

RAPID COMMUNICATION

## Correlation between *Saccharomyces cerevisiae* DNA in intestinal mucosal samples and anti-*Saccharomyces cerevisiae* antibodies in serum of patients with IBD

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remain relatively stable thereafter in immunological susceptible persons.

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### Abstract

**AIM:** To investigate the correlation between ASCA and presence of mucosal *S. cerevisiae* DNA in a population of CD, ulcerative colitis (UC) patients and controls.

**METHODS:** *S. cerevisiae*-specific primers and a fluorescent probe were designed for a 5' exonuclease real time PCR (TaqMan™) assay, which is a homogenous system using a fluorescent-labelled probe for the detection of PCR product in real time. We analyzed the relation of the PCR results with the ASCA findings in a group of 76 inflammatory bowel disease (IBD) patients (31 CD, 45 UC) and 22 healthy controls (HC).

**RESULTS:** ASCA (*IgA* or *IgG*) were positive in 19 (61%) patients with CD, 12 (27%) with UC and none of the HC. PCR amplification was inhibited and excluded from the final results in 10 (22%) UC patients, 7 (22%) CD patients, and 6 (30%) HC. In only 15 of the mucosal samples, *S. cerevisiae* DNA was detected by real time PCR, including 7 (29%) in CD, 7 (19%) in UC, 1 (6%) in HC. In 4 CD and in 4 UC patients, ASCA and mucosal *S. cerevisiae* were positive. Mucosal *S. cerevisiae* was present in combination with negative ASCA *IgA* and *IgG* in 3 UC, and 3 CD patients.

**CONCLUSION:** We conclude that since the presence of *S. cerevisiae* in colonic mucosal biopsy specimens is very rare, ASCA is unlikely to be explained by continuous exposure to *S. cerevisiae* in the mucosa. Therefore, ASCA formation must occur earlier in life and levels

### INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by a chronic relapsing intestinal inflammation. The causes of IBD are unknown but genetic, environmental, immunological, and microbial factors may be involved. Results from studies on animals suggest that the intestinal flora participates in the initiation and perpetuation of IBD<sup>[1]</sup>.

*Saccharomyces cerevisiae* is the most common species of the genus *Saccharomyces*. It is used in baker's yeast and readily finds its way into our foods<sup>[2]</sup>. Several case-series describe serious *S. cerevisiae* infections in immune-compromised patients, usually following treatment with broad-spectrum antibiotics<sup>[3-6]</sup>. Opportunistic infections by viral and fungal agents have been described as occurring in rare cases of ulcerative colitis (UC). Only one case describes diarrhea associated with *S. cerevisiae* cultured in the stool specimen of an UC patient<sup>[7]</sup>. So far, no studies have been published concerning the presence of *S. cerevisiae* in the intestinal tissue of patients with IBD. *S. cerevisiae* specific primers and a fluorescent probe were designed for a 5' exonuclease real time PCR (TaqMan™) assay. This method provides high specificity and sensitivity for detecting *S. cerevisiae* DNA but is hampered by the difficulties arising from DNA extraction of *Saccharomyces* species.

In 1988, the presence of anti-*Saccharomyces cerevisiae* antibodies (ASCA) in patients with Crohn's disease (CD) was firstly described<sup>[8]</sup>. The ASCA test for diagnosing CD has a sensitivity of 72% and a specificity of 82%<sup>[9-12]</sup>. The underlying cause of generating *S. cerevisiae* antigens

supporting the specific antibody response in CD is still unknown. ASCA are thought to result from a specific antibody response to the *S. cerevisiae* cell wall mannan (phosphopeptidomannans). It is unknown whether this is a direct response towards the yeast itself or an epiphenomena with a similar immunologic response towards another antigen. It is postulated that the yeast wall cell mannan may mimic a high mannose-containing molecule towards which the antibody is directed inducing a hypersensitivity reaction during inflammation<sup>[12,13]</sup>.

