UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: INCIDENCE OF VULVOVAGINAL CANDIDIASIS IN WOMEN OF THE IMBABURA PROVINCE

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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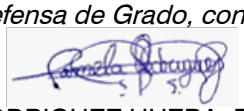

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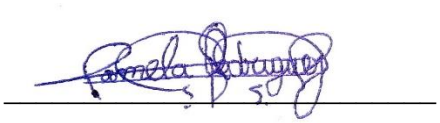

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Dedication

To my parents Rosa Huera and Wilson Rodriguez for your understanding, and unconditional support to reach my goals and dreams.

Evelin Pamela Rodriguez Huera

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To God for allowing me to achieve this aspired goal after so many efforts, defeats, among other things, that I have had during my professional training.

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Finally, last but not least, to my family, for giving all the support and love.

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RESUMEN

La candidiasis vaginal es un tema relevante en la actualidad y también se ha convertido en una de las enfermedades más prevalentes en la mujer. La candidiasis vulvovaginal (VVC) es una infección causada por el hongo *Candida*, el cual usualmente forma parte de la microbiota comensal intestinal. Se convierte en un problema de salud cuando el hongo coloniza la mucosa vaginal en una persona inmunodeprimida, embarazada o después de un tratamiento con antibióticos. Los hongos pueden crecer rápidamente, superando a los microorganismos protectores y provocando la colonización en el hospedador. Los antimicóticos son medicamentos antimicrobianos recetados que detienen el crecimiento de los hongos o los matan. Sin embargo, el hongo puede adquirir resistencia a los tratamientos y suponer un grave riesgo para la salud de muchas mujeres. El propósito de este proyecto es determinar la prevalencia de CVV en una población de mujeres, identificar las especies de *Candida* y evaluar la sensibilidad de los hongos frente a los antifúngicos comúnmente recetados. Este estudio fue realizado desde noviembre de 2019 hasta febrero de 2021 en la provincia de Imbabura. En la presente investigación, se obtuvieron en centros de salud primaria ciento treinta y cuatro muestras de exudados cervicales vaginales de mujeres con síntomas de CVV, que fueron posteriormente analizados en los laboratorios de la Universidad de Investigación de Tecnología Experimental Yachay. Realizamos cribados de hongos patógenos con el fin de aislar, identificar especies de *Candida* y determinar la susceptibilidad del hongo a los antifúngicos clínicamente más utilizados. De las 30 muestras positivas, 96.7% fueron *C. albicans*, seguido de *C. parapsilosis* (3.3%) mientras que no se encontraron *C. glabrata* y *C. tropicalis*. Además, *C. albicans* fue identificada en mujeres entre los 18 y 29 años. La mayoría de aislados fueron sensibles al clotrimazol y voriconazol, y algunos resistentes al fluconazol. Nuestros resultados sugieren que la identificación de las especies de *Candida* y las pruebas de sensibilidad antifúngica deben realizarse de forma rutinaria para lograr un tratamiento adecuado y resultados clínicos correctos.

Palabras clave: resistencia, *Candida*, infecciones, antibióticos, mujeres.

ABSTRACT

Vaginal candidiasis is a relevant topic nowadays and has also become one of the most prevalent diseases in women. Vulvovaginal candidiasis (VVC) is an infection caused by a fungus of the genus *Candida,* which usually forms part of our commensal gut microbiota. It becomes a health problem when the fungus colonizes the vaginal mucosa in an immunocompromised, pregnant person or follows antibiotic therapy. The fungi can proliferate, surpassing protective microorganisms and causing colonization in the host. Antimycotics are prescribed antimicrobial drugs that stop the growth of fungi or kill them. However, the fungus can acquire resistance to the treatments and pose a severe health risk for many women. The purpose of this project is to determine the prevalence of VVC in a population of women, identify the *Candida* species causing them, and evaluate the sensitivity of the fungi to commonly prescribed antifungals. This study was carried out since November 2019 to February 2021 in the Imbabura province. In the present investigation, one hundred and thirty-four samples of vaginal cervical exudates from women with symptoms of VVC were obtained in primary care health centers and were analyzed in the laboratories of Yachay Tech University. We conducted screens for fungal pathogens in order to isolate, identify C*andida* species, and determine the fungus susceptibility to antifungals clinically most used. Among all samples, 31,34% were culture positive. Out of the 30 positive samples, 96.7% were *C. albicans*, followed by *C. parapsilosis* (3.3%) while *C. glabrata* and *C. tropicalis* were not found. Moreover, *C. albicans* were identified in women between the ages of 18 and 29. Most isolates were sensitive to clotrimazole and voriconazole, and few were resistant to fluconazole. Our results suggest the identification of *Candida* species and their antifungal sensitivity testing should be routinely performed to achieve proper treatment and appropriate clinical results.

Keywords: resistance, *Candida*, infections, antibiotics, women.

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VVC	Vulvovaginal Candidiasis
RVVC	Recurrent Vulvovaginal Candidiasis
BV	Bacterial Vaginosis
Spp.	Species
FLU	Fluconazole
CLO	Clotrimazole
VOR	Voriconazole
TJ	Tomato Juice
YPD	Yest Extract Peptone Dextrose
PAMP	Pathogen-Associated Molecular Pattern
GlcNAc	N-acetylglucosamine
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
CFU	Colony-Forming Units

1 Introduction: Theoretical framework

1.1 Vulvovaginal candidiasis (VVC)

Vulvovaginal candidiasis (VVC) is one of the most frequent infections in women, affecting 75% of them, with at least one episode during their lifetime; 40% to 50% of these women have recurrent events (El-Houssaini, Elnabawy, Nasser, & Elkhatib, 2019), (Zhai et al., 2018). Symptomatic vaginitis (inflammation of the vagina and/or vulva) is caused mainly by *Candida albicans* (Spence, 2010) but also by non-*albicans* species. This vaginal inflammation is characterized by signs and symptoms of local redness, severe itching, and vaginal discharge. Many risk factors that facilitate the colonization of *Candida* species, such as diabetes, pregnancy, being immunocompromised, and having disorders of the immune system. Also, VVC is related to hormonal changes; thus, it is more frequent during the reproductive years (18-45 years of age) (Blostein et al., 2017).

