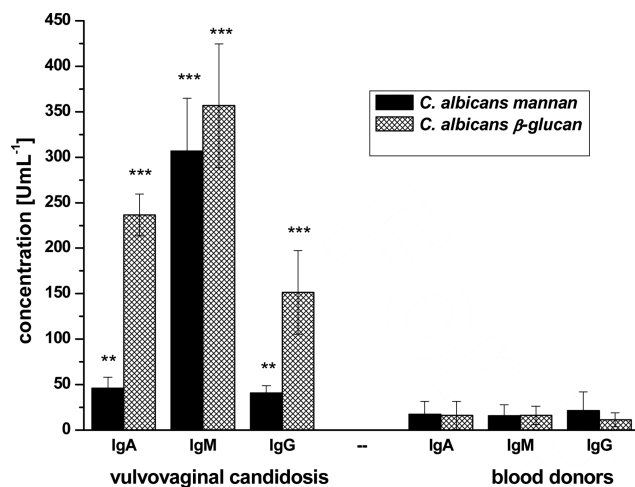


Figure 5. Class distribution of specific anti-mannan and anti- β -glucan antibodies concentrations in patients with RVC vs healthy blood donors. The experimental data are expressed as geometric means of double measurements $\pm$ SD. Levels of significance: ***, $0 < P < 0.001$, **, $0.001 < P < 0.01$. Differences were considered significant where $0.01 < P < 0.05$.

against *C. albicans* β -glucan. The overall anti- β -glucan response overcomes that of the anti-mannan; for IgA the ratio of anti- β -glucan to anti-mannan antibodies was 5.16 ($P < 0.01$), with IgM, it was 1.16 (ns) and for IgG 3.72 ($P < 0.05$). Simultaneously, with high-concentration immunoglobulin levels of anti- β -glucan antibodies, the number of positive results over range of reference values was revealed with anti- β -glucan antibodies too. The assayed concentrations resulted into the relationship $\text{IgM} > \text{IgA} > \text{IgG}$ (Fig. 5). IgM isotype culminated with 97.83% of positive results, compared to healthy blood donors ($P < 0.001$). The frequency of positive results of anti-mannan antibodies has been apparently lower, especially with IgA (34.3%) and IgG (30.13%) isotypes, although statistically significant in comparison with control group consists of healthy blood donors ($P < 0.01$).

Cell wall mannan and β -glucan represent the mostly abundant *Candida* glycans (Masuoka 2004; Ruiz-Herrera et al., 2006;

Shibata, Kobayashi and Suzuki 2012). Glycan exposure at the surface varies with fungal morphotype; studies with Dectin-1 have revealed that β -D-glucan, normally masked by thick layer of mannoprotein, is exposed during budding growth at discrete patches on the cell surface (Goodridge, Wolf and Underhill 2009). This knowledge is essential for the discrimination between importance of β -D-glucan or anti- β -D-glucan antibodies and mannan or anti-mannan as relevant serologic biomarkers related to severity and follow-up of *Candida* infection. Moreover, not only expression of *Candida* surface glycans reflected the fungus–host interactions, PAMP–PRR interactions, yeast–hyphae transition and stage of disease, but also the similar conditions have been established with protein serodiagnostically important antigens, identified as virulence-associated and/or hyphal-regulated as Als1, Als3, Hwp1, Hyr1, etc. (Mochon et al., 2010).

Variety of conventional in vitro diagnostic tests based either on *Candida* cell wall glycans and proteins determination to provide antigenemia or to identify sera antibodies levels have been developed with various specificity and sensitivity (Lain et al., 2008).

Concerning that β -D-glucan is expressed predominantly on the surface of yeast or budding cells and mannan is dominant in the hyphal morphoform, we have performed correlation analysis to compare both immunogens with CR3-RP that is expressed on the yeast as well as mycelial form of relevant *Candida* sp. The highest Pearson's correlation coefficient has been revealed with anti-CR3-RP IgM and anti- β -D-glucan IgM ($r^2 = 0.624$) followed by isotype IgA ($r^2 = 0.381$). Specific anti-CR3-RP IgG and anti- β -D-glucan IgG reflected only weak correlation ($r^2 = 0.011$). The similar correlations were obtained with anti-mannan antibodies; the highest correlation was revealed between anti-CR3-RP IgM and anti-mannan IgM ($r^2 = 0.478$) and for IgA isotype antibodies ($r^2 = 0.37$). As with β -D-glucan antibodies, the lowest correlation has been assessed for anti-mannan and anti-CR3-RP IgG, i.e. $r^2 = 0.141$. High prevalence of anti-CR3-RP IgM, anti- β -D-glucan IgM and anti-mannan IgM positive sera and the correlation between them reflected the exposure to *Candida* antigenic trigger and onset of infection and/or reinfection, as the IgM antibodies are regarded as the predominant immunoglobulins involved in early infections. Frequently, upon reinfection, levels of IgM antibody may not be as elevated as in earlier infections. High sera levels of specific IgA antibodies against CR3-RP, β -D-glucan and mannan are associated with infected mucosa, as they reflect rather superficial *Candida* infection than systemic one. This immunoglobulin class often occurs in combination with IgG antibodies, which are indicative of past or ongoing infections. In our patient cohort, positive IgG results, especially with β -D-glucan and CR3-RP, were lower than IgA and IgM, thus reflecting the recurrent *Candida* infection.

Presumably, with respect to obtained results of seroprevalent antibodies against CR3-RP in comparison with *Candida* cell wall anti-mannan and anti- β -D-glucan ones in patients with RVC, it could be concluded that novel *Candida*-derived antigen exerts immunobiological efficacy as prospective sensitive diagnostic tool interesting for serodiagnostics of *Candida* infections.

ACKNOWLEDGEMENTS

The authors are grateful to the company Biogema Ltd., Košice, Slovakia, for giving ELISA anti-*Candida* II diagnostic testing kits as a gift.

FUNDING

This contribution is the result of the project Centre of Excellence for Glycomics, IT MS26240120031, supported by the Research & Development Operational Program funded by ERDF. This publication is the result of the project implementation: Comenius University in Bratislava Science Park supported by the Research and Development Operational Program funded by the ERDF Grant number: ITMS 26240220086. This work was founded by the grant VEGA 1/0966/12 and VEGA 2/0026/13 supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic.

Conflict of interest statement. None declared.

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