## MATERIALS AND METHODS

### Patients

Seventy-six patients with IBD (45 UC, 31 CD) and 22 healthy age- and sex-matched controls regularly visiting the Departments of Gastroenterology from the VU University Medical Center, Amsterdam, the Netherlands, a third line referral center, and St Anna Hospital, Geldrop, the Netherlands, a regional hospital, were enrolled in the study. IBD patients with clinical complaints compatible with active inflammation of the mucosa were screened for the study, but both IBD patients with active inflammation as well as quiescent disease assessed by macroscopic endoscopic findings were included in the study. The diagnosis of CD and UC was based on standard endoscopic, histological, and radiographic features<sup>[14]</sup>. Disease localization and activity, demographical data and medication were documented. Fifty-four percent of UC patients and 42% of CD patients was treated with immunosuppressive medication (Table 1). None of the patients was treated with probiotics containing *S. boulardii*. However, they were not requested to use a diet low of *S. cerevisiae* either.

During sigmoidoscopy, 2 mucosal biopsy specimens were obtained from the sigmoid and directly snap frozen in liquid nitrogen and then stored at -18°C until further analysis. In addition, blood samples were drawn for detection of ASCA antibodies.

This study was approved by the Medical Ethical Committee of the VU University Medical Center, Amsterdam, the Netherlands.

### ASCA ELISA

ASCA IgA and IgG were determined by commercially available ELISA kits (Inova, Uniprom Diagnostics BV, Krimpen aan de IJssel, the Netherlands). The antigen consists of phosphopeptidomannan (PPM) extracted from *S. cerevisiae*. ASCA ELISAs were performed according to the manufacturer's instructions. ASCA results were expressed as arbitrary units with a cut-off for positivity of 25 U/mL as advised by the manufacturer and described elsewhere<sup>[15]</sup>. Serum was considered positive if either IgA or IgG or both were positive. Serum was considered negative if both IgA and IgG ASCA were negative.

### DNA isolation

Biopsy specimens were pretreated with 200 µL of 5 g/L collagenase A (Roche Diagnostics, Almere, Nederland B.V.) and incubated at 37°C overnight with continuous shaking. Subsequently, each biopsy was divided into two equal cell

fractions. One fraction was spiked with 20 colony forming units/µL *S. cerevisiae*. Both fractions were incubated for 90 min at 37°C with 600 µL of sorbitol buffer and 200 U of lyticase (Sigma-Aldrich, Steinheim, Germany), prior to the isolation of chromosomal DNA with the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The end volume after the extraction of the DNA from the biopsy specimen was 100 µL. The control fraction of the biopsy specimen contained 20 CFU equivalents/µL (elution concentration).

*S. cerevisiae* specific primers and a fluorescent probe for a 5' exonuclease RT PCR (TaqMan™) assay were designed with the Primer Express software package (Applied Biosystems, Foster City, CA, USA). This assay is a homogeneous system with a fluorescent double-labeled probe for the quantitative detection of PCR product. This provides a rapid automated combined PCR amplification and detection system with no post-amplification manipulation of amplicons, thereby considerably reducing the risk of contamination. Coupled with a quick, simple DNA extraction method, this protocol allows for rapid specification of clinical isolates. Amplification reactions were carried out in a total volume of 25 µL. Reaction mixtures contained 1x universal master mix (Applied Biosystems), 300 nmol of *Saccharomyces* forward primer (5'-GAA ATG CCA CCG TGA ATG C), 300 nmol of *Saccharomyces* reverse primer (5'-CTT TGG TGG TGA TCC TCT ATG ATT G) and 100 nmol of labeled probe (FAM - TGG CAC CAT GAA CCC TAG CGT CGT T - TAMRA), and 5 µL of DNA. To prevent inhibition, 5 g/L bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) was added to the PCR mixtures. Amplification was carried out after an incubation for 2 min at 50°C and for 10 min at 95°C, followed by 45 cycles at 95°C for 15 s, and at 60°C for 1 min. These reactions were performed on an ABI Prism 7000 Sequence Detection System (SDS) (Applied Biosystems, Figure 1).