Most of the women have only one or two episodes of VVC in one year, but a subset of those patients experience a chronic infection. This is called recurrent vulvovaginal candidiasis (RVVC) and is defined as four or more episodes of VVC within a 12-month period (Sobel, 2016). Recurrence of candidiasis is somewhat due to lack of effectiveness in treating the previous infection, allowing the same *Candida* strain to grow back after some time. In other words, antifungals did not kill all fungus and the same strain becomes more resistant to it (Ignjatović et al., 2020).

Diagnosis is needed by women who have signs and symptoms of vaginitis because approximately 10%-20% of them harbor *Candida* species (Bitew, 2018) as a commensal organism. That is to say; in such cases, there is not a mycosis caused by *Candida* spp. Moreover, these women are asymptomatic since the acidic pH of the vagina, the immune system, and the presence of a healthy vaginal bacterial microbiota prevent the overgrowth of *Candida* spp. Vulvovaginal candidiasis only occurs when there is any disorder in the immune system associated with antibiotics consumption, pregnancy, diabetes, and number of sexual partners.

The followings methods can identify the fungi in vaginal exudates: I) saline preparation; II) 10% KOH, used to identify the presence of yeast and hypha cells; III) vaginal culture, used in symptomatic women with a pH of 4.0- 4.5 (Sobel, 2016) IV), “germ tube test”; and VII) molecular techniques. The most sophisticated method to identify *Candida* is vaginal culture and its molecular techniques. The treatment for vulvovaginal candidiasis depends on

the severity of this infection. It is commonly treated with broad-spectrum antifungals such as 1% clotrimazole, voriconazole, fluconazole, miconazole, and in severe cases, amphotericin B.

1.2 Candida albicans

Some species of *Candida* can produce infections in people, but the most common is *Candida albicans,* which is the leading cause of morbidity and mortality in immune-compromised individuals. It belongs to the Phylum Ascomycota; order Endomycetales, where we can find the family *Saccharomycetaceae*; however, *Saccharomyces cerevisiae* and *C. albicans* do not share the same behavior. This genus includes 150 species, but the most pathogenic species that affect the human host are *C. albicans, C. glabrata, C. tropicalis*, and *C. parapsilosis* (Table 1) (Cervera, 2003) (Oliver et al., 2020). Among all the *Candida* species, *C. albicans* is the most frequent, representing 73.8% of all cases (De Bedout et al., 2003) (Table 1). *C. glabrata* and *C. parapsilosis* species can also cause vulvovaginal candidiasis, but these cases are rare and tend to manifest a milder clinical picture.

C. albicans is an opportunistic pathogen. It is an asexual diploid fungus; as a commensal, it colonizes many areas of the body such as the gastrointestinal tract (gut), female reproductive tract, and even the skin (Maria Antonia De la Parte, Mireya Mendoza, 2006) (Cervera, 2003). However, it becomes a problem when disturbances change the environment inside the vagina to encouraging its growth. For instance, a change in the pH of vagina, high levels of estrogens produced by pregnancy and menstruation, the concurrence of circumstances that alter the vulvovaginal ecosystem, and/or depress the immune system (Kashem et al., 2015).

Candida infection typically affects the skin and the mucous membranes of the mouth, intestines, and vagina. In rare cases, *Candida* yeast cells enter in the bloodstream through catheters or contaminated prostheses. This may lead to the spread to other parts of the body like heart, brain, blood, eyes, and bones. This can undoubtedly cause severe, life-threatening infections (Polke, Hube, & Jacobsen, 2015).

C. albicans can respond and live in many environmental conditions (Polke, Hube, & Jacobsen, 2015). Thus, it is a model organism most studied and isolated for studying fungus that are pathogens to humans.

Table 1. *Candida species were clinically significant.*

Candida spp.	**Incidence**	**Morphology**	**Description**	**Ref.**
Candida albicans	73.8%	Yeast, hyphae, pseudohyphae, chlamydospores	The major cause of VVC. Can colonize many areas of our body.	(De Bedout et al., 2003), (Yapar, 2014)
Candida glabrata	13.8%	Yeast (closely related to *S. cerevisiae*). No hyphae or pseudohyphae	Common infection in older, neoplastic patients and during menopause. Often it causes recurrent vulvovaginal candidiasis, candidemia, and candiduria.	(Calderone R.A and Clancy C. J., 2012), (Ignjatović et al., 2020)
Candida tropicalis	2.2%	Oval yeast cells and true hyphae	Infection in leukemia and neutropenic patients.	(Zhang et al., 2016)
Candida parapsilosis	1.3%	Round, oval or elongated yeast cells and as pseudohyphae	The common pathogen in catheter-related infections. Causes candidemia in young infants.	(Yapar, 2014), (Calderone R.A and Clancy C. J., 2012)

1.3 Morphogenesis

C. albicans is a multimorphic fungus that can grow as rounded buds, pseudohyphae, true hyphae, and chlamydospores, depending on their environmental conditions. In particular, *C. albicans* experiences two relevant morphological switchings. The first is the transition between yeast to hyphal growth modes, an essential factor for its virulence that contributes to its survival, escaping the macrophages' engulfment (Forche et al., 2008). The other switch is between white and opaque cells. This switching is bi-stable, heritable, and epigenetically regulated (Du & Huang, 2015).

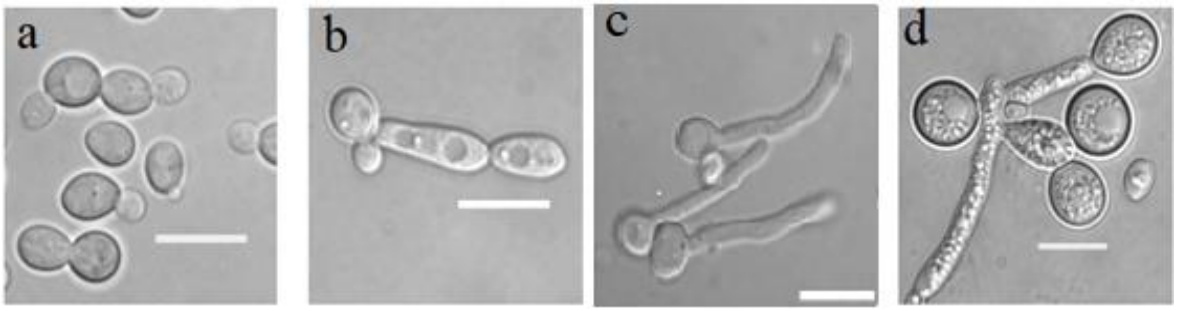


Figure 1. Morphologies of *C. albicans:* a) Yeast cells, b) Pseudohyphae, c) Hyphae, d) Chlamydospores. (from F. J. Álvarez and adapted). Bar scale. 10µm.

The round buds, also known as yeast cells, are the most common morphology similar to that of *Saccharomyces cerevisiae* buds, although they are a little more elongated. Yeast divides through mitosis, or budding pattern (the nuclei replicate and half of the genetic material moves into the bud; then the bud grows in size and eventually separates from the mother cell). Under certain environmental circumstances, the yeast cells may not be wholly separate and may continue dividing in this way, and form long chains of yeast cells known as pseudohyphae (Azadmanesh, Gowen, Creger, Schafer, & Blankenship, 2017). This is also considered a filamentous form, where the chains of elongated cells are constricted at their septae. The other filamentous form is true hyphae, with parallel-sided walls without constrictions at the septation sites and apical growth (unidirectional growth from the tip only). Hence, hyphae and pseudohyphae are both elongated but easy to distinguish by microscopic analyses (Figure 1).

Chlamydospores are large (6-10 µm), with think-walled, and refractile cells. The role of this morphological form in the life cycle and pathogenicity of *C. albicans* is currently unknown. Recent studies have shown that *EFG1* and *HOG1* genes control the chlamydospore production, while the quorum sensing molecule farnesol promotes chlamydospore induction (Citiulo et al., 2009).

As mentioned before, *C. albicans* can exist in numerous cellular forms, but the most relevant morphologies for infection are elongated filamentous cells and rounded yeast-like cells (Figure 1). The yeast form is well suited for liquid environments, such as for dissemination in the bloodstream. In contrast, hyphae and pseudohyphae forms are well suited for invading solid substrates (Polke et al., 2015a), like in the colonization of organs. *C. albicans* infects its host as hyphae, which allows for tissue penetration during the early stages of infection, then occurs the division of yeast cells by sexual and asexual reproduction (Sudbery, Gow, & Berman, 2004). The morphological switching of hypha from budding cells depends on the presence of extracellular nutrients as well as the environmental conditions.

For instance, a temperature of 37 °C, neutral pH, high levels of CO_2, serum, nitrogen deprivation, certain amino acids and sugars stimulate cells to form hyphae. The presence of high albumin levels, N-acetylglucosamine (GlcNAc), amino acids and seminal fluid or semen stimulate filamentous growth in *C. albicans* (Alvarez at al., 2015), contributing to its virulence. Interestingly, GlcNAc represses the filamentous growth of *C. tropicalis.*

Another phenotypic switch that *C. albicans* exhibits are the morphological variation known as the white/opaque (w/o) switching system, which is regulated by epigenetic factors. White colonies contain oval, budding cells that are found in systemic infections. On the other hand, opaque cells are oblong-shaped cells, characteristically larger, more giant, have a rough surface, and are found in mucosal infections. Both white and opaque cells can be homozygous for the mating locus (a/a or α/α). Still, only opaque cells homozygous for that locus are competent to undergo mating (Blankenship & Heitman, 2005) (Sudbery et al., 2004). It is essential to mention that *C. albicans* cells, like *S. cerevisiae* cells, have a mating locus implicated in sexual reproduction. However, unlike *S. cerevisiae*, *C. albicans* do not have a complete meiosis.

During the mating process occurs the secretion of mating pheromones, which prompts the cells to form a conjugation tube that grows following those pheromones´ direction (Alvarez, 2007). As a result, the mating of two diploid opaque cells (2n) occur, which gives rise to a tetraploid progeny (a/α) (4n). These cells regain diploidy (or an aneuploidy) through a random chromosome loss process via a parasexual mechanism. Finally, white opaque switching can affects *C. albicans* survival in the host through mating (Polke, Hube, & Jacobsen, 2015), generating great genetic and phenotypic diversity. As a result, this influences the immune recognition by the host and increases the resistance to azoles.

1.4 Cell wall structure

The *C. albicans* cell wall maintains the cell's integrity and interacts with the exterior, protecting it environmental stress conditions such as temperature (Beltran et al.,2019), pH, and osmotic permeability. It also has high plasticity to allow different morphologies and easily adheres to the surface through adhesins. In addition, interact with host immune cells. Therefore, it plays an essential role in virulence and pathogenesis. The cell wall is composed of proteins, glucans, and chitin (Gow, Latge, & Munro, 2017); however, it is divided into two parts, which are skeletal (inner surface of the wall) and the matrix component (outer wall) of *C. albicans* cell wall (Figure2).

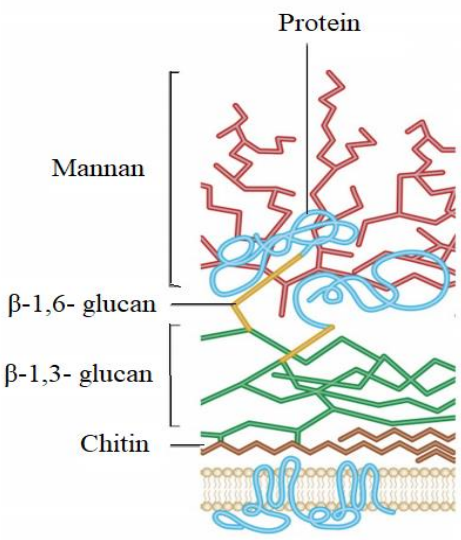


Figure 2. Structure of the fungal cell wall. (From "*The Fungal Cell Wall : Structure, Biosynthesis and Function"*, by Gow, Latge, & Munro, 2017).

Furthermore, the skeletal component of the cell wall of *Candida* species includes β-glucans and chitin, which handle the cell wall's shape and strength (Garcia-rubio, Oliveira, Rivera, Niño-vega, & Hall, 2020). Chitin is a β-(1,4)-linked polymer of N-acetyl glucosamine (GlcNAc) (Netea, Brown, Kullberg, & Gow, 2008). The β-1,3 glucan is covalently linked to β-(1,6)-glucan. Furthermore, hydrogen bonds stabilize β-1,6 glucans, which also act as a linker-molecules, binding proteins to the β-1,3- glucan-chitin core through the glycosylphosphatidylinositol (GPI) proteins (Rubio et al., 2020). Consequently, these polymers and polysaccharide chains form a tough three-dimensional network of microfibrils.

On the other hand, the outer wall contains glycosylated proteins with α and β-linked oligomannosyl residues by mannosyltransferases that use Guanosine diphosphate mannose (GDP-mannose) as a substrate (Gow et al., 2017). In *C. albicans*, cell wall proteins are (GPI)-anchor-dependent cell wall proteins (GPI-CWPs) (Hall et at., 2013), which are attached through a GPI remnant to β-(1,3)- glucan- chitin by β-(1,6)-glucan linker (Netea et al., 2008). The CWPs are usually highly glycosylated with mannose-containing polysaccharides and carbohydrates, which account for up to 90% of their molecular mass. Many CWPs have Ser/Thr-rich polypeptide, stabilizing the cell wall by O-linked mannan side chains (Netea et al., 2008).

C. albicans make up of one to five mannose (Man) sugars that are α (1,2)- linked. These structures define the different serotypes of *Candida* spp. Mannans are less rigid than β-glucan and chitin; therefore, they have low permeability, which encourages the action of antifungals. The outer mannan layer plays an essential role in immune evasion because it masks β-1,3

glucans, evading the immune system. These mannans are considered pathogen-associated molecular pattern (PAMP) ligands recognized by cells receptors, following interleukins´ activation.

One of the most relevant recognition in the cell wall is the detecting of β-(1,3) glucan by C-type lectin Dectin-1 (Gow et al., 2017). This polysaccharide is often masked by the hyphae cells´ outer layers, followed by the shielding of mannans (Erwig & Gow, 2016). In this way, the immune system does not recognize the pathogen and prevents it from being killed by macrophages. Hence, yeast switching to hyphal cells plays an important role in virulence, promoting colonization and invasion of epithelium by *C. albicans* cells. Some experimental research shows that lactate (carbon source) induces the masking of β-(1,3) glucan (Gerwien et al., 2020). Moreover, some strains have more glucans to activate a hyperinflammatory response. Finally, various antimycotics are focused on enhancing the signaling of leucocytes via Dectin-1, a receptor found in macrophages (Gow et al., 2017) (Wheeler & Fink, 2006).

1.5 Vaginal microbiota

A healthy vaginal microbiota is composed of many good bacteria such as *Lactobacillus* species, but can also harbor opportunistic pathogens. *Lactobacilli* are found on the vaginal epithelium and contribute to women's health by maintaining a low pH. *Lactobacillus* spp. are fermentative bacteria that produce lactic acid from glycogen, which maintains the acidic pH 3.8 - 4.8 (Mendling, 2016), considered normal. Also, they produce H_2O_2, which prevents bacterial vaginosis (BV); it also releases bacteriostatic compounds that inhibit the proliferation of pathogens and competes with opportunistic microorganisms preventing adhesion to the mucous membranes (Monin, Whettlock, & Male, 2020), and secrete antibacterial peptides.

Hence, *Lactobacillus* species prevent the invasion of pathogens by maintaining a low pH and a normal population of opportunistic microorganisms. However; the disruption of vaginal ecosystems facilitates an overgrowth of *Candida* spp. and other bacteria, which causes VVC or bacterial vaginosis (BV). Sexual hormones, menstruation, pregnancy, immunosuppressive drugs, sexual activity, menopause, and other factors can affect the vaginal microbiota, allowing the colonization of pathogenic organisms. Some studies have showed how lactobacilli strains inhibit the expression of hypha-related genes of *C. albicans* (Jeng, Chee, Chew, Thian, & Than, 2020).

1.6 Pathogenesis

Candidiasis arises because the *C. albicans* cells that already existed in your body found ways to overcome our body's defenses and managed to multiply uncontrollably. *Candida* that exists in the gastrointestinal tract can colonize the perianal region; then, it can migrate through the perineum to reach the vagina and colonize this area if immune system is depressed.

Adhesion of *C. albicans* is the first step in colonization and invasion of mucocutaneous tissues, which is probably mediated by the interaction of yeast surface glycoproteins with the host epithelial cell (Gow et al., 2017). Then, there is the formation of germ tubes, hyphae, or pseudohyphae, which penetrate directly into the epithelial cell through adhesins. Adherence continues with the production of hydrophilic enzymes such as proteinases, phosphatases, and phospholipases (Höfs, Mogavero, & Hube, 2016). Then, the cells activate mechanisms that allow them to penetrate, inducing endocytosis and penetration. Once the cell crosses the epithelium, *C. albicans* will cause damage and loss of epithelium integrity.

1.7 Clinical manifestations.

The most frequent vulvovaginal candidiasis symptoms are vulvar itching, redness in the vagina and vulva, and white or yellow vaginal discharge “cheese-like” without odor may be present. Painful urination and dyspareunia (pain during sexual intercourse), and pain in the vaginal area are common symptoms. These complications may be accompanied by dysuria. Although most vaginal candidiasis is mild, some women can experience severe infections, including redness, swelling, and cracks in the vagina’s wall. However, none of these symptoms described above are unique to *Candida* vulvovaginitis. Some vulvovaginal infections like BV and Trichomoniasis can cause similar symptoms (Ignjatovic et al., 2020).

1.8 Risk factors

Some factors increase the risk of vulvovaginal candidiasis, such as pregnancy, cancer therapy, HIV infections, neutropenia, organ transplantation, contraception use, uncontrolled diabetes, sexual activity or number of sexual partners, antibiotic therapy, and treatments with cytostatics and corticosteroids (Erwig & Gow, 2016). Moreover, circumstances that alter the vulvovaginal ecosystem and/or depress the immune response are also risk factors.

The high levels of reproductive hormones such as estrogen and progesterone´s secretion during pregnancy (Odabasi & Mert, 2020) enhance *Candida* growth. These two hormones have an inhibitory effect on the immune system (neutrophils), inhibiting the activity of vaginal epithelial cells against pathogens. First, estrogens promote the increase of glycogen

content in the vaginal tissue, and a decrease immunoglobulins secretion (Tsega & Mekonnen, 2019). Also, estrogens produce a decrease in antimicrobial peptides and pattern recognition receptor (PRR) expression. While estrogens increase the levels of glycogen, progesterones enable its release. This glycogen serves as a carbon source, favoring the adherence of *C. albicans* to vaginal epithelial cells (Zhai et al., 2018), activating the germ tube formation and increasing hyphal length (Höfs et al., 2016). In addition, the pH changes from acidic to neutral inside the vagina. Something similar happens during menstruation and the intake of contraceptive drugs. Finally, pregnant women are more susceptible to vaginal candidiasis. Therefore, they must be tested often because *C. albicans* not only causes an infection in the women but also can pose a threat to their fetus.

Second, candidiasis is common in women taking antibiotics. Broad-spectrum beta-lactams such as ampicillin, and tetracycline, kill healthy bacteria or bacterial competitors from the vagina, leading to fungal overgrowth. Besides, the breakdown of the epithelium´s anatomical barriers is because antibiotic use favors the penetration and colonization of fungi (Calderone and Clancy, 2012). Antibiotics kill the bacterial pathogens and the healthy bacteria of the vagina such as *Lactobacillus acidophilus* (Salinas et al., 2020), which causes a change in the pH. Also, this facilitates the colonization of fungi and favors the morphological transition of *C. albicans*, from yeast to hyphal cells.

1.9 Antifungal drugs

There are many classes of antifungal drugs used to treat *Candida* infections. Polyenes, azoles, and echinocandins (Whaley et al., 2007) are used depending on infection type and site. Since azoles are the only option among antifungals as oral and prophylaxis treatment against invasive fungal infections, the emergence of their resistance represents a serious global problem. In addition, azoles are considered the antifungal drugs most used against *Candida* infections (Gubbins & Anaissie, 1981). The azoles target the production of ergosterol, a central component of the plasma membrane of fungi.

The azole antifungal agents of clinical use are categorized into N-1 substituted imidazoles comprehending two nitrogen atoms in the azole ring such as ketoconazole, miconazole, **clotrimazole**; triazoles contain three nitrogen atoms in the azole ring, for example, itraconazole and **fluconazole** (Francois, Aerts, Cammue, & Thevissen, 2005) (Figure 3). The second generation of triazole antifungals include **voriconazole** and posaconazole.

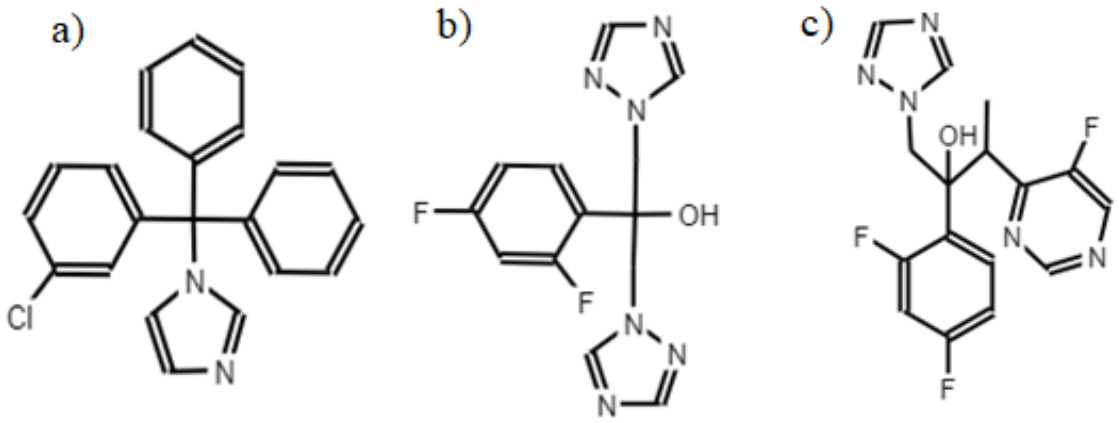


Figure 3. Antifungals clinically most used: a) Clotrimazole; b) Fluconazole; c) Voriconazole.

Furthermore, the action mechanism of different antifungal drugs depends on the place where they act within the fungal cell, which is closely related to the drug's chemical structure. Some studies show that the hidden nitrogen of the azole ring binds to the target side of *C. albicans*, which is the iron of Erg11p (CYP551 protein of *C. albicans*) (François, Aerts, Cammue, & Thevissen, 2005). As a result, this binding prevents the activation of oxygen and suppresses lanosterol´s demethylation (Berkow & Lockhart, 2017) which inhibits ergosterol biosynthesis. Besides, the N-1 nitrogen is a determinant of the efficacy and efficiency of the antifungal. And for this reason, all azoles share the same action mechanism against fungi.

1.9.1 Clotrimazole (CLO).

It is a broad-spectrum synthetic imidazole. It is effective in the treatment of oral and vaginal candidiasis and also for systemic mycosis. The structure of clotrimazole is illustrated in Figure 3. The action mechanism is the same for all azoles, the inhibition of ergosterol biosynthesis (CYP450 inhibition), and other mechanisms. These include the inhibition of sarcoplasmic reticulum Ca^{2+} ATPase, depletion of intracellular calcium (Khatter and Khan, 2020), blocking of calcium and potassium channels (Crowley & Gallagher, 2014). It is administered orally, topically, and intravaginally, but its oral intake may cause abnormal liver function.

1.9.2 Fluconazole (FLU).

It is a second-generation fluorinated triazole (Figure 3) with high bioavailability and low toxicity, which makes it one of the most used drugs to treat vulvovaginal candidiasis and almost all fungal infections. It can be administered intravenously and orally, because it has high absorption (Corrêa & Salgado, 2011). But it causes side effects such as diarrhea, stomach pain, rash and liver problems. Because of its easy administration and low toxicity, it

is used as a prophylactic treatment in those patients at high risk of suffering from invasive candidiasis, such as immunosuppressed patients. For this reason, *Candida* spp. can develop resistance to fluconazole. According to Berkow and Lockhard, *C. albicans* has a low incidence of resistance to fluconazole, while *C. tropicalis, C. parapsilosis,* and *C. glabrata* have a higher level of resistance to it (Berkow and Elizabeth, 2017). Moreover, *C. krusei* has intrinsic resistance, and *C. glabrata* has a lower sensitivity to this antifungal. It has already been observed that *Candida* spp. can acquire fluconazole resistance through multiple mechanisms, which will be discussed in the next section.

1.9.3 Voriconazole (VOR).

It is a synthetic derivative of fluconazole, but it has a broad antifungal spectrum. The structure is like that of triazoles, but the triazole ring is replaced by fluorinated pyrimidine and an additional α-methyl group (Figure 3) (Saravolatz et al., 2003). The mechanism of action is the same for all azoles. VOR has a broad spectrum and has high bioavailability and biodistribution as well as relatively low toxicity (Saravolatz, 2003). The administration route is orally and intravenously. VOR has good activity in *vitro*, against those strains resistant to fluconazole, such as *C. krusei* and *C. glabrata*. Although, this does not always occur since cross-resistance of fluconazole and voriconazole has been observed. In one study, voriconazole completely inhibited ergosterol synthesis and accumulation of its precursors, while fluconazole partially inhibited ergosterol synthesis (Rodrigues et al., 2017). Then, voriconazole is more effective than fluconazole.

1.10 Mechanism of action

Azoles inhibit ergosterol biosynthesis by blocking the key enzyme lanosterol 14- α-demethylase, an enzyme of Cytochrome P450- Erg11p (or Cyt51p) that converts lanosterol to ergosterol, encoded by *ERG11* (a component of the *Candida* cell membrane) (Figure 4) (François et al., 2005), (Whaley, Berkow, Rybak, Nishimoto, Barker, Rogers, et al., 2017). This inhibition of cell walls occurs due to an accumulation of toxic sterol (14α-methyl-3, 6-diol) produced by *ERG3*. These sterols alter both the cell membrane's structure and function, inhibiting the growth, reproduction, and morphology of the cell.

1.11 Antibiotic and antifungal resistance

Nowadays, antimicrobial resistance has been increasing at high levels all over the world. Each year in the U.S., at least 2.8 million people get an infection resistant to drugs, and over 35,000 people die from it (Centers of disease control and prevention, 2020). Resistance to antimicrobials is the ability of a microorganism (bacteria, fungus) that develops to elude the

antimicrobials designed to kill them (WHO, 2020). Therefore, they continue living within the host, activating new strategies or mutations that allow them to survive. As a result, this causes serious medical problems, because the treatment becomes less effective, requiring alternative drugs at high dosages.

Drug resistance can be due to many factors such as 1) self-medication or long-term medication; which brings an evolution in resistance with it; 2) the treatment is not complete or patients do not finish their medication. Some of these bacteria die but others do not, acquiring resistance to this; 3) poor medication in hospitals and clinics accompanied by incomplete diagnostic. This occurs in small communities, where medicines are used without identifying the infection cause; 5) poor hygiene and sanitization; 6) some strains are naturally resistant to some drugs. Finally, the use of fungicides in agriculture to treat fungal diseases in crops can also contribute to resistance in people exposed to these drugs.

Resistance to antifungals can be defined from both a microbiological and clinical point of view. Microbiological resistance is a reduced susceptibility of the fungus to an antifungal agent. It can be primary (intrinsic) or secondary (acquired). Primary resistance is usually found among certain fungi that were not exposed to the drug. It can be passed down to the next generation; thus, all isolates of that species can share this characteristic. On the other hand, secondary resistance develops among some susceptible strains that were exposed to the antifungal agent, and it is usually related to an altered gene expression (Kanafani & Perfect, 2008).

Due to vulvovaginal candidiasis being the second most common cause of vaginal infections (the first is bacterial vaginosis), most women may have been self-diagnosed or treated, increasing the potential for development of antifungal resistance. The incidence of antifungal resistance among invasive *Candida* species remains low, with statistics reported of 1.0%–2.1% in *C. albicans*, 0.4%–4.2% in *C. parapsilosis*, and 1.6% in *C. tropicalis* (Kanafani & Perfect, 2008). However, *C. glabrata* is resistant to fluconazole with a value of 14% (Perlin, Rautemaa-richardson, & Alastruey-izquierdo, 2017).

According to the “European Committee on Antimicrobial Susceptibility Testing” (EUCAST) and the “Clinical and Laboratory Standards Institute” (CLSI), antifungal susceptibility in *vitro* is based on a standardized assessment method for yeast, *Candida* species (NCLS, 2004) (EUCAST, 2020), whose main utility is to allow the detection of resistance and the susceptibility to drugs. Both methods make it possible to discriminate wild susceptible strains (which have not gained resistance) and resistant strains (which exhibit intrinsic or acquired resistance mechanisms).

1.12 Azole resistance in *Candida* spp.

One of the most prescribed antifungals to treat *Candida* species are the azoles. Many published studies have documented that *C. albicans* has developed high resistance to fluconazole (Whaley et al., 2017). This is because fluconazole is used to combat a local variety of infections or as a prophylactic measure. Moreover, some strains naturally acquire resistance to fluconazole, which is the case of *C. krusei*. Consequently, this facilitates the development of resistance mechanisms, such as the increase of efflux pumps, mutations in *ERG11*, *ERG3*, and overexpression of *ERG11*, which will discuss in the next section.

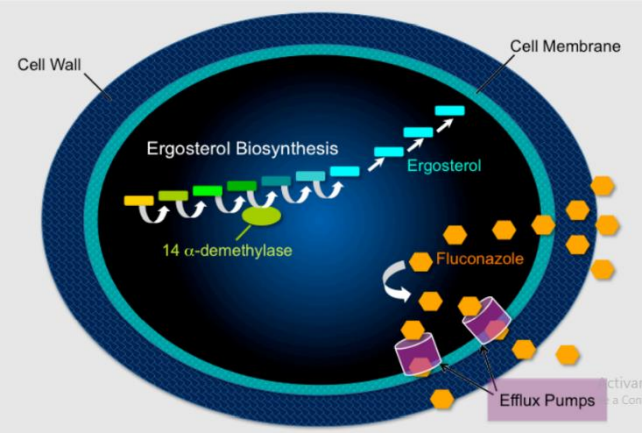


Figure 4. Ergosterol biosynthesis and mechanisms of azole resistance.

1.12.1 Overexpression of efflux pumps

The reduced level of drug accumulation inside the cell is because of efflux pumps, which discharge the drugs outside the cell through "ATP-binding cassette (ABC) transporters" (Figure 4). Besides, efflux pumps are encoded by *CDR1*, *CDR2* and *MDR1* genes and regulated by the transcription factors Tac1 and Mrr1 in *C. albicans* (Revie, Iyer, Robbins, & Cowen, 2018) and CgPdr1 in *C. glabrata* (Perlin et al., 2017). In some studies, overexpression of *CDR1*, *CDR2*, and *MDR1* genes in *C. albicans* generates resistance to fluconazole (Kanafani & Perfect, 2008).

1.12.2 Mutations in ERG11

Because of amino acid substitutions in Erg11p, the azole´s binding site (nitrogen) cannot bind the fungal target (iron group). François et al. demonstrated that replacement of the amino acids G129A, Y123H, S405F, G464S, and R467K cause fluconazole resistance in clinical isolates of *C. albicans* (Francois et al., 2005). Thus, the azole cannot inhibit ergosterol biosynthesis with high affinity.

1.12.3 Mutations in ERG3

Inhibiting fungal cell growth is by depleting ergosterol and the accumulation of toxic sterols inside the cell. A replacement of 14α-methyl fecosterol instead of 14α-methyl-3, 6-diol, which are encoded by the *ERG3* gene during deficiency of sterol Δ5, 6-desaturase (François et al., 2005) (El-houssaini, Elnabawy, Nasser, & Elkhatib, 2019) produce antifungal resistance. Besides, mutation of their *ERG3* gene and the loss of function of Erg3p stimulate fungal growth during azole treatment because the non-toxic sterols lead to a functional cell membrane. This has been demonstrated with deletions and insertions in *ERG3* in *C. albicans* isolates (Morschhäuser, 2016), resulting in depletion of ergosterol next to cross-resistance to azoles and polyenes.

1.12.4 Overexpression of ERG11

ERG11 is the most important gene in ergosterol biosynthesis. It is regulated by the zinc cluster transcription factor (*UPC2*) and is activated when there is not ergosterol (Kanafani & Perfect, 2008). Therefore, if there are gain of expression of *UPC2*, overexpression of *ERG11* in the cell makes it capable of producing much more ergosterol. Besides, some research demonstrates that a missense mutation in *UPC2* is associated with fluconazole resistance (Goldman et al., 2004) (Whaley, Berkow, Rybak, Nishimoto, Barker, & Rogers, 2017).

Finally, *C. albicans* is a commensal organism that affects us when there is a disturbance in our immune system or when the vaginal microbiota is altered because of many risk factors. The early diagnosis of *Candida* infections is key to choosing the appropriate treatment. The first-line drugs used are azoles (clotrimazole, fluconazole, and voriconazole) in the treatment of candidiasis, due to their low toxicity and the possibility of being administered orally. However, some strains of *C. albicans* can develop resistance to fluconazole through modification of genes that ensure the overexpression of efflux pumps, the mutation in *ERG3* and *ERG11*, and overexpression of *ERG11*. In addition, recent studies showed that *Candida* species can develop intrinsic resistance favoring the fitness of *C. albicans*, and azole resistance is a problem of critical importance in the world.

2 Problem statement

Vulvovaginal candidiasis is an infection caused by *Candida* species that produces inflammation symptoms (itching, irritation, and vaginal discharge). *Candida* is an organism that lives as commensal in the skin, gut, and can colonize the reproductive organs. However, this balance can be disturbed, causing an overgrowth of *Candida* spp (Sobel., 2016). This is because of the hormonal changes produced by oral contraceptives, long-term use of

antibiotics, uncontrolled diabetes, pregnancy, and other risk factors. Candidiasis is relatively frequent among women in their reproductive years, that is 18-45 years old.

The “Ministerio de Salud Pública” (MSP) has estimated 15277 cases of vulvovaginal infections caused by *Candida* species in 2017, this is a significant number for a disease at the Nacional level (David, & Icaza., 2019). Clinical data are often not right or not enough to make a good diagnosis; thus, laboratory studies are required to arrive at the etiological agent. It should be noted that VVC symptoms are similar to those of other types of vaginal infections, causing severe health problems.

In Ecuador, vaginal candidiasis is the second cause of vaginitis in women, of which approximately 80% is caused by *C. albicans* (de Bedout et al., 2003). In a recent study, Salinas et al analyzed the vaginal microbiota of the women who had vaginal infections, and tested the types of vaginal infections without identifying the *Candida* species colonizing the vagina (Salinas et al., 2020). However, a proper identification of the species is of utmost importance.

The low incidence of clinical reports of Candidiasis in Ecuador is caused by the absence of materials required for the identification of *Candida* spp., and testing the susceptibility of the fungus to antifungals. This factor makes clinicians incapable of identifying the main pathogenic fungus at the specie level; therefore, they cannot prescribe the correct treatment because some strains generate intrinsic resistance. Undoubtedly, this failure in medical diagnostics may trigger antifungal resistance, and increase the risk of complications in the patient's health. Another disadvantage in many Ecuadorian Health Centers is the lack of antifungals, so the patients are treated with a general antimycotic without testing the fungus' antifungal susceptibility causing the infection. As a result, the pathogen can develop more resistance to it, leading to serious complications.

3 Hypothesis, general and specific objectives

3.1 Hypothesis

An early diagnosis, combined with the identification of fungal species, and antifungal susceptibility tests at our university biology laboratories can help the local doctors to prescribe the proper treatment for patients, reducing the possibility of developing resistance to the most commonly used antifungals.

3.2 General objective

To determine the prevalence of fungus, identify *Candida* species from vaginal exudates of women, and evaluate their sensitivity to frequently used antifungals to provide a precise diagnosis and efficient treatment, through isolation of the fungus, antibiogram test use, and genotyping the strain by digestion of PCR products.

3.3 Specific objectives

- Determine the prevalence of fungal infection in women.
- Determine the *Candida* species present in the isolated clinical samples.
- Determine the susceptibility of isolated strains to most commonly used antifungals.
- Perform PCR reactions to amplify the DNA of the fungus.
- Perform the digestion of PCR products to identify *Candida* species.

4 Methodologies

4.1 Strains and media

A total of 134 clinical samples were obtained from patients with signs of vulvovaginal infections in the Imbabura Province of Ecuador from December 2019 to February 2021. With the collaboration of the Gynecologist participating in this study. All strains were cultured on YPD liquid or solid medium (1% yeast extract, 2% peptone, 2% dextrose, with the addition of 2% agar for solid medium) (Sherman, 1991) tubes or plates with 100 µg/ml Ampicillin and incubated at 30 °C for 24 h. Pure cultures were stored in 15% glycerol, at -80 °C, in the School's Laboratory of Biological Sciences and Engineering, at Yachay Tech University in San Miguel de Urcuquí, Imbabura, Ecuador.

For the morphology analysis, human serum (albumin, globulins), were used to induce hyphae formation. RPMI 1640 (glucose, salts, amino acids, vitamins, and a pH indicator) a synthetic medium, tomato juice (*Solanum lycopersicum*) medium ((V8, agar if solid medium), yeast extract, dextrose, and $NaCO_3$) (Shiyamalee & Panagoda, 2020), and YPD were used as culture media.

4.2 Sample collection

The number of colony-forming units (CFU) that appeared on the YPD plates was counted: a number of 100 CFU per plate indicative of fungal infection (Bauters, Dhont, Temmerman, & Nelis, 2002). If fungal cells appeared, they were grown in liquid YPD + Ampicillin at 37 °C for 8 to 12 hours at 2 g-force for species determination and for antifungal sensitivity tests. Figure 5 shows the workflow of the whole process.

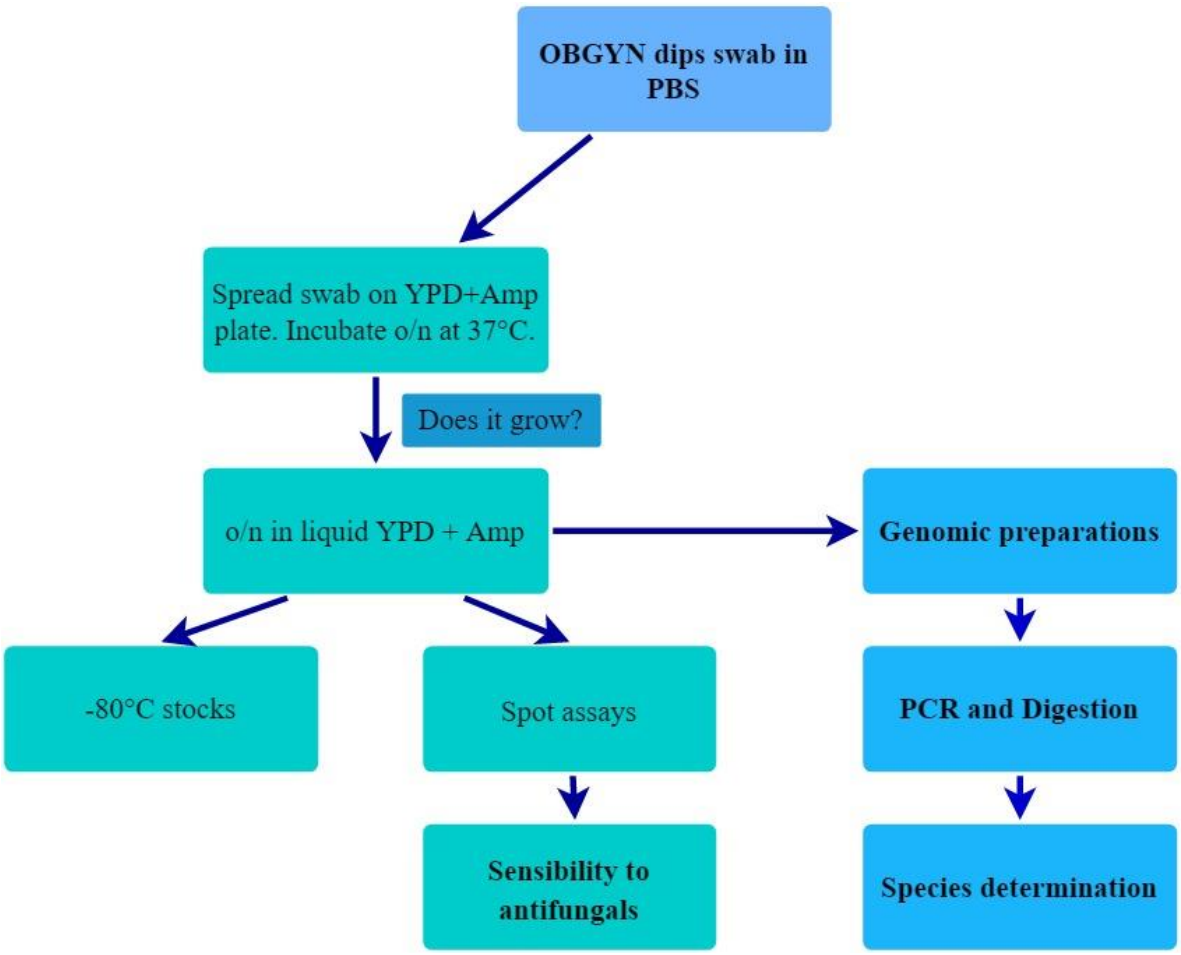


Figure 5. Flowchart of sample processing.

4.3 DNA extraction

Genomic DNA of *Candida* spp. was extracted using the bead bashing method according to Mirhendi, et al (2005). Cell wall disruption occurs by mechanical grinding with glass beads followed by purification using Phenol: Chloroform: Isoamyl alcohol (24:24:1). Firstly, each strain kept at -80 °C was reactivated in 1 ml YPD with ampicillin, for 8 to 12 hours under shaking at 37 °C, with an agitation of 2 g-force or until obtaining over 100 million cells/ml. The cells were collected by centrifugation at 435 g-force for 2 min in a phenol-resistant microcentrifuge tube (Eppendorf), then washed with dsH_2O. Later, cells were resuspended in 200 µl of lysis buffer "TENTS" (2% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA). After adding glass beads (0.5 mm in diameter) under the meniscus, samples were vortexed for 1 min to disrupt the cells completely. Carefully, 200 µl of phenol: chloroform/isoamyl alcohol were added, and the mix was vortexed for 1 min. Samples were subsequently centrifuged at 18360g-force for 5 min and the upper aqueous layer was extracted into a clean tube. The supernatant (DNA) is removed and precipitated in 2 volumes

of 95% EtOH, and the tubes were incubated at -20 °C for 30 min. Next, samples were centrifuged at a speed of 21293 g-force, at 4 °C, for 5 min. Then, the pellet was washed with 70% ethanol, centrifuged 1 min at 24 °C, and the supernatant was removed. Finally, the DNA was suspended in 50 µl of TE (10mM Tris-1 mM EDTA) