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Genetic Differentiation of Fungi of the Genus *Candida* Isolated from Patients with Inflammatory Bowel Diseases

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1. Introduction

Inflammatory bowel diseases (IBD) constitute a group of incurable, inflammatory GI diseases of unknown etiology and the chronic course, with periods of spontaneous disease remissions and aggravations (Muszyński, 2001). This group of diseases includes Crohn`s disease and ulcerative colitis. They are characterized by inflammatory reactions and changes in the structure of intestinal mucosa (Rzeszutko, 2006). IBD etiopathogenesis is not clearly defined. The most essential etiological issues include environmental factors, genetic conditioning, abnormalities of intestinal immunological mechanisms and the bacterial flora (Konturek, 2001). The inflammation which develops in the intestine is mostly due to improper, low-fiber diet which is based on highly processed products and on the poor physical activity. Familial diseases confirmed in 10% of cases speak for the genetic source. Immunological mechanisms which develop in response to food and bacterial antigens are extremely significant (Muszyński, 2001; Polińska et al., 2009). A markedly increased interest has been observed in the intestinal microflora which is believed to be essential in inducing IBD development and in relapses of its clinical symptoms. Bacteria which are normally resident in the GI tract determine the proper work of intestines and protect from excessive proliferation of undesired microorganisms. The contribution of the intestinal flora to the development of the biofilm, which prevents the colonization of pathogens including fungi of the genus *Candida* in the GI tract, is extremely significant. The area of interest in fungi which are a part of microflora and constitute a potential source of systemic dissemination by colonizing the GI tract has greatly increased lately (Berhardt & Knoke, 1997). In the normal environment fungi are in balance with the bacterial flora and as commensals do not induce the inflammation. The main cause of its development, however, is the immune system impairment and a decrease of immunity. It affects patients with neoplasms and transplant recipients (Pfaller & Diekema, 2007; Warnock & Campbell, 1996). Other diseases causing inflammations are: diabetes and other endocrinopathies, infections due to HIV virus and also surgical procedures, damaged tissues and inflammations of the

mucous membrane of the alimentary tract. Aggressive therapies with antibiotics, steroids and immunosuppressants disturb the endogenic bacterial flora and as a result contribute to the development of mycoses (Budak et al., 2003; Schelenz, 2008). The most essential factors affecting the increase of the frequency of fungal infections, especially those with *Candida* etiology, are commonly used invasive medical devices introduced into the human body, such as various catheters, tracheotomy tubes, stents, prostheses, implants and pacemakers which develop the biofilm on their surfaces due to fungi. The formation of the biofilm due to fungi which cause the inflammation has an essential clinical effect because it increases their resistance to drugs as well as the ability of cells which are inside the biofilm to defend themselves againt the immunological reaction of the host. The biofilm formed on the surface of medical devices which have been introduced into the body decreases their effectiveness and is the source of future inflammations (Jain et al., 2007; Ramag et al., 2006).

In the considerable number of healthy individuals fungi of the genus *Candida* colonize the oral cavity and pass through the esophagus, the stomach and the small intestine to reside in the large intestine. A number of healthy carriers of *Candida* in the oral cavity amounts to 30-50%, where *C.albicans* is a predominant species, constituting 70-80%. The most frequent site of candidiasis is the large intestine. Erosions, extensive ulcerations covered by false membranes and thrush-like changes develop within the mucous membrane. The fungal penetration into the submucosal membrane, muscularis and blood vessels is also possible. It includes vascular inoculation and the formation of microabscesses in various organs and tissues. Vascular invasion can lead to the obstruction of the artery and eventually to myocardial infarction.

Fungi of the genus *Candida* which are present in the large intestine were considered as sources of chronic and recurrent infections which can lead to the development of inflammations. Intestinal inflammations are likely to produce favorable conditions for the fungal invasion (Zwolińska-Wcisło et al., 2006). It is difficult to show the border between the colonization and the infection caused by fungi of the genus *Candida* within the oral cavity because asymptomatic carriers frequently develop the abundant increase of fungi in the cultured oral cavity and throat smears. It means that the presence of fungi on the mucous membrane of the oral cavity cannot be treated only as the saprophytic flora. In the healthy individual the stomach and the small intestine have the function of the passage of the fungal flora. In patients from the risk group the initial segment of the small intestine can be the site of the pathological colonization of fungi and the fungal infection develops in the ileocecal segment. Affected patients experience stomach cramps, flatulence, diarrhea and GI bleeding i.e., symptoms frequently reported by patients with acute IBD in the medical history (Mokrowiecka & Małecka-Panas, 2007).

It is difficult to explain the relation between the occurrence of fungi and the development of inflammations because candidiasis of the oral cavity and further segments of the GI tract is not the frequent subject of investigations. Recent studies on the role of fungi of the genus *Candida* in inflammatory bowel diseases were performed in the group of patients with IBD whose clinical samples for mycological investigations were collected by means of colonoscopy (biopsy taken from the large intestine, aspirate from the intestinal contents and brush-smears), as well as the throat smear and the examination of feces. Samples were taken from patients in various periods of symptom aggravation. It was revealed that despite the abundant increase of fungi in throat and feces cultures no essential settlement of fungi on the mucous membrane of the large intestine was noted. However, while performing regular mycological studies of the group of patients with the history of the 5-year- course of the

disease fungi were isolated from all clinical samples in each acute stage of the disease. It was shown that the presence of fungi in the oral cavity of this group of patients could affect the significantly frequent fungal colonization of the mucous membrane of the large intestine in the active phase of the disease. The possibility of the transmission of the fungal flora of the oral cavity to other segments of the GI tract was confirmed on the basis of 100% affinity of *C.albicans* strains isolated from the same patient, examined by means of the PCR-RAPD method (Trojanowska et al., 2010).

In the most recent studies the diagnostics of invasive fungal inflammations includes molecular methods based on DNA and RNA analyses. The polymerase chain reaction (PCR) with its various modifications is routinely used. One of them is RAPD (*Random Amplified Polymorphism DNA*), commonly used in epidemiological investigations because of its rapid and simple procedure and the possibility of the simultaneous comparison of a large number of strains.

Strain typing based on PCR-RAPD enables the determination of genetic affinity of investigated strains or their distinctiveness in the particular time. The RAPD method is based on the reaction of amplification using short starters with the randomized base sequence, which bind to the analysed DNA in various sites, depending on the investigated strain. The presence or the lack of differences in the electrophoretic partition signifies the differentiation or compatibility of strains. It is used to perform epidemiological investigations which aim at determining the source of infection and the clonal characterization of distinguished isolates. In addition, it is possible to watch the transmission of strains which cause the inflammation, or monitor the therapy. In case of the infection relapse the analysis of isolated strains enables to detect if it is caused by the same strain which can suggest the ineffective therapy or if it is due to other strains suggesting the recurred infection (Dzierżanowska, 2006).

C.albicans is the most commonly isolated species from the GI tract and as the opportunistic flora can easily adapt itself to many sites of the body. However, as the non-pathogenic intestinal flora it can be replaced by rarely isolated but more virulent non-albicans strains, such as: *C.glabrata*, *C.tropicalis* and *C.krusei*. It is significant to explain if non-albicans species isolated from various clinical samples of the same patient can constitute one clone transferred from the oral cavity to the lower segments of the GI tract or if various factors affect the genetic diversity of isolated strains?

2. The aim of the study

The aim of the study was the determination of the genetic diversity of *Candida spp.* strains isolated from clinical samples of various segments of the GI tract of the same patient using PCR-RAPD method and the assessment of the transmission of non-albicans strains in the alimentary tract based on the analysis of obtained results of genetic investigations.

3. Materials and methods

The material for the study consisted of 39 *Candida spp.* strains isolated from clinical samples taken by means of colonoscopy and from the oral cavity and feces of patients diagnosed in the Department of Gastroenterology, Hepatology and Infective Diseases of Jagiellonian University Collegium Medicum in Cracow. The strains came from 7 patients. In four of them ulcerative colitis was diagnosed and three of them developed Crohn`s disease. In six

patients non-albicans strains were cultured on all clinical samples and in one patient *C.albicans* strains were cultured on samples taken from the GI tract twice, at one-year-interval. Clinical samples for mycological examinations were taken in various periods of symptom aggravation. The smear and feces were examined before preparing the patient for colonoscopy during which the biopsy was taken from the affected mucous membrane of the large intestine as well as the aspirate of intestinal contents and the brush-smear.The identification of fungi was carried out using the CAN2 chromogenic base (bioMerieux) and API Candida tests (bioMerieux). Genetic examinations were performed using the PCR-RAPD technique for 10 *C.albicans* strains isolated from 1 patient, 20 *C.glabrata* strains from 4 patients, 5 *C.tropicalis* strains from 1 patient and 4 *C.krusei* strains from 1 patient.

3.1 DNA isolation

From the 48-hour-culture of strains on the Sabouraud agar (bioMerieux) the suspension in 0.85% NaCl was compounded. Genomic DNA was isolated using Genomic Mini AX YEAST (A&A Biotechnology). DNA concentration was marked spectrophotometrically at the wave length equal to 260nm.

3.2 PCR-RAPD reaction

In the PCR-RAPD reaction 3 primers were used: CD16AS(5`-CTC TTG AAA CTG GGG AGA CTT GA-3`), ERIC2 (5`-AAG TAA GTG ACT GGG GTG AGC G-3`) and HP1247 (5`-AAG AGC CCG T-3`). The reaction was conducted using Promega reagents. The reaction mixture in its final volume of 25 ul contained: 5 ul 5x colourless Go Taq Flexi Buffer (ph 8.5), 1.5 ul 25mM MgCl2, 100 uM of each nucleotide, 100pmol primer and 0.625 U Go Taq DNA polymerase. The following RAPD reaction conditions were stated for the examined strains: initial denaturation at 94^{o}C for 5 min, 45 cycles comprising the true denaturation at 94^{o}C for 1 min, primer attachment at 36^{o}C for 1 min, elongation at 72^{o}C for 1 min and final prolongation at 72^{o}C for 7 min.

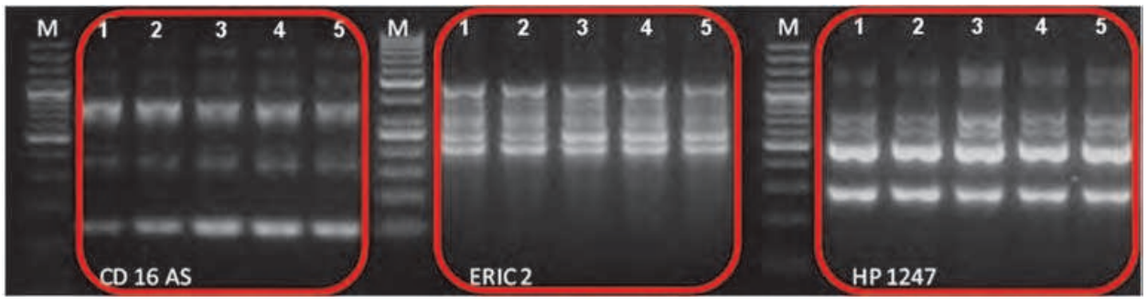
3.3 Electrophoresis in the agarose gel

Reaction products were detected during electrophoresis in the 2% agarose gel (Sigma), in the TBE buffer (Tris-Borate Buffer) and the differentiation of the electrophoresis image of PCR-RAPD reaction products in the BIO-PROFIL Bio-ID++ program was analysed (Vilber-Lormat, France).

4. Results

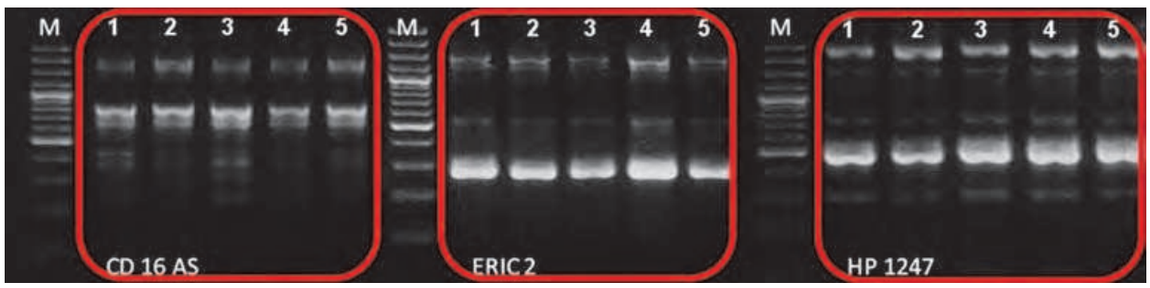
The assessment of the *Candida spp.* strain transmission in the GI tract was performed on the basis of the PCR-RAPD reaction product analysis comprising the determination of a degree of affinity between strains isolated from particular segments of the GI tract of the same patient. The examinations included strains isolated from the following clinical samples: throat smear (1), biopsy of the affected wall of the large intestine (2), aspirate of intestinal contents (3), brush-smear from the intestine (4), feces (5). *C.glabrata* was isolated from clinical samples of 4 patients, *C.tropicalis* and *C.krusei* were isolated from other examined patients and *C.albicans* was isolated from the same patient twice, at one-year-interval. The selective amplification of the genetic material obtained from examined strains with CD16SA, ERIC2 and HP1247 primers confirmed 100% homology among *C.albicans* strains isolated for

the first time from the patient No.I (Fig.1) and *C.glabrata* strains from the patient No.II (Fig.2).



1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 1. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.albicans* strains isolated from patient No.I (the first isolation).



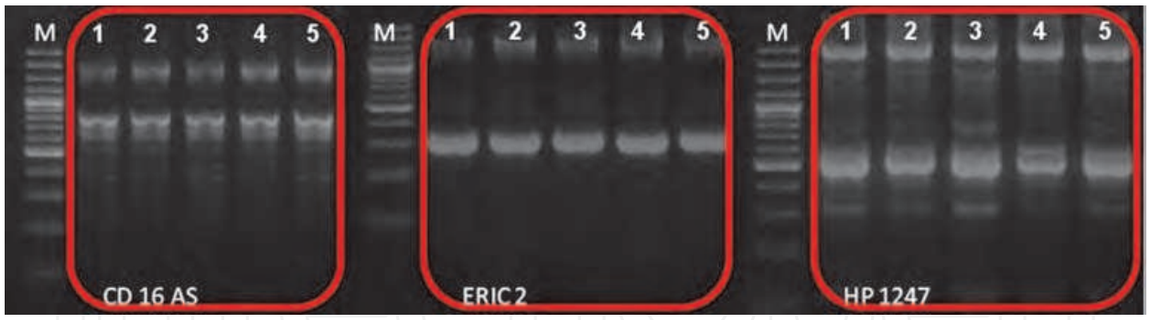
1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 2. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.glabrata* strains isolated from patient No.II.

100% compatibility was also revealed with two primers (CD16AS and ERIC2) in case of *C.glabrata* from the patient No.III and the HP1247 primer showed 100% compatibility between strains taken from the throat, biopsy, aspirate and feces as well as 80% strain homology from the brush-smear (Fig.3). Similarly, 100% homology with CD16AS and ERIC2 primers was obtained in case of *C.tropicalis* from the patient No.VI and over 80% homology with the HP1247 primer was achieved (Fig.7).

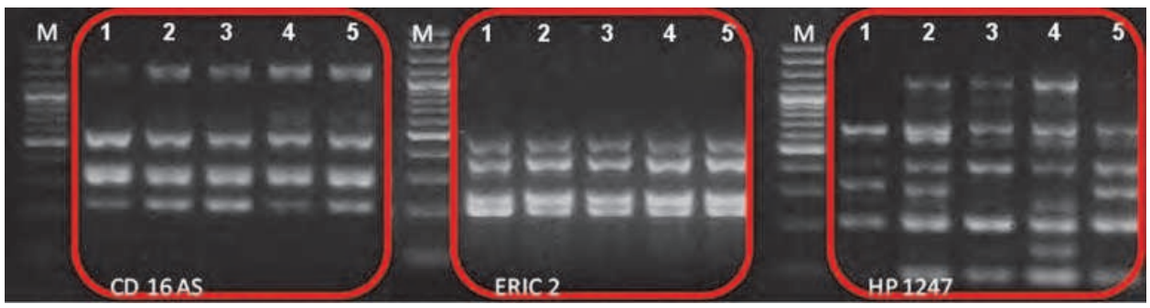
The homology lower than 90% with three primers was found among *C.albicans* cultured a year after the first isolation from the patient No.I (Fig.5), *C.glabrata* derived from the patient No.IV (Fig.6) and the patient No.V (Fig.7) as well as *C.krusei* isolated from the patient No.VII (Fig.8).

A degree of homology equal to or exceeding 90% was assumed as a criterium of the lack of genetic strain diversity (Speijer et al., 1999) (Tab.1).



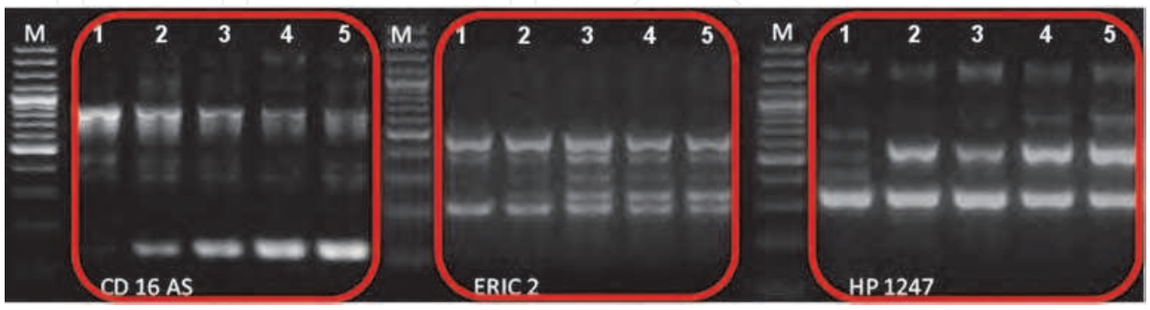
1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 3. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.glabrata* strains isolated from patient No.III.



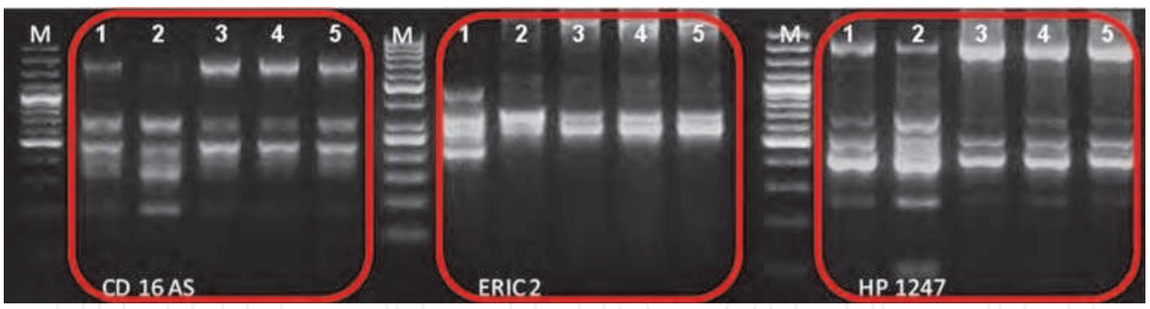
1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 4. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.tropicalis* strains isolated from patient No.VI.



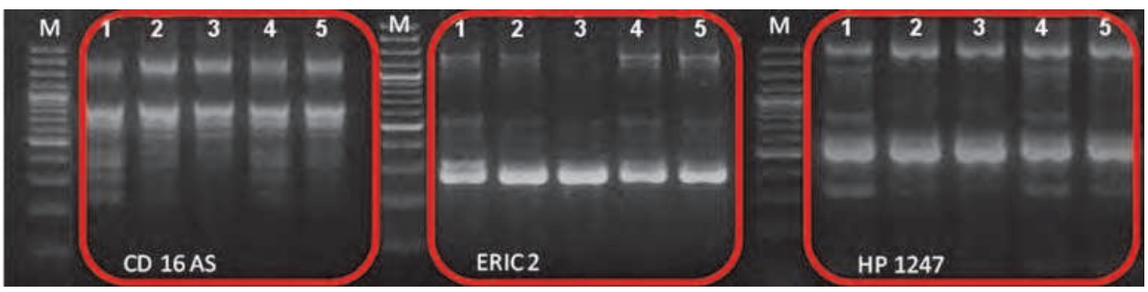
1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 5. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.albicans* strains isolated from patient No.I (second isolation).



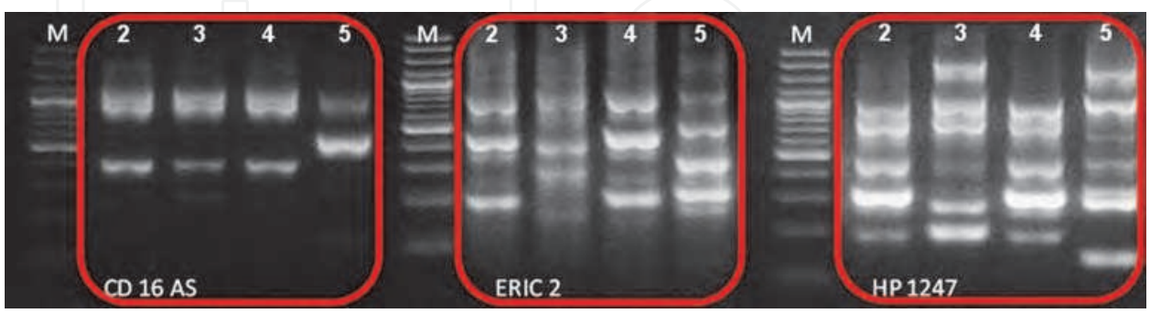
1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 6. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.glabrata* strains isolated from patient No.IV.



1- throat smear, 2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 7. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.glabrata* strains isolated from patient No.V.



2- colonic biopsy, 3- aspirate of intestinal contents, 4- brush-smear from the intestine, 5- feces, M- marker (100bp)

Fig. 8. Distribution of the products of PCR-RAPD reaction with three primers (CD 16 AS, ERIC-2, HP 1247) on agarose gel, showing similarities between *C.krusei* strains isolated from patient No.VII.

Patient	Strain	Degree of homology
I	*Candida albicans*	>90%
I	*Candida albicans*	<90%
II	*Candida glabrata*	>90%
III	*Candida glabrata*	>90%
IV	*Candida glabrata*	<90%
V	*Candida glabrata*	<90%
VI	*Candida tropicalis*	>90%
VII	*Candida krusei*	<90%

Table 1. A degree of homology among *Candida spp.* strains isolated from clinical samples from patients with IBD.

5. Discussion

Results of earlier investigations performed in a group of patients with IBD confirmed the possibility of the oral cavity fungal flora transmission to further segments of the GI tract on the basis of 100% genetic affinity among isolated *C.albicans* strains in the PCR-RAPD method (Trojanowska et al., 2010). It was an impulse for performing current investigations which include the analysis if non-albicans strains isolated from various clinical samples, taken from the same patient turn out to be one clone or not and if not, which factors cause the genetic diversity among these strains?

The analysis of *C.albicans* genotypes isolated from the patient No.I in the first examination of the acute stage of the disease revealed 100% homology of all strains. The passage of *C.albicans* is highly probable due to the properties of this fungal species. It is the most common opportunistic species having a great ability to adapt itself to various environments of the body, which is determined by the high expression of virulent factors responsible for the pathogen invasion (enzymes, phenotypic variability, adhesion) (Dzierżanowska, 2006). It is extremely interesting that the analysis of *C.albicans* strains isolated from the same patient after one-year-observation showed the larger variability of strains, especially the strain isolated from the oral cavity whose similarity to other strains amounted to 61-90%, depending on the used primer. The change could have been affected by the used therapy and in the result by the selection of the more resistant clone. This finding is confirmed by investigations performed by Tanaka, which enabled to divide strains isolated during the course of candidiasis into three groups. One of them included strains which were replaced by other strains during the course of the disease (Tanaka , 1997). In our investigations, which analysed non-albicans strains, 100% similarity was confirmed in *C.glabrata* strains isolated from patients No.II and III as well as the patient No.IV (*C.tropicalis*), using CD16AS and ERIC2 primers while the HP1247 primer turned

out to be more differentiating for investigated strains. Other patients (No.IV, V, VII) developed the larger differentiation between strains. The attention should be focused on the fact that some differences in the *Candida spp.* strain homology can be determined by the limited repeatability of the PCR-RAPD method as well as its considerable susceptibility to changes of reaction conditions, such as: the concentration of the primer and other reaction constituents (Wolinowska, 2002). Moreover, slight genome mutations affecting the RAPD profile change may emerge during the frequent strain passage and a long-term culture (Lehmann et al., 1992). The patient`s condition, susceptibility to infection and the used therapy are also significant in the selection and differentiation of strains.

Inflammatory processes which accompany the main disease are essential factors predisposing to the invasive growth of *Candida* in patients with IBD. These patients develop pathological factors in the structure of the GI wall, disturbed immunological mechanisms and a disproportion in the proper intestinal flora composition, which promote the excessive fungal development (Zwolińska-Wcisło et al., 2009). Patients from this group undergo the complex therapy including glucocorticoids and immunosuppressants which inhibit inflammatory reactions in intestines but suppress the immunity of the body leading to the spread of the fungal flora in areas which are specially favorable for its multiplication.

Inflammatory bowel diseases are still the subject of many publications. It is interesting because of theoretical and cognitive issues in the aspect of discovering new etiopathogenetic mechanisms as well as practical ones concerning the use of new diagnostic and therapeutic methods. The knowledge of IBD mechanisms enables the more precise intervention into particular stages of the inflammatory process and the elaboration of methods to control the inflammation. It also allows to minimize adverse effects and eventually to improve the quality of life of patients affected by these diseases. The chronic character of IBD, periodic aggravations of the disease, the high activity of the inflammatory process as well as not always adequate response to the introduced treatment make these diseases serious clinical problems (Montgomery & Ekbom, 2002; Colombel et al., 2008). Variety of clinical symptoms, complications and systemic effects make that the differential diagnostics comprises numerous diseases (Grzymisławski & Kanikowska, 2010).Apart from some bacterial infections, other inflammatory diseases with the clinical picture resembling IBD, such as: tuberculosis as well as parasitic and fungal inflammations should be taken into consideration (Radwan et al., 2009).

Investigations confirm (Trojanowska et al., 2010) that acute stages of these diseases reveal the presence of fungi in all clinical samples taken from the GI tract. As the endogenic flora fungi can be the cause of abnormal immunological mechanisms in IBD and in consequence can cause the development of systemic candidiasis (Radwan et al., 2009). The diagnostics of GI fungal diseases is based on imaging techniques (endoscopy), microscopic methods (histological preparations), and mycological, serological and genetic investigations (Biliński et al., 2008).The molecular analysis enables to detect the presence of genes determining factors of fungal virulence and genes resistant to antifungal drugs (Wolinowska, 2002). Nowadays, there is an increase of the number of inflammations due to *C.krusei* and *C.glabrata* strains resistant to fluconazole, the drug which is mostly used in the prevention of candidiasis in patients with IBD.

Fungi of the genus *Candida* constitute the essential etiological factors of opportunistic inflammations. The development of medicine and the introduction of new therapeutic strategies paradoxically contribute to the increase of fungal inflammations. It is mostly connected with an increasing number of patients with immunosuppression and disturbed homeostasis (Schelenz, 2008).

6. Conclusions

The transmission of *Candida spp*. strains in the GI tract, especially the most frequently isolated *C.albicans* and *C.glabrata* strains, is possible. Therefore, the fungi in the oral cavity of patients with IBD cannot be regarded exclusively as saprophytic flora, which in the active phase of IBD, can multiply and disseminate to further parts of the GI tract. This fact suggests justifiability of using antifungal therapy, wich can provide the relief of symptoms of candidiasis or decrease in their intensity.

This knowledge can be used in prophylaxis, diagnostics and in monitoring the treatment of GI tract candidiasis. The confirmation of candidiasis with the *C.glabrata* or *C.krusei* etiology requires the elimination of fluconazole which is commonly used in the prevention and the treatment of candidiasis. The introduced therapy which causes the selection of the more resistant clone can affect the genetic diversity of strains.

7. References

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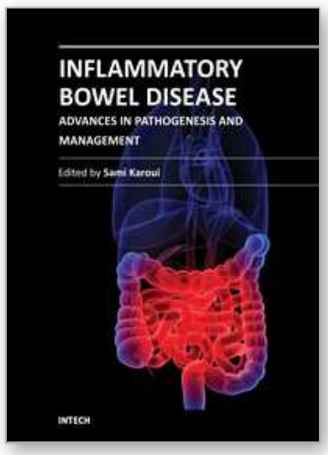

Inflammatory Bowel Disease - Advances in Pathogenesis and Management

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This book is dedicated to inflammatory bowel disease, and the authors discuss the advances in the pathogenesis of inflammatory bowel disease, as well as several new parameters involved in the etiopathogeny of Crohn's disease and ulcerative colitis, such as intestinal barrier dysfunction and the roles of TH 17 cells and IL 17 in the immune response in inflammatory bowel disease. The book also focuses on several relevant clinical points, such as pregnancy during inflammatory bowel disease and the health-related quality of life as an end point of the different treatments of the diseases. Finally, advances in management of patients with inflammatory bowel disease are discussed, especially in a complete review of the recent literature.

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