### Statistics

SDS software was used for analysis of the data obtained from RT-PCR results, and *t*-test with separate variance estimates were used to test differences between patients with CD, UC and controls.

## RESULTS

ASCA (IgA or IgG) were identified in 19 (61%) CD patients, 12 (27%) UC patients and none of the HC. Of the patients, 42% of CD patients and 54% of UC patients were using immunosuppressive medication (Table 2).

To determine the sensitivity of real time PCR, *S. cerevisiae* cells were cultured and the amount of colony forming units (CFU) were counted. After DNA isolation a dynamic range, based on the related amount of DNA (CFU equivalents) was made from 10 to 10<sup>5</sup> (Figure 2).

Specificity of the PCR for *S. cerevisiae* was examined by comparing the results of pure *S. cerevisiae* DNA and *S. cerevisiae* DNA mixed with DNA isolated from 10 different bacteria (*Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Clostridium difficile*, *Lactobacillus acidophilus*, *Propionibacterium acnes*, *Actinomyces viscosus*, *Enterococcus faecalis*, *Fusobacterium*

**Table 1 Medication at time of harvest of biopsy specimens**

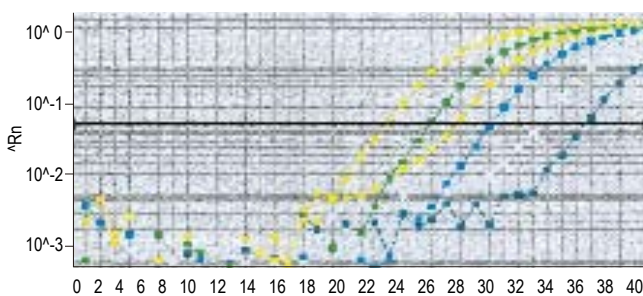
	Medication	No of patients (%)
UC	Prednisone	12/37 32
	AZA	11/37 30
	ASA	8/37 22
	Ciclosporine	4/37 11
	No medication	12/37 32
	Any immunomodulating drug	20/37 54
CD	Prednisone	6/24 25
	AZA	5/24 21
	ASA	2/24 8
	Ciproxin	1/24 4
	Anti-TNF	2/24 8
	No medication	13/24 54
	Any immunomodulating drug	10/24 42

**Table 3 ASCA and RT-PCR detection of *S. cerevisiae* in CD patients**

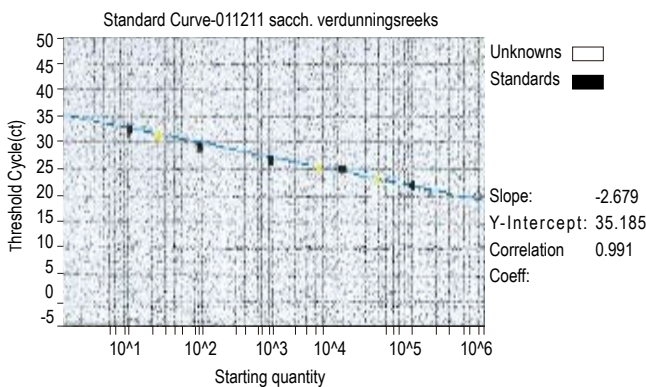
	ASCA positive	ASCA negative	Total
RT-PCR positive	4	3	7
RT-PCR negative	12	5	17
Total	16	8	24

**Table 4 ASCA and RT-PCR detection of *S. cerevisiae* in UC patients**

	ASCA positive	ASCA negative	Total
RT-PCR positive	4	3	7
RT-PCR negative	8	22	30
Total	12	25	37



**Figure 1** Annealing cycles of *S. cerevisiae*



**Figure 2** Dilution series of *S. cerevisiae*

*nucleatum*, *Escherichia coli*, *Streptococcus pyogenus*, *Listeria monocytogenes*, *Listeria ivanovi*), belonging to the normal gut flora and 6 different *Saccharomyces* species (*S. boulardii*, *S. unisporus*, *S. kluyveri*, *S. pastorianus*, *S. paradoxus*, *S. servazzi*). In addition, this DNA mixture neither influenced the sensitivity nor the specificity of the *S. cerevisiae* amplification (data not shown). Among the 15 mucosal biopsies, *S. cerevisiae* DNA was detected in 7 (29%) CD patients, 7 (19%) UC patients, 1 (6%) HC. The amount of *S. cerevisiae* DNA was low and varied. Positive values were to be found between 37-38 CFU equivalents. Inhibition during amplification occurred in 8 (18%) UC patients, 7 (22%) CD patients and 6 (30%) HC.

Furthermore, 25% (4/16) CD patients and 33% (4/12) UC patients showed positivity for both ASCA and mu-

cosal *S. cerevisiae* DNA. In 12% (3/25) UC and 38% (3/8) CD patients, mucosal *S. cerevisiae* DNA was present in combination with negative ASCA *IgA* and *IgG* (Tables 3 and 4).

## DISCUSSION

ASCA (*IgA* or *IgG*) were positive in 19 (61%) CD patients, 12 (27%) UC patients and none of the HC. Out of the 15 mucosal biopsies, RT PCR demonstrated the presence of *S. cerevisiae* DNA in 7 (29%) CD patients, 7 (19%) UC patients, and 1 (6%) HC. No significant correlation could be found between ASCA and the presence of *S. cerevisiae* DNA isolated from mucosal biopsies.

Frequencies of ASCA reported in literatures range from 39-76% for CD patients, 5-15% for UC patients to 0-5 % in controls<sup>[8, 11, 12, 16-23]</sup>. In this study ASCA positivity in the UC group was high (27%) as compared to earlier ASCA studies from our group and reported frequencies in literature.

The presence of ASCA appears not to be related to disease activity<sup>[23]</sup>. Therefore, we assume that our selection of patients (clinical complaints compatible with active disease) is not likely to influence ASCA status or the presence of *S. cerevisiae*. Forty-two percent of CD patients and 54% of UC patients were using immunosuppressive agents in this cohort (Table 1). This high percentage of patients using immunosuppressive agents reflects the overall disease activity of our patient population, being relatively severe. However, use of immunosuppressive agents does not explain the high frequency of ASCA in our UC population since most ASCA-positive UC patients did not use this type of medication. Therefore, it can be hypothesized that healing of the mucosa with the use of immunosuppressive agents will lead to decreasing ASCA levels, as has been reported in the treatment of children with active CD<sup>[22]</sup>. ASCA were detected with well-validated commercially available kits that have been described in previous studies<sup>[9]</sup>.

Mucosal permeability appears increased in active CD as a consequence of direct effects of pro-inflammatory molecules and transmigrating neutrophils. However, considerable controversy exists regarding a primary genetically



Table 2 RT-PCR and ASCA results in relation to medication

	Medication	RT-PCR + (%)	RT-PCR - (%)	ASCA + (%)	ASCA - (%)
UC	Prednisone	6/7 86	6/30 20	5/12 42	7/25 28
	AZA	1/7 14	10/30 33	2/12 17	9/25 36
	ASA	3/7 43	5/30 17	0	8/25 32
	Ciclosporine	1/7 14	3/30 10	1/12 8	3/25 12
	No medication	0	12/30 40	7/12 58	6/25 24
CD	Prednisone	2/7 29	4/17 24	4/16 25	2/8 25
	AZA	2/7 29	3/17 18	2/16 13	3/8 38
	ASA	1/7 14	1/17 6	1/16 6	1/8 13
	Ciproxin	1/7 14	0	1/16 6	0
	Anti-TNF	2/7 29	0	2/16 13	0
	No medication	2/7 29	11/17 65	8/16 50	5/8 63

determined defect in epithelial barrier function<sup>[24-26]</sup>.

In a small study<sup>[27]</sup>, intestinal permeability, tested by a cellobiose/mannitol test, was raised in 6 (37%) of the CD patients and in 11% of their relatives. This altered intestinal permeability was unrelated to sex, age, disease activity, localization, duration, treatment schedule, as well as to serum ASCA positivity, a rather stringent subdivision in such a small patient group. Although an interesting finding, this makes interpretation difficult. Furthermore, genetic polymorphisms were not taken into account.

ASCA is not related to mucosal disintegrity, because ASCA is independent of disease activity<sup>[28]</sup>. Elevated serum levels of ASCA did not primarily seem to result from a defect of the gut barrier<sup>[29]</sup>. These data fit in with the hypothesis that a relationship between antibody responses toward microbial antigens and complicated small bowel diseases reflect the interplay between a genetically susceptible host and relevant luminal flora, as has been suggested before, relating intestinal permeability to environmental factors, and ASCA generation to genetic predisposition<sup>[23]</sup>.

To our knowledge, this is the first study describing the presence of *S. cerevisiae* DNA in intestinal mucosa of IBD patients by using RT-PCR. The presence of ASCA antibodies could not be correlated with mucosal *S. cerevisiae* DNA in biopsy specimens taken during sigmoidoscopy although the RT-PCR test is highly sensitive and specific. Every individual sample was checked for inhibition by a simultaneous spiked amplification. Inhibition during amplification occurred in 8 (18%) UC patients, 7 (22%) CD patients and 6 (30%) HC. Extraction of DNA from yeast requires special enzymes to remove the cell wall<sup>[30]</sup>. Theoretically, excluding a number of patients from the total patients due to inhibition of the RT-PCR amplification may influence the results, particularly taking into account the relatively small study population. However, amplification problems were equally distributed in all groups. Furthermore, one could hypothesize that the presence of *S. cerevisiae* is higher in ileal mucosa than in the sigmoid since higher ASCA levels were detected in CD patients with ileal localization of disease<sup>[28]</sup>. Preliminary RT-PCR data from our group show comparable numbers of *S. cerevisiae* in the left and right side of the colon consistent with an equally distributed presence of *S. cerevisiae* throughout the (distal) intestinal tract (Akol, personal communication). Interbiopsy variability between multiple biopsies taken at the same

localization was low. Therefore, our results were unlikely to be biased by neither the number of biopsy specimens nor the localization from where the biopsies were obtained.

The presence of mucosal *S. cerevisiae* DNA with concomitant negative serum ASCA IgA and IgG in one UC and two CD patients can not be explained by the intestinal leakage hypothesis. This finding corroborates the hypothesis that ASCA formation is an epiphenomenon (in genetically susceptible individuals) rather than the result of antigenic challenge by intestinal presence of *S. cerevisiae* in leaking mucosa. On the contrary, we postulate that since the presence of *S. cerevisiae* DNA in mucosal biopsy specimens is very rare, presence of ASCA can not be explained by antigenic exposure to *S. cerevisiae*.

Another hypothesis is that everyone encounters *S. cerevisiae* early in life. In some patients, the (transitory) *S. cerevisiae* presence may lead to ASCA formation, particularly in people prone to develop CD. Only in a small percentage of people, life-long colonisation may be the result, comparable to what has been documented in other species such as *Clostridium difficile*. Although intra-individual, longitudinal determination values of ASCA were not tested in this study, ASCA formation may be initiated early in life and perpetuate thereafter as described in adult populations<sup>[31]</sup>. In contrast, ASCA levels decrease after treating active inflammation in children with CD<sup>[22,32,33]</sup>. These differences between stable ASCA values in an adult population and decreasing ASCA values in a paediatric population have recently been described for a population with coeliac disease<sup>[34]</sup>, pointing at differences in antigen handling/immunologic response in children and adults.

In conclusion, our study demonstrates no any correlation between the presence of ASCA antibodies and mucosal *S. cerevisiae* DNA. Although the study population was small, this finding underscores the hypothesis that ASCA antibodies are not solely formed as a reaction on the mannan from the yeast cell wall but rather is an epiphenomenon with a similar immunologic response towards another, yet unidentified antigen.

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