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Prevalence of anti-gliadin and anti-tissue transglutaminase antibodies in women with Vulvovaginal Candidiasis and relationship with celiac disease

A thesis

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بسم الله الرحمن الرحيم

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Dedication

To the messenger of Allah

(Allah preys upon him)

To the eye of life

(Imam al-Mahdi)

To my family for their preying, supporting and encouragement

To everyone who help me I dedicate this work

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Summary

Gliadin is an important protein of gluten that is responsible for the development of celiac disease (CD). Therefore, detection of anti-gliadin antibodies is an old test for diagnosis of CD which is replaced now by other serological tests such as anti-tissue transglutaminase (anti-tTG) test. *Candida* spp. may induce production of anti-gliadin antibodies which proposed to be in correlation with CD.

A case-control study was designed to evaluate the possibility of vulvovaginal candidiasis to elevate the levels of anti-gliadin antibodies in patients without CD. It included 90 subjects divided into two groups: the first was 50 patients with vulvovaginal candidiasis and the second group was 40 healthy women. Swab samples were collected from all subjects for diagnosis of *Candida* infection in the vagina. Serums of all subjects were analyzed for detecting anti-gliadin IgA and IgG and also anti-tTG IgA antibodies by ELISA assay.

Vulvovaginal candidiasis infection was determined among patients and found that age group 24-32 years represented a high number of infected patients (40%), followed by aged 15-23 years (36%). The duration of infection was mostly found within the range from less than 1 week to 2 weeks (66%),especially in age 24-32 years (30%), while less duration was from 9 to 13 weeks (2%).

Treatment of vulvovaginal candidiasis was registered among 31 infected women, especially those aged 15-23 years and 19 without treatment. Most patients were used antifungal treatment for one week (38%) and less number was treated for 4 to more than 5 weeks (8%).

Measurement of anti-gliadin, IgA and IgG, and anti-tTG IgA antibodies revealed negative results among all patients and control groups. A high titer with positive results of anti-gliadin IgA was only observed in 4 patients (8%) and a moderate elevation of this antibody was found among 7 patients (14%). The age of 4 positive patients with anti-gliadin IgA was distributed between 2 patients at age 24-32 years (4%) and other two was found in age group 15-23 years and in group 33-41 years. The other 78% of negative results to anti-gliadin was found more frequently in age group 15-23 years (32%) and in group 24-32 years (26%).

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Lists of Abbreviations

Abrevation	Details
Anti-GA	Anti-gliadin antibodies
Anti-tTG	Anti-tissue transglutaminase
BSA	Bovine serum albumin
CAL	Calibrator
CD	Celiac disease
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysial autoantibodies
ESPGHAN	European Society of Pediatric Gastroenterology and Nutrition
HLA	Human leukocyte antigen
Hwp1	Hyphal wall protein
IgA	Immunoglobulin alpha
IgG	Immunoglobulin gamma
IIF	Indirect immunofluorescence
IL-9	Interleukin 9

kDa	Kilo Dalton
NaCl	Sodium chloride
PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
RVVC	Recurrent vulvovaginal candidiasis
SD	Stander deviation
SDA	Sabouraud’s dextrose Agar
SDB	Sabouraud’s dextrose broth
TG	Transglutaminase
TMB	Tetramethylbenzidine
TMB/H_2O_2	Tetramethybenzidine and hydrogen peroxide
tTG	Tissue transglutaminase
VVC	Vulvovaginal candidiasis

Chapter one

Introduction

1.Introduction

Vulvovaginal candidiasis (VVC) is a high prevalence and incidence infection among women worldwide that caused by pathogenic activity of *Candida* spp. (Gonçalves *et al.*, 2016). The most common species of *Candida* causing VVC is *C. albicans* which has the ability to infect women in all ages (Fidel, 2007). This type of yeast living as a member of normal flora in the vagina and can alter into pathogenic fungi under specific conditions (Gonçalves *et al.*, 2016). In addition to VVC, *Candida* spp. can associate with many other diseases in the human body such as celiac disease (CD).

The CD is one of autoimmune disease with inflammatory characters triggered by ingestion of gluten-containing foods (Fasano, 2005; Bizzaro*et al.*, 2013). It is usually diagnosed by several serological tests as well as a histological examination of intestinal biopsy (Green *et al.*, 2005). Two classes of anti-gliadin antibodies, IgA and IgG, are mostly measured by anti-gliadin test for diagnosis of CD (Brusca, 2015). This test is become non significant today due to the production of anti-gliadin antibodies is not specific to CD and it can produce under the effect of other diseases (Bizzaro *et al.*, 2013). Thus, anti-tissue transglutaminase (anti-tTG) is now acceptable for diagnosis of CD, especially the type of human recombinant TG (Reif and Lerner, 2004; Hill and McMillan, 2006; Lewis and Scott, 2006;Korponay-Szabó *et al.*, 2015; Brusca, 2015).

A possible correlation between *Candida* spp. and CD has been suggested by many studies. This correlation is built on sharing of many pathophysiological characters such as production of anti-gliadin and anti-tTG antibodies (Renga *et al.*, 2019; Aaron and Torsten, 2020).*C. albicans* has a specific protein in its cell wall called Hwp1 protein that

has a similar amino acids sequences of gliadin protein which stimulate production of anti-gliadin antibodies (Nieuwenhuizen *et al.,* 2003). In the absence of CD, two cases of chronic mucocutaneous candidiasis were mentioned to have elevation in the levels of anti-gliadin antibodies (Garcia *et al.*, 2002; Brinkert *et al.*, 2009). On the other hand, infection with *C. albicans* was found encouragement in patients with CD (Harnett *et al.*, 2017; Aaron and Torsten, 2020).

Aims of the study:

1- Evaluation the ability of *Candida* spp. for stimulating production of anti-gliadin antibodies in women with vaginal candidiasis.
2- Determine false positive diagnosis of celiac disease in infected patients with vulvovaginal candidiasis in depending on measurement of anti-gliadin antibody only.

Chapter two

Literature review

2. Review of literatures:

2.1 Vulvovaginal candidiasis

Occurring of *Candida* spp. as a member of normal flora in the human body can be changed from commensally into infectious state under specific conditions (Spampinato and Leonardi, 2013; Surain and Aggarwal, 2020; d'Enfert *et al.*, 2020). Candidal infection could be either superficial in the skin and mucous membranes with no threat on the human life or invasive with life-threatening (Spampinato and Leonardi, 2013). The common type of invasive infection by *Candida* spp. are development in immunocompromised patients (Surain and Aggarwal, 2020). The most medical important species of *Candida* are seven, including *C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. stellatoidae, C. krusei*, and *C. kyfer* (Kewaunee and Allen, 2007). *C. albicans* is the common infectious species with a high virulence to infect human due to its obligatory association with warm blood animals (McCullough *et al.*, 1996; d'Enfert *et al.,* 2020;Surain and Aggarwal, 2020). It can produce fungal infections in various location of the human body which all called candidiasis that can take the name of site of infection, such as oral candidiasis in oral cavity and vulvovaginal candidiasis (VVC) in the vagina (Fidel, 2007; d'Enfert *et al.*, 2020). Other species of *Candida*, including *C. glabrata, C. tropicalis* and *C. parapsilosis*may associate with increasing candidal infection into 95% (Surainand Aggarwal, 2020).

Vulvovaginal candidiasis (VVC) is one of common type of *Candida* infections in the human body with a high prevalence rate that can reach to a millions every year (Gonçalves *et al.*, 2016). Under normal condition, the vagina controlled the growth of its content of *Candida* spp. by the

activities of *Lactobacillu*s bacteria and cellular and humoral immunity (Ferrer, 2000). Thus, any disturbance in any of these controlling factors will encourage *Candida* spp. to overgrowth and cause VVC (Gonçalves *et al.*, 2016). In addition to the normal living *Candida* spp. in the vagina, it can be getten from other sources such as perineum, gastrointestinal tract or from sexual transmission (Surain and Aggarwal, 2020). *C. albicans* is the most common species causing Vulvovaginal candidiasis and it represented a significant problem in early age women (Fidel, 2007). Other non-*Candida albicans* Candida species are also diagnosed as a causative agents of Vulvovaginal candidiasis, especially by *C. glabrata* (Gonçalves *et al*., 2016).

Several factors can associate with increasing the development of VVC, including pregnancy, a long treatment with antibiotics or using estrogen oral conceptive, pills or corticosteroids, diabetes and immunodeficiency state (Surain and Aggarwal, 2020). Thus, recurrent of Vulvovaginal candidiasis is more common due to the presence of such predisposing factors which may reach to four episode or more per year (Matheson and Mazza, 2017). Generally, cases of VVC can be categorized into complicated (10%) and uncomplicated (90%) depending on clinical features, microbiological findings, significant of therapy and host factors (Ilkit and Guzel, 2011).

The Vulvovaginal candidiasis infection may show variable symptoms ranging from most common such as vulvar pruritus and less such as vaginal discharges which are cottage cheese-like with a watery or homogeneously thick characters, and other symptoms vary such as irritation, vaginal soreness, vulvar burning, external dysuria and dyspareunia (Sobel, 2005).

Diagnosis of Vulvovaginal candidiasis is mainly depending on the clinical features and microbiological tests for direct Microscopical fungal detection and culturing (Ilkit and Guzel, 2011).

2.2 Celiac disease

Celiac or coeliac disease (CD) is one of the immunomediated chronic disease with an inflammatory nature resulting from ingestion of food containing gluten protein by genetically susceptible individuals (Fasano, 2005; Bizzaro *et al.*, 2013). It affects 1% of the general population all over the world in both children and adults (Nardecchia *et al.*, 2019; Caio *et al.*, 2019). The prevalence of Celiac disease is around one case in every 100-200 individuals (Bizzaro *et al.*, 2013). The ratio of Celiac disease in male to female is 1:2.8 (Brusca, 2015). Gluten, which is mainly found in many plant grain such as wheat, rye, barley, triticale and others has a role to trigger T-cell mediated autoimmune enteropathy after adherence on mucous layer of stomach in susceptible individuals leading to variable effects on the intestinal layers, including villous atrophy, damage of intestinal layers by inflammation, and several other effects in extraintestinal regions (Silano *et al.*, 2009; Elzoghby *et al.*, 2015; Biesiekierski, 2017; Brusca, 2015; Sharma *et al.*, 2020). Thus, symptoms of Celiac disease are variable between intestinal related symptoms including malabsorption syndrome (weight loss, abdominal distention and chronic diarrhea) and extra-intestinal related symptoms affecting other organs of the human body (Fasano, 2005; Nardecchia *et al.*, 2019). The tolerance of gluten by CD patients is usually variable between 10 mg to 34-36 mg of gluten per day (Akobeng and Thomas, 2008).

The vast majority susceptible individuals for Celiac disease are expressed two main types of HLA class II antigen-presenting receptor called HLA-DQ2 protein, which is most common in patients with CD

(90%-95%), and HLA-DQ8 which is less common protein (5%-10%) (Fasano, 2005; Silano *et al.*, 2009; Bizzaro,*et al.*, 2013; Caio *et al.*, 2019; Sharma *et al.*, 2020). After study the structure of gluten protein, it suggest that a specific 33-mer peptide of gluten structure in α gliadin type of gluten that represented epitope is the trigger of intestinal inflammation to gluten in patients with CD and this peptide is stable to the destruction effect of gastric, pancreatic and intestinal brush border proteases (Shan *et al.*, 2002; Silano *et al.*, 2009; Brusca , 2015; Biesiekierski, 2017;Barone *et al.*, 2014).

Diagnosis of CD mainly depends on different criteria, including symptoms and serological tests and the results of these tests can determine the decision to make a third step of diagnosis represented by histological examination of duodenal biopsy to identifing tissue change due to reduction in villous height (Green *et al.*, 2005). Detection of positive result with ant-tissue transglutaminase (anti-tTG) is the most significant indicator to move to the next step of histological analysis of biopsy (Reif and Lerner, 2004). Small-bowel biopsy is considered a diagnostic key for detection CD since 1950 (Rauhavirta *et al.*, 2019). In general, an important serological tests for CD are detecting of antibodies against reticulin, deamidated gliadin, transglutaminase 2 and antiendomysium antigens (Hill and McMillan, 2006; Korponay-Szabó *et al.*, 2015; Brusca, 2015). These tests were revealed higher specificity and sensitivity for diagnosis of CD in patients after compared with a control group (Huebener *et al.*, 2015).

2.3 *Candida* species

Candida is an important genus of yeast with 200 known species which all related to the kingdom of fungi and represented 1% from all 1500 known fungal species (Hameed *et al.*, 2018). It is dimorphic fungi

with many morphological forms ranging as in *C. albicans* from yeast, pseudohyphae to true hyphae forms (McCullough *et al.*, 1996; Kobayashi and Cutler, 1998;Surain and Aggarwal, 2020). The recent taxonomy of *Candida* species after discover sexual stage of some species as follow; Kingdom: Fungi, Phylum Ascomycota, Class: Ascomycetes, Order: Saccharomycetales, Family: Saccharomycetaceae, Genus: *Candida* (Hameed *et al.*, 2018).

Most *Candida* species lives in commensalism with animals and human body, while some of them are saprophytic in environment (Hameed *et al.*, 2018; d'Enfert*et al.*, 2020; Surain and Aggarwal, 2020). They can find as a colonized fungi on the surface of skin and other mucous membrane of the host (Kewaunee and Allen, 2007). A great number of *Candida* species can't tolerate 37° C which makes them non pathogens, while only thirty species have like this temperature tolerance (Hameed *et al.*, 2018). The most important species of *Candida* for the human body is *C. albicans*, followed by other species such as *C. glabrata, C. parapsilosis, C. krusei, C. tropicalis* and *C. kefyr* (Turner and Butler, 2014;Hameed *et al.*,2018).

Morphological characteris of *Candida* spp. is mainlly depended on the form of this fungi such as they take round cells in yeast form, elongated and ellipsoid cells with a branching pattern in pseudohypha form, and long without constrictions between cells in hyphae form (Surain and Aggarwal, 2020). Name of *Candida* is originally derivative from the Latin term "Candidus" which means glowing white as indicator for the white-creamy colony of this fungi when growing on culture media (Hameed *et al.*, 2018).

2.4 *Candida* and CD

An association between CD and fungi has been demonstrated by many studies. Serological markers play an important role to prove such correlation. *C.albicans* is the most fungal species shows a correlation with CD (Aaron and Torsten, 2020). Multiple pathophysiological characters are sharing between *C. albicans* and CD, including stimulates production of several serological markers such as anti-gliadin and anti-tTG in either of *C. albicans* or CD, in addition to produce IL-9 with evoke of T and B cells activities (Aaron and Torsten, 2020). The factor responsible for such type of sharing is hyphal wall protein (Hwp1) of *C. albicans* cell wall which has homologous amino acides sequences to α- and γ-gliadin and it has a role in facilitation adhesion of fungi with the epithelial tissue (Nieuwenhuizen *et al.*, 2003). Thus, the humoral immunity can stimulate production antibodies against gliadin when exposure to Hwp1 of *C. albicans* due to a cross reactivity between it and gliadin protein (Corouge *et al.*, 2015; Aaron and Torsten, 2020; Boutrid *et al.*, 2019). The Hwp1 is considered a substrate to TG enzyme of fungi or in other containing organisms like a human (Staab *et al.*, 1999; Aaron and Torsten, 2020). Thus, infection with *C. albicans* can promote development of CD by fungal content of Hwp1 protein as analogous of gliadin protein , In a case control study included patients with either systemic *C. albicans* infection or with CD and a healthy control group, higher levels of anti-gliadin, anti-Hwp1, and anti-tTG IgA were found in both groups of patients compared with control group (Corouge *et al.,* 2015).

On the other hand, patient with CD can encourage infection with *C. albicans*. The fungus *C. albicans* was successfully isolated from 33% of patients with CD, while it was 0% in control group (Harnett *et al.*,

2017). The clinical short damaged enterocytes of CD patient become easy to infect with intestinal *C. albicans*(Aaron and Torsten, 2020). Immunological factors of cytokine IL-9 and mast cells induced by CD have a role in converting intestinal *C. albicans* from commensal into pathogenic fungi (Renga *et al.*, 2019). A hypothesis assumed by Nieuwenhuizen *et al.,* (2003) that Hwp1 of *C. albicans* which homologous to gliadin T-cell epitopes, can induce CD through covalently link with TG releasing from intestinal epithelial cells under specific condition. This conjucated between Hwp1 and TG will stimulate immune cells to produce antibodies against gliadin represented by Hwp1 and also against TG enzyme.

2.5 Anti-gliadin test

Gluten is a complex protein composed from a hundred of related different proteins (Bizzaro *et al.*, 2013; Biesiekierski, 2017). It is mainly found in wheat plant as a storage protein (Brusca, 2015;Biesiekierski, 2017). Another similar protein to gluten within the same prolamins group also have the same name such as secalin in rye, hordein in barley and avenins in oats (Silano*et al.*, 2009;Brusca, 2015). Commonly used of gluten is as an additive in foods to improve the texture, flavor and moisture retention and the average daily consumed of gluten in Western diet is about 5-20 g/day (Biesiekierski, 2017). Gliadin and glutenin are the most common types of gluten proteins (Qi *et al.*, 2006; Bizzaro*et al.*, 2013;Brusca, 2015; Biesiekierski, 2017).

Gliadin is a single polypeptide chain of gluten with a molecular weight of 25-100 kDa linked by disulfide bonds (Qi *et al.*, 2006; Elzoghby *et al*., 2015). It represented 40-50% of the total protein in wheat seeds (Qi *et al.,* 2006). Gliadin consists of about 50 fractions that grouped

into four classes based on biochemical analysis; α, β, γ, and ω, while glutenin consists from low and high molecular weight protein (Qi *et al.*, 2006; Bizzaro*et al.*, 2013).

The complex structure of gluten can induce production several types of antibodies in the human body. Gliadin as the main component of gluten will modified by tissue transglutaminase (tTG) enzymes to have negatively charged glutamic acid that facilitated binding of gliadin with HLA-DQ2 or DQ8 complex on the T cell surface and induce cell and humoral response to produce antibodies against gliadin (anti-gliadin) and tTG (anti-tTG) (Shan *et al.*, 2002; Bizzaro, *et al.*, 2013).The amino acid glutamine consists of 35% gliadin structure and it may represent the center of gliadin to induce CD (Reif and Lerner, 2004). Antibodies against gluten were first mentioned in the late of 1950 in the serum of patient with CD (Bizzaro *et al.*, 2013). Thus, a diagnostic kit of gluten epitopes was prepared to detect gluten antibodies in the serum, saliva and small intestinal aspirate samples of patients with CD (Kelly *et al.,* 1991; Brusca, 2015). Such a kit could also used to clarify needed for biopsy from patient with CD (Bahia *et al.*, 2001). Anti-gliadin test with its two classes IgG and IgA have been used for decades in immunoassay methods for diagnosis of CD (Brusca, 2015). Most applicable methods used to detect two classes of anti-gliadin are indirect immunofluorescence (IIF) and ELISA (Bizzaro *et al.*, 2013). The sensitivity of anti-gliadin for diagnosis of CD by ELISA was about 90.9% for IgG and 95.5% for IgA, while, specificity was 97.8% for IgG and 95.7% for IgA (Bahia *et al.*, 2001). IgA anti-gliadin is the most valuable type of antibodies to diagnosis or follow up CD than IgG with a 84% sensitivity and 95% specificity (Kelly *et al.*, 1991).In the recent systematic review by the European Society of Pediatric Gastroenterology and Nutrition (ESPGHAN) Working Group on CD Diagnosis in 2012, show sensitivity

of IgA anti-gliadin ranged from 60.9% to 96%, and specificity ranged from 79.4% to 93.8% (Bizzaro *et al.*, 2013).

The large content number of epitopes in gliadin structure make anti-gliadin test less accurat to detect CD due to non specific antibodies production (Bizzaro*et al.*, 2013; Brusca, 2015). Production of IgG and IgA of anti-gliadin in patients with other than CD is another reason to limit the specificity and sensitivity of anti-gliadin test for CD (Bizzaro*et al.*, 2013). Factors related to serum contents of globulins, enzymes, hormones and nutrient are also have a role to give a false positive results of anti-gliadin test (Ortiz *et al.*, 2011). Thus, other serological tests have been suggested for diagnosis of CD such as anti endomysial autoantibodies (EMA), and later of anti-tTG(Bizzaro*et al.*, 2013). Deamidated gliadin antibodies (IgA and IgG) is found more sensitive and specific tool for diagnosis of CD than anti-gliadin test and in similar values with anti-tTG test (Rashtak *et al.*, 2008). A complex antigen of tTG and gliadin peptide in a third generation kit is another choose to increase the diagnostic accuracy of anti-gliadin compared with use of gliadin or tTG antigens alone (Hill and McMillan, 2006).

2.6 Anti-tissue transglutaminase(anti-tTG) antibody

Transglutaminase-2 (TG2) or also called tissue transglutaminase-2 (tTG2) is a protein related to the family of eight enzymes of transglutaminase group that have a function on irreversible catalyze the cross-linked protein containing glutamine amino acid to a protein with lysine residue (Hill and McMillan, 2006; Brusca, 2015). The tTG2 which is found in almost all cell types has many other functions in addition to enzymatic activity such as its association with cell adhesion, cell signaling, G-protein activities, tissue repair, and removal of cell debris

after cell apoptosis (Carroccio *et al.*, 2001; Reif and Lerner, 2004; Brusca, 2015).

The state of tTG2 is usually inactive within intracellular region of the tissue and become active under the effects of many mechanical or inflammatory factors (Hill and McMillan, 2006). Activation factors may include the absence of lysine, that needed by cells, in which it makes tTG2 act on deamidates a protein like gliadin enriched with glutamine to produce a negative charged glutamic acid (Hill and McMillan, 2006). Thus, gliadin will cross-linked with tTG2 to form a complex form taken by B-cells to promote its production of autoantibodies against CD, including tTG2 (anti-tTG2) and endomysial antibodies (EMA) (Carroccio*et al.*, 2001; Reif and Lerner, 2004; Hill and McMillan, 2006). The T-cells are also associated with the production of tTG2 antibodies by B cells through recognition of gluten complex on HLA-DQ peptide in patients with CD and that what makes production of anti-tTG2 is more specific to CD (Reif and Lerner, 2004). These antibodies used as a specific tests for diagnosis of CD since 1997 (Carroccio *et al.*, 2001; Reif and Lerner, 2004; Hill and McMillan, 2006; Korponay-Szabó *et al.*, 2015). Test for tTG2 and endomysial antibodies have been revealed more sensitivity (93% for both) and specificity (>99% for EMA and >98% for anti-tTG2) for diagnosis of CD (Lewis and Scott, 2006). The IgA antibodies for both tTG and EM also showed to have 90% of sensitivity and specificity (Green *et al.*, 2005).In general, anti-tTG test have more advantages than EMA due to that anti-tTG is more easy to standardization and automated detection without need to use a primate tissue (Hill and McMillan, 2006). Thus, detection of anti-tTG become a first-line assay for diagnosis of CD and could also more specific than histological analysis (Reif and Lerner, 2004;Korponay-Szabó *et al.*, 2015).

The first use of tTG as antigen for anti-tTG test was that extracted from the liver of guinea pig, then replaced with a purified human tTG or human recombinant tTG (Hill and McMillan, 2006). Nowadays, human recombinant TG is more preferable to use than from guinea pigs due to the high sensitivity and specificity of the first one in diagnosis of CD in children and adults (Hill and McMillan, 2006; Lewis and Scott, 2006; Brusca, 2015). Also, human recombinant TG have an ability with a number of economic and practical advantages for diagnosis asymptomatic CD patients (Lewis and Scott, 2006). Moreover, results of anti-tTG of guinea pig can give a false positive result in patient with chronic liver diseases without CD due to the content of diagnostic kit from liver proteins of guinea pig, while this is not found in recombinant human kit (Carroccio *et al*., 2001). IgA anti-tTG is more sensitive and specific marker for diagnosis of CD by using ELISA assay (Reif and Lerner, 2004). The IgA-tTG of human recombinant has 90.2% sensitivity and 95.4% specificity in both adults and children, while it has a sensitivity ranged from 95% to 100% and specificity ranged from 97% to 100% in adult (Rostom *et al,*. 2005;Brusca , 2015). In the review of 5 studies about diagnostic value of anti-tTG showed that IgA-tTG of guinea pig liver in adult showed 88-100% sensitivity and 92-97% specificity and in children showed 93.1% sensitivity and 96.3% specificity, while human recombinant anti-tTG in adult showed 98.1% sensitivity and 98% specificity and in children about 95.7% and 99%, respectively (Rostom *et al.*, 2005).

It is difficult to obtain 100% sensitivity and specificity from serological test of CD (Hill and McMillan, 2006). This is also true with anti-tTG test, in which false positive or negative results is noted in some cases with diseases not related to the CD. The IgA and IgG classes of anti-TG2 has been observed in patients with viral infection, inflammatory

bowel disease or those with end-stage heart failure (Rauhavirta *et al*., 2019). Chronic liver diseases and myeloma could give a false positive result with IgA-tTG, while negative false result can also observed in patients who have a positive histological and other serological markers of CD (Hill and McMillan, 2006). In such cases, a biopsy analysis should clarified the results (Reif and Lerner, 2004).

2.7 *Candida* and anti-gliadin

The hypothesis of Nieuwenhuizen *et al.* (2003) about the role of *C. albicans* in the production of anti-gliadin antibodies after binding of its Hwp1 protein with TG of enterocytes as a trigger of CD is more acceptable explanation now days. Strong evidences have been introduced to support this hypothesis after finding of higher levels of anti-gliadin, anti-Hwp1, and anti-TG antibodies in patient with CD or *C. albicans* than healthy control (Renga *et al*., 2019; Lerner and Matthias, 2020). Investigation for the anti-gliadin antibodies in patient with CD and infected with *C. albicans* has been shown a positive result (Aaron and Torsten, 2020). A higher level of anti-gliadin without a significant difference is found in both patients with Celiac disease and those with *C. albicans* infection compared to healthy control (Corouge *et al.,* 2015).

Infections with *C. albicans* in patient without CD could have a role to promote production of anti-gliadin antibodies even in the patients have no signs of CD. Two cases of candidiasis showed an elevation in anti-gliadin levels in patient without CD. A 13-year-old boy with chronic mucocutaneous candidiasis and without clinical features of Celiac disease or dermatitis herpetiformis revealed elevation in anti-gliadin levels (Garcia *et al*., 2002). Another case of 4-year-old immunocompromised boy infected with chronic mucocutaneous candidiasis, but without Celiac

disease also had high levels of anti-gliadin during infection period (Brinkert *et al.,* 2009). Thus, infection with *Candida* in both immunocompetent and immunocompromised people could be expected to induce production of anti-gliadin antibodies without the presence of CD.

Chapter three

Materials and Methods

3. Materials and Methods:

3.1.Materials:

3.1.1. Equipments and apparatus

The equipments and apparatus used during the study are listed in Tables (3-1 and 3-2):

Table 3-1: Equipments used in the present study

Instrument	Company	Origin
Autoclave	Harayma	Japan
Bunsen burner	Germany	Jenway
Oven	Memmert	Germany
Sensitive balance	Sartorius	Germany
Multi-channel micropipette	Hamilton company	U.S.A.
Ordinary centrifuge	Hittich	Germany
Incubator	Fisher scientific,	U.S.A.
EISA system	Biotest	Germany
Refrigerator	Concord	Italy
pH Meter	Hanna	Romania
Compound microscope	Leica	Germany
Biological safety cabinet	Lab Tech	Korea

Table 3-2: Apparatus used in the present study

Equipment and Apparatuses	Manufacture company	Origin
Conical flasks	BBL	USA
Disposable Petri dishes	BBL	USA
Disposable Loop	Loop Shandon	England
Eppendorf tube	Sigma	England
Filter paper	Difco	USA
Gloves	TG medical	Malaysia
Glass tubes	BB	USA
Sterile cotton swabs	MEDI	China
Slides and cover slides	BBL	USA
Sterile syringes	MEDI	China
Cotton	AlanaamPharma	Iraq
Micropipette tips (different size)	Volac	England
Pipettes with different size	Volac	England.
Plane tubes	AFCO	Jordan
Measuring cylinder (50,100ml).	Germany	Marienfeld

3.1.2. Chemical and Biological Materials

The chemical and biological materials used during the study are summarized in Table (3-3):

Table 3-3: Biological and chemical materials were used in the present study

Materials	Manufacture company	Origin
Alcohol (70%)	AlanaamPharma	Iraq
Sabouraud's dextrose Agar (SDA)	Himedia	India
Sabouraud's dextrose broth (SDB)	Himedia	India
Transport media	Himedia	India
Gram stain	Himedia	India

3.1.3 Diagnostic Kits

Table (3-4) Kits using in the study

No.	Kit name	Manufacture company	Origin
1	ELISA to IgG anti-gliadin	Wendelsheim	Germany
2	ELISA to IgA anti-gliadin	Wendelsheim	Germany
3	ELISA to IgA anti-tTG	Wendelsheim	Germany

3.1.4. Kit Contents and Reagents

Table (3-5): Content of IgG or IgA anti-gliadin kit

Reagents	Content
96 well ELISA Microtiter plate	Microplates coated with the specific antigen
Sample Buffer	Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Wash Buffer	Tris, NaCl, Tween 20, sodium azide< 0.1% (preservative)
Negative Control	Human serum (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Positive Control	Human serum (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Cut-off Calibrator	Human serum (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Calibrators	Concentration of each calbrator: 0, 3, 10, 30, 100, 300 U/ml. Human serum (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Conjugate, IgG or IgA	Containing: Anti-human immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA)
TMB Substrate	Stabilized tetramethybenzidine and hydrogen peroxide (TMB/H2O2)
Stop Solution	1M Hydrochloric Acid

Table (3-6): Content of IgA anti-tTG kit

Reagents	Content
96 well ELISA Microtiter plate	Microplates coated with the specific antigen
Sample Buffer	Tris, sodium chloride (NaCl), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Wash Buffer	Tris, NaCl, Tween 20, sodium azide< 0.1% (preservative)
Negative Control	Control material (diluted), bovine serum albumin (BSA), sodium azide<0.1% (preservative)
Positive Control	Control material (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Cut-off Calibrator	Calibration material (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Calibrators	Concentration of each calbrator: 0, 3, 10, 30, 100, 300 U/ml. Calibration material (diluted), bovine serum albumin (BSA), sodium azide< 0.1% (preservative)
Conjugate, IgA	Containing: immunoglobulins conjugated to horseradish peroxidase, bovine serum albumin (BSA)
TMB Substrate	Stabilized tetramethybenzidine and hydrogen peroxide (TMB/H2O2)
Stop Solution	1M Hydrochloric Acid

3.2. Methods

3.2.1 Culture media

3.2.1.1 Sabouraud's dextrose agar (SDA)

Sabouraud's dextrose agar (SDA) was prepared according to the manufacturer's instructions by suspending 65 gm of SDA powder in 1 liter of distilled water. Final pH was adjusted to 6.8 and then 0.05 g/L of chloramphenicol antibiotic was added after autoclaving. This medium was used for cultivating candida spp from clinical sample. About 0.05 g/L of chloramphenicol was added to prevent growth of contaminating bacteria (AL-Janabi, 2011).

3.2.1.2 Sabouraud's Dextrose Broth (SDB)

This medium was prepared by dissolving 65 gm of SDB powder in one liter of distilled water. The pH was adjusted to 6.8 by pH meter and then sterilized by autoclave at 121 C° temperature and 15 Pa pressure for 15 minutes. The medium used to activate *Candida* isolates (AL-Janabi, 2011).

3.2.2. Preparation of Gram stain

Gram staining is a common method in microbiology laboratory for distinguishing between two large classes of bacteria based on their cell wall constituents. Also Gram staining can use to identify yeast. The stain consists of crystal violet (purple or blue), iodine (modrant), ethanol (95%) decolorization to distinguish Gram-positive from Gram-negative, and safranin (counter stain-red dye) to staining isolates and examination under the microscope (Tille, 2014).

3.2.3. Patients

A total of 50 infected women with vaginal candidiasis (age rang; 15-59 years) and 40 healthy women as control group (age rang; 20-30 years) were enrolled in this case-control study during attended AL-Zahraa

Hospitals in AL-Najaf Governorate from November 2020 to February 2021. Vulvovaginal Candidiasis (VVC) was clinically diagnosis in patients group by the Gynecological consultant of the hospital as the first step. Infection with *Candida* spp. was later confirmed by microscopically identification of the presence of *Candida* spp in the vaginal wet smear and growing on culture media. Patients with history of any autoimmune diseases such as CD, rheumatoid arthritis and type 1diabetes and those under hormone treatment were excluded from this study.

3.2.3.1. Collection of serum samples

About 5 ml of venous blood was collected from every involved subject. Blood was left to coagulate in the tube without EDTA. Coagulated samples were centrifuged at 4000 rpm for 5 minutes. Serum was collected in a new plane tube which was labeled with the patients name, age and sex**.** About 1.5 ml of collected serum was kept in freeze (-20°C) for detection later, not more than 12 weeks.

3.2.3.2. Collection of yeast samples

A double swab from the vaginal area was collected from every involved subject. Direct Microscopical examine was performed to one swab for detecting live *Candida* species. A swab was mixed with a drop of distilled water on glass slide and left to dry. Smear was stained with Gram stain and tested under the light microscope to determine the morphological characters of positive present of yeast. Another collected swab was cultured on transport media to immediately transfer to the laboratory to culture on plate with SDA. Inoculated plates were incubated at 37ºC for 24-48 hr. Identification of yeast species was performed by manual method.

3.2.4. Morphological identification

3.2.4.1.Identification Candida on Sabouraud Dextrose Agar Medium

All collected samples were cultured on Sabouraud dextrose agar (SDA); the colonies of Candida spp. were cream colored to yellowish, grow rapidly at 24-48 hr., the morphology of the colony is smooth, glistening or dry depending on the species. These results were agreed with (Bhavan et al., 2010).

3.2.4.2.Microscopic identification by Gram stain of Candida Species

Microscopic examination is a preliminary test to diagnose the candida ssp. Each sample was stained with gram stain and examined microscopically.

Smears from the vaginal swap sample were prepared on slides cleaned with alcohol . They then heat-fixed , staining was done by flooding the smears with crystal violet solution for 1 min and then with iodine for 1 min. After washing,the smears were decolorized with 95 % ethanol and counter strained with safranin stain . The slide was subjected to observation of *Candida* morphology under oil immersion objective lens (100 x) of a positive result Bright Field microscope (Bhavan et al., 2010).

3.2.5. Serological Tests

The sera of patients and controls are assessed for the level of two antibodies types of anti-gliadin (IgA and IgG) and one of IgA anti-tTG using of ELISA method. Principle of all three tests that serum sample was diluted 1:101 and incubated in the microplates coated with the specific antigen. Patient´s antibodies, if present in the specimen, will bind to the antigen. The unbound fraction is washed off in the following step. Afterwards anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are incubated and react with the antigen-antibody

complex of the samples in the microplates. Unbound conjugate is washed off in other step.

Addition of TMB-substrate can generate an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The intensity of color formation from the chromogen is a function of the amount of conjugate bound to the antigen-antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample.

3.2.5.1 Assessment serological diagnosis of antibodies to anti-gliadin and anti-tTG

Highly purified alpha-gliadin was used in ELISA kit to detect IgG or IgA antibodies against gliadin protein in human serum. Serological test for anti-tTG is depended on the detection of IgA antibodies against neoepitope of tissue transglutaminase (tTG) in human serum. A new generation kit of tTG was used that contain a mixture of human recombinant transglutaminase cross-linked with gliadin-specific peptides. This kit can give more significant sensitivity and specificity for diagnosis of anti-tTG antibodies.

3.2.5.2 General Protocol

All of three serological tests have the same protocol as described by the manufacturer company which is as followed:

1- Solutions of reagents and buffers were all prepared based on the manufacture instructions.

2. A 100 µl of either of calibrators (CAL A to CAL F), cut-off calibrator, positive control, negative control, or patient serum was added separately in a single well of the microplate.

3- Microplate was incubated for 30 minutes at 20-32°C, then washed three time with 300 µl washing buffer (diluted 1:50).

4- A 100µl of conjugate was added into each well.

5- Microplate was incubated for 30 minutes at 20-32°C, then washed three time with 300 µl washing buffer (diluted 1:50).

6- A 100 µl TMB substrate was added into each well.

7. Microplate was incubated for 30 minutes at 20-32°C with protection from intense light.

8- A 100 µl stop solution was added into each well, using the same order as with the substrate, and then incubated for 5 minutes minimum.

9- Microplate was agitated carefully for 5 sec.

10- Absorbance was read at 450 nm (recommended 450/620 nm) within 30 minutes.

11- Concentration of antibodies was automatically calculated by the ELISA instrument.

12- The normal range recorded by the manufacture company of each three types of antibodies (IgG and IgA anti-gliadin and IgA anti-tTG) was the same: < 12 U/ml is normal value, 12-18 U/ml is equivocal value, and > 18 U/ml is a positive value.

3.3. Statistical Analysis

Data of all tests were analyzed statistically with one-way ANOVA by using Excel application of Window 7. The minimum level of (*p*) value was < 0.05 concerts as significant level.

Chapter four

Results

4. Results:

4.1. Duration of infection with VVC

VVC was primarily diagnosed in involved patients depending on clinical features and confirmed by microscopic and cultured characters. The duration of infection was determined for all patients depending on age factor. Age group from 24 years to 32 years was represented a high number of infected patients (40%), followed by aged 15-23 years (36%). Meanwhile, older patients (51-59 years) showed low percentage of infection (4%). Moderate number of infection was observed in patients aged from 33 to 50 years (Table 4-1).

Most patients were suffered from infection for less than one week to two weeks (66%), while those with nine to 13 weeks were found in less number of patients (2%). Among patients with < 1-2 weeks of infection, those at age 24-32 years were the most infected group (30%) with a significant difference from other groups at $p < 0.05$, followed by age group 15-23 years (22%), while less age group with infection within this duration time was 51-59 years (2%). A single patient at age 24-32 years was suffered from VVC for a long time (9 to 13 weeks), while one from the same age and another from age group 33-41 years were suffered from infection for 6 to 8 weeks (Table 4-1). Thus, infection with *Candida* spp. in the vagina could not be continued for a long time and it may decrease with increasing of age.

Table (4-1): Duration of the infection with vulvovaginal candidiasis

Age (year)	No. of patients				Total No. (%)
	Duration of infection (week)				
	< 1-2	3-5	6-8	9- 13	
15-23	11*,** (22%)	7 (14%)	0	0	18 (36%)
24-32	15*,** (30%)	3 (6%)	1 (2%)	1 (2%)	20 (40%)
33-41	4* (8%)	1 (2%)	1 (2%)	0	6 (12%)
42-50	2 (4%)	2 (4%)	0	0	4 (8%)
51-59	1 (2%)	1 (2%)	0	0	2 (4%)
Total No. (%)	33 (66%)	14 (28%)	2 (4%)	1 (2%)	50

* Significant differences between duration of infection in the same age group

** Significant differences between ages in the same duration of infection

4.2 Treatment duration of vulvovaginal candidiasis

Patients with VVC were treated with antifungal agents for a various periods of time. There were 31 women from 50 involved patients had a treatment against VVC, while 19 patients had no treatment. High number of patients under treatment was used antifungal therapy for no more than one week (38%) and less number was treated from 4 to 5 weeks (8%). Patients aged 15-23 years were the most frequency group treated against infection (12 patients), while only one patient at older age (51-59 years) was under treatment (Table 4-2).

Most patients under treatment were aged 15 years to 23 years with duration time from less than one week to one week (14%) with a significant difference from other groups at $p < 0.05$, followed by age group 33- 41 years (8%). Treatment from 4 to more than 5 weeks was observed in two patients at age group 24-32 years (4%) and in one (2%) from each of 15-23 years and 33-41 years. There was no patient at age 33-41 years and 51-59 years treated for 2-3 weeks and also at age 51-59 years treated for 4 to more than 5 weeks (Table 4-2).

From untreated patients, those at age 24-32 years were represented high frequency age group (22%) with a significant difference from other age group, followed by patients aged 15-23 years (12%). There was no patient at aged group 42-50 years could be included within untreated member (Table 4-2).

Table (4-2): Duration of treatment from vulvovaginal candidiasis

Age (year)	No. of patients				Total No. (%)
	Treatment duration (week)			Without treatment	
	< 1-1	2-3	4-5		
15-23	7** (14%)	4 (8%)	1 (2%)	6 (12%)	18 (36%)
24-32	5 (10%)	2 (4%)	2 (4%)	11** (22%)	20 (40%)
33-41	4** (8%)	0	1 (2%)	1 (2%)	6 (12%)
42-50	2 (4%)	2 (4%)	0	0	4 (8%)
51-59	1 (2%)	0	0	1 (2%)	2 (4%)
Total No. (%)	19 (38%)	8 (16%)	4 (8%)	19 (38%)	50

** Significant differences between ages in the same duration of infection

4.3. Detection diagnostic antibodies of celiac diseases in VVC patients

Specific serological tests, including detection of antibodies against gliadin and tissue transglutaminase that used for diagnosis of CD were tested in the blood of infected women with VVC who had no clinical characteristics of CD. The results of the present case-control study showed that all involved patients with VVC had negative results for two serological tests of anti-gliadin IgG and anti-tTG IgA. Antibodies for gliadin IgA was found truly significantly positive results in only four patients (8%), while 7 patients (14%) showed none certainly positive results with anti-gliadinIgA (Table 4-3).

Non-infectious women in the control group all shown a negative result. Meanwhile, there was 78% of infected patients showed negative results for all of the tested antibodies (Table 4-3).

Table (4-3): Detection of anti-gliadin IgA, and IgG and anti-tTGIgA in patients with VVC

Subject groups	No. of patients					Total No.
	Anti-GliadinIgG	Anti-gliadin IgA		Anti-tTGIgA	Negative	
		Positive	Equivocal			
Patient	0	4*,** (8%)	7 (14%)	0	39 (78%)	50 (55.55)
Control	0	0	0	0	40 (100%)	40 (44.44)
Total No. (%)	0	4 (4.44%)	7 (7.77%)	0	79 (87.77%)	90

** Significant differences between ages in the same duration of infection

4.4. Relation of anti-gliadinIgA positive results to the age of patients with VVC

Most four patients with VVC who showed a certain positive result with anti-gliadin IgA was found in two patients at age group 24-32 years (4%), while other two positive results distributed between one patient in age group 15-23 years and another in group 33-41 years. The equivocal positive results of anti-gliadin IgA who were 7 patients was detected in high number at age group 24-32 years (10%) with a significant difference from other patients, while other two were singly observed in age group 15-23 years and 42- 50 years (Table 4-4).

From all 78% negative results of patients with VVC, negative results of anti-gliadin IgA was found more frequently in age group 15-23 years (32%) and in 24-32 years (26%). The other negative results distributed in equally between other age groups (Table 4-4).

Table (4-4): Anti-gliadin IgA levels in patients with Vulvovaginal candidiasis

Age (year)	No. of patients			Total No. (%)
	Anti-gliadin IgA (U/ml)			
	Positive (> 18)	Equivocal (12 – 18)	Negative (<12)	
15-23	1 (2%)	1 (2%)	16** (32%)	18 (36%)
24-32	2 (4%)	5** (10%)	13** (26%)	20 (40%)
33-41	1 (2%)	0	5 (10%)	6 (12%)
42-50	0	1 (2%)	5 (10%)	6 (12%)
Total No. (%)	4 (8%)	7 (14%)	39 (78%)	50

** Significant differences between ages in the same duration of infection

Chapter five

Discussion

5. Discussion

5.1. Duration of infection with Vulvovaginal candidiasis

Patients with VVC were chosen in this study and the results revealed that VVC can be appeared in various time with highly detection in the first week of infection. Vulvovaginal candidiasis (VVC) is common genital disease that affects millions of women every year (Gonçalves *et al.*, 2015). About three-quarters of all women are affected by Vulvovaginal candidiasis during their reproductive age (Dovnik *et al.*, 2015). A high prevalence of Vulvovaginal candidiasis usually cost millions of dollars due to prescription medicine or physical office visits (Fidel, 2007). The VVC could be found in a majority of non pregnant women (78%) (Yano *et al.*, 2019), while it was in 90.38% of pregnant women (Nelson *et al.*, 2013). About 29% of another group of 120 pregnant women was also registered to have Vulvovaginal candidiasis (Alsharifi, 2017). Among 100 women at reproductive age, 22% have VVC (Rati*et al.*, 2015). However, the VVC can be symptomatic or asymptomatic disease. Vaginal yeasts were successfully isolated from 22.2% of asymptomatic patients and from 42.4% of symptomatic patients where 92.1% of them had *C. albicans* (Mathema *et al.*, 2001).

Candida spp. are the most causative agent of Vulvovaginal candidiasis (Beigi *et al.*, 2004; Nelson*et al.*, 2013; Gonçalves *et al.*, 2015; Rati *et al.*, 2015). A positive culture of *Candida* spp. was found in 42% of 219 sexually active women when 15% of them were asymptomatic (Rylander *et al.*, 2004). It was estimated that 75% of all women can have Vulvovaginal candidiasis in their life time and 90% of them caused by infection with *C. albicans* (Nyirjesy, 2001). *Candida* spp. was also isolated from 27% of suspected patients with Vulvovaginal candidiasis

(Brandolt e*t al.*, 2017), while it isolated from 25.5% of 94 high school students (Essel *et al.*, 2014).

The Vulvovaginal candidiasis can be divided into uncomplicated and complicated depending on the clinical severity of infection (Sobel, 2005; Gonçalves *et al.*, 2015; Dovnik *et al.*, 2015). The uncomplicated type is characterized by mild to moderate severity with recurrent of infection fewer than four episodes per year and it mainly caused by *C. albicans* (Sobel, 2005; Gonçalves *et al.*, 2015). Whereas, complicated type that caused by non-*Candida albicans* species characterized by more severity with recurrent more than four episodes in year (Gonçalves *et al.*, 2015; Dovnik *et al.*, 2015). The uncomplicated type is the most common VVC, while only 10-20% of women suffer from complicated type (Sobel, 2005).

From the results of this study, Vulvovaginal candidiasis was diagnosed with more frequent in age group 24-32 years and aged 15-23 years, while it was low in older age (51-59 years). This was also found among pregnant women in Tikrit province of Iraq where VVC was in higher frequently at age group 25-35 years (57.1%) and least at age above 45 years (5.7%) (Alsharifi, 2017). In general, VVC is usually affected women of all ages with worldwide distributed (Lema, 2017) and a risk of VVC increases in women in age 26-35 years and decrease at older age above 45 years (Vermitsky *et al*., 2008;Nelson *et al.*, 2013; Zeng *et al.*, 2018). There is a 50% chance to get Vulvovaginal candidiasis in any time in women at age more than 25 years (Ringdahl, 2000). Thus, age greater than 26 years, in addition to other factors such as smoking, condom use, and sexual activity are all considered predisposing factors to Vulvovaginal candidiasis (Beigi *et al.,* 2004). Salvi (2019) found that VVC was in higher number among women aged 26-30 years, followed by 31-40 years and decreased in age more than 40 years. About

82.4% of patients with VVC in South of Brazil were at age 30 years or younger (Brandolt *et al.*, 2017).Although no significant correlation was observed between the prevalence of Vulvovaginal candidiasis and age among Nigerian non-pregnant women, VVC was higher in age group 20-30 years and least among those less than 20 years and greater than 40 years(Emiribe *et al.*, 2015). The high incidence of VVC was found in 21 years (50%) and 20 years (45.5%) of High School girls in Ghana (Essel *et al.*, 2014). Dominant of VVC was also reported in age 21-25 years, followed by those at 16-20 years (Swaminathan *et al.*, 2017). However, VVC caused by *C. albicans* still considered a significant problem in women of childbearing age (Fidel, 2007). In contract, Rati (2015) found that majority of infected women with Vulvovaginal candidiasis was in age belonged to 26-35 years.

5.2 Treatment duration of Vulvovaginal candidiasis

According to the results of this study, great number of patients with VVC was used local antifungal treatment for less than 1 week, while those used for more than 5 weeks were represented fewer numbers. Treatment of Vulvovaginal candidiasis is often complicated and usually gives variable results. Generally, therapy may include either topical form such as cream, lotions and vaginal suppositories or orally such as one of azole agents (Gonçalves *et al.*, 2015). Suitability of treatment is mainly depending on the clinical features of the Vulvovaginal candidiasis when it is complicated or uncomplicated. For uncomplicated VVC, short-term local azoles for up to 3 days can treat 90% of cases with disappearing of symptoms within 2-3 days (Sobel, 2005; Dovnik *et al.*, 2015). Otherwise, complicated VVC can take a long time of treatment with at least 1 week of local azole or multiple doses of oral fluconazole (Sobel, 2005; Dovnik *et al.*, 2015). Other treatment choices of topical gentian violet, boric acid

or nystatin suppositories are not effective for treatment of VVC and they could give 60-80% cure rate (Sobel, 2005). However, the differentiation of VVC from other bacterial or parasitic vaginal infection is more important to grantee positive treatment results (Sobel, 2005; Rathod *et al.*, 2012). The suggested treatment for cases with both VVC and bacterial vaginitis should be started with ornidazole, then by fluconazole and follow up treatment by collecting vaginal samples after 9 to 16 days after the treatment (Liu *et al.*, 2013).

Long duration of treatment for more than 6 months can stimulate development of recurrent infection with *Candida* spp. (Lema, 2017). Recurrent Vulvovaginal candidiasis (RVVC) is a term called on infected women suffering from episodes of VVC by four or more per year and it represented one type of complicated VVC (Nyirjesy, 2001; Gonçalves *et al.*, 2015; Lema, 2017). Long-term therapy of Vulvovaginal candidiasis may be related to the recurrent infections that resulting from resistant of *Candida* spp. to the antifungal agents or other predisposing factors (Lema, 2017). Treatment with antifungal for 4 to 12 months found not effective on the present of *Candida* in the vagina of 1248 non-pregnant women (Beigi *et al.*, 2004). Drug resistant species of *Candida* and non– *C. albicans* can induce by repeated treatment with specific antifungal agent (Ringdahl, 2000; Lema, 2017).

The patients of this study who need 4 to 5 weeks of treatment were more frequent in age group 24-32 years compared to other groups. This may indicator that those women at the risk to develop RVVC. About 22% of adolescent women aged 12 to 22 years were showed to have RVVC (Rylander *et al.*, 2004). A significant higher number of RVVC was found among women in the range of 26 to 40 years than in groups of 18-25 years and 41-55 years (Yano *et al.*, 2019). The rate of RVVC is

often small compared to uncomplicated Vulvovaginal candidiasis. A bout 75% of women can suffer from one episode of VVC in their reproductive year, while RVVC with more than 4 episodes has been estimated in 5-10% of them (Nyirjesy, 2001; Lema, 2017). Only 4% of 709 asymptomatic young women showed colonized the vagina with yeasts at 4 visits, while 70% had yeasts at one visit (Beigi *et al.*, 2004). From three months study of visiting women with VVC, 28% of them diagnosed with Vulvovaginal candidiasis at the next visit (Rathod *et al.,* 2012) .The RVVC represented by suffering from more than 10 time episodes of VVC was found in a lower number compared with those with one episode (Yano *et al.,* 2019).

Although RVVC can develop in women with healthy and good immunity (Lema, 2017), several predisposing factors may promote development of such infection like pregnancy, diabetes, antibiotics, contraceptives, sexual transmission, lupus, thyroid diseases and immunosuppression (Ringdahl, 2000; Gonçalves *et al.*, 2015; Salvi, 2019;Sheary *et al.*, 2020).The reason for development of RVVC is not clearly identified (Sheary *et al.*, 2020). From 284 non-pregnant women, 50% of them do not know the reasons of their RVVC (Yano*et al.*, 2019).

Many theories had been proposed to explain the sources trigger of RVVC development. Relapse after long-term treatment or reinfections are two proposed theories, in which relapse is more acceptable theory (Sheary *et al.*, 2020). Fidel (2007) in two published papers (2004 and 2007) hypothesized that RVVC is mainly results from the activities of innate immune system and not from adequate immune system. Based on one of the studies, it was clarified theory that increases the number of *Candida* in the vagina due to predisposing factors will send signals to stimulate PMN to make inflammation and develop Recurent Vulvovaginal candidiasis (Fidel ,2007).

The suitable treatment of Recurent Vulvovaginal candidiasis is not easy to determine until now and it could be more difficult to treat than uncomplicated VVC (Ringdahl, 2000; Sheary *et al*., 2020). About 71% of women with Recurent Vulvovaginal candidiasis required a long-term treatment with antifungal agents and maintenance to control symptoms (Yano *et al.*, 2019). The recommended treatment program for RVVC starts with induces therapy with azole agents up to weeks followed by maintenance therapy for up to six months (Lema, 2017). Treatments of RVVC caused by *C. albicans* can manage by initial course of 14 days oral azole to induce clinical remission followed by six month maintenance regimen with ketoconazole (100 mg daily), itraconazole (100 mg daily) and fluconazole (100-200 mg weekly)(Nyirjesy, 2001).

5.3 Detection diagnostic antibodies of celiac diseases in Vulvovaginal candidiasis patients

Serological testing for anti-gliadin and anti-tTG antibodies in addition to antiendomysium antigens and reticulin are considered the most important diagnostic tests for CD (Hill and McMillan, 2006; Korponay-Szabó *et al.*, 2015; Brusca, 2015). Detection of anti-gliadin antibodies with its two classes IgG and IgA was the first and old test used for diagnosis of CD since 1950 and now it become less significant test (Bizzaro, *et al.*, 2013; Brusca, 2015). The development of new test of anti-tTG antibodies since 1997 makes it the first-line with more accuracy for diagnosis of CD (Reif and Lerner, 2004; Korponay-Szabó *et al.*, 2015). However, infection with candidiasis could be considered another factor to produce anti-gliadin antibodies in individuals without CD (Garcia *et al*., 2002; Brinkert *et al*., 2009).

The results of this study demonstrated the absence of abnormal levels of anti-tTG IgA and anti-gliadinIgG in all patients with VVC. Meanwhile, four patients had higher levels of IgA anti-gliadin. A positive results of anti-gliadin in patients with candidiasis was discovered in only two cases of chronic mucocutaneous candidiasis until now (Garcia *et al.*, 2002; Brinkert *et al.*, 2009). The first case was detected a slightly elevated in anti-gliadin antibodies in boy with chronic mucocutaneous candidiasis and declined later(Garcia *et al.*, 2002). In the second case of chronic mucocutaneous candidiasis, the levels of anti-gliadinIgG antibodies was higher (365 U/L), but there was no elevation in titer of anti-tTG or anti-gliadin IgA (Brinkert *et al.,* 2009).

The levels of IgA anti-gliadin is found higher among our patients with VVC. This is also the case with the diagnosis of CD when the sensitivity and specificity of IgA anti-gliadin is usually more higher for diagnosis of CD than IgG antibodies (Kelly *et al.*, 1991; Bizzaro, *et al.*, 2013). However, antibodies to anti-gliadin as with anti-tTG could be the sharing key between CD and many species of *Candida*, especially *C. albicans* (Aaron and Torsten, 2020; Corouge*et al.*, 2015). Human epithelial tissue of several organs contain a variable amount of TG such as intestinal and buccal epithelial tissues (Sundstrom *et al.*, 2002; Nieuwenhuizen*et al.*, 2003;Ponniah *et al.*, 2007) . Also it found as one components of cell wall of *Candida albicans* (Aaron and Torsten, 2020).

The connection between the presences of *Candida* spp. and CD antibodies is mainly dependent on the fungal Hwp1 cell wall protein. Hwp1 is a specific protein of hyphal cell wall of *Candida albicans* that can be work as a substrate to human TG (Staab *et al.,* 1999; Sundstrom *et al.*, 2002; Nieuwenhuizen*et al.,* 2003; Corouge *et al.,* 2015). Binding of human TG with Hwp1 can produce a complex that has the ability to

stimulate development of CD and production of anti-tTG antibodies (Nieuwenhuizen *et al.*, 2003; Aaron and Torsten, 2020). This proposal mechanism has been proved by finding higher levels of specific antibodies to anti-Hwp1, anti-gliadin and anti-tTG in the presence of *Candida* spp. in patient with CD compared with healthy individuals (Renga *et al.,* 2019). In addition to other antibodies, anti-tTG IgA is found in high levels in either patients with CD or with *C. albicans* infection than in healthy individuals (Corouge *et al.,* 2015). Thus, *C. albicans* can be one of causative agent of CD (Corouge *et al.*, 2015; Aaron and Torsten, 2020). The TG of human tissue could activate by *Candida* spp. to cause tissue damage through production of reactive oxygen species (ROS) (Shrestha *et al.*, 2017).

5.4 Relation of positive results of IgA anti-gliadin to the age of patients with VVC

From the results of this study, positive IgA anti-gliadin was mostly observed in two VVC patients in age group 24-32 years, while other patients were singly diagnosed in a group of 15-23 years and 33-41 years. These results indicate that the high level of IgA is not related to the specific age and could be found in every age. Antibodies of IgA specific to gliadin protein is one class of anti-gliadin that used for diagnosis of CD a decade ago (Brusca, 2015). Its sensitivity and specificity for diagnosis of CD is mostly more than the second class of anti-gliadin (IgG) (Bahia *et al.*, 2001). Levels of IgA anti-gliadin usually increased with age as found among Pakistani patients with CD where the positive results of IgA were higher among young age (29.3% in age $\leq$ 15 years) and gradually decreased with old age (20% in 16-30 years, 17.1% in 31-45 years and 16.3% in 46-60 years)(Jamila*et al.*, 2018). Measurement of IgA anti-gliadin in 150 Iraqi children with CD showed that younger age (1-5

years) with high levels of IgA was the more frequent (73.8%) than other ages (70.47% and 64.1% for 6-10 years and 11-15 years, respectively) (Mohammed, 2013). A positive result with IgA anti-gliadin (61%) was also found among Italian children with CD aged 5-10 years (Verma *et al.,* 2018). Positive IgA with higher levels was measured in salivary and serum of children with CD who aged 9 months to 14 years compared with control group (Hakeem *et al.,* 1992). On the other hand, a positive high levels of IgA anti-gliadin also observed in adults. High titer of IgA anti-gliadin was clearly shown among individuals without CD aged 60-69 years and become lower in age 15-19 years (Uibo *et al.,* 1993).

Conclusions and Recommendation

Conclusions

1- Women at middle age (24-32 years) is more susceptible to infect with Vulvovaginal candidiasis.
2- Duration of infection with VVC is commonly from less than one week to two weeks, especially in those aged 24-32 years.
3- With progress in age the infection with VVC it may decrease .
4- A great number of patients were under antifungal treatment for one week, especially those in age 15-23 years.
5- Usage of anti-gliadin IgG and anti-tTG IgA is not useful in diagnosis of VVC.
6- Anti-gliadin IgA could be used to detect VVC, but with insignificant results.
7- Positive results with anti-gliadin IgA were not restricted in a specific age.

Recommendations:

1. Different types of *Candida* infections need to evaluate in correlation with anti-gliadin test.
2. Prove the significant application of anti-gliadin IgA for detection VVC need more studies.
3. Predisposing genetic factor may be usefle to study in a positive VVC patients with anti-gliadin IgA.

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الخلاصة

الكلايدين هو من اهم بروتينات الكلوتين المسؤول عن تطور مرض السيلاك ، لذلك فان الكشف عن الاضداد المضادة للكلايدين يعد من اقدم الفحوصات المستخدمة لتشخيص مرض السيلاك وقد استبدل حاليا بفحوصات مصلية اخرى مثل فحص الاضداد المضادة للانزيم النسيجي ترانسكلوتامينز، ومن المحتمل ان تحفز خميرة الكانديدا افراز مضادات الكلايدين مما يجعلها مع علاقة مع مرض السيلاك.

صممت دراسة معتمدة على مقارنة الحالة المرضية مع السيطرة لغرض تقييم تاثير مرض المبيضات المهبلية على رفع مستوى مضادات الكلايدين عند مرضى ليس لديهم مرض السيلايك، وتضمنت الدراسة 90 امرأة مقسمة الى مجموعتين تتضمن الاولى 50 مريضة لديهن داء المبيضات المهبلية والمجموعة الثانية تتضمن 40 امرأة سليمة من المرض. جمعت عينات المسحات من جميع النساء لغرض تشخيص الاصابة بالكانديدا لديهن في منطقة المهبل كما جمع عينات المصل من المشتركين لغرض الكشف عن مضادات الكلايدين نوع IgA و IgG وكذلك الاضداد المضادة للانزيم النسيجي ترانسكلوتامينز نوع IgA باستخدام فحص الاليزا.

حدد مرض المبيضات المهبلية عند النساء، ووجد بان الفئة العمرية 24-32 سنة الاكثر عددا من الاصابات (40%) يتبعها الفئة العمرية 15-23 سنة (36%)، وان اكثر طول فترة للاصابة كانت ضمن معدل الاقل من اسبوع الى اسبوعين (66%)، خاصة عن العمر 24-32 سنة (30%)، بينما اقل فترة للاصابة كانت من 9 الى اكثر من 13 اسبوعا (2%).

سجلت معالجة مرض المبيضات المهبلية عند 31 امرأة مصابة ، وخاصة عند اللاتي اعمارهن 15-23 سنة، بينما 19 مريضة كانت بدون معالجة، كما ان اغلب

المرضى استخدموا المضادات الفطرية لفترة اسبوع واحد (38%)، بينما اقل عدد عولج للفترة من 4 الى اكثر من 5 اسابيع (8%).

اظهر قياس كلا النوعين من مضادات الكلايدين (IgA, IgG) ومضادات الانزيم النسيجي ترانسكلوتامينز IgA نتائج سالبة بين مجاميع المرضى والسيطرة، بينما كانت نتائج مضادات الكلايدين للنوع IgA بتراكيز ايجابية عالية عند 4 مرضى (8%) والتراكيز المتوسطة لهذا الضد عند 7 مرضى (14%)، وان اعمار المرضى الاربعة الموجبة نتائجهن مع مضادات الكلايدين للنوع IgA توزعت بين مريضتين اعمارهم 24-32 سنة (4%) والاثنين الاخرتين توزعوا بشكل مفرد بين الفئة العمرية 15-23 سنة والفئة 33-41 سنة، اما بقية 78% من النتائج السالبة لمضادات الكلايدين فقد كان تكرارهم عالي عند الفئة العمرية 15-23 سنة (32%) والفئة 24-32 سنة (26%).

جمهورية العراق
وزاره التعليم العالي والبحث العلمي
جامعه كربلاء/ كليه الطب
فرع الاحياء المجهرية

سيادة الأضداد المضادة للكلايدين و المضادة للانزيم النسيجي ترانسكلوتامينز عند النساء المصابات بالمبيضات المهبلية وعلاقتها مع مرض السيلاك

رسالة مقدمة الى
مجلس كلية الطب جامعة كربلاء
كجزء من متطلبات نيل شهادة الماجستير في الاحياء المجهرية الطبية

من قبل
ميثم جاسم محمد العرباوي
بكالوريوس التقنيات الصحية والطبية/جامعه الكوفة (2016)

بأشراف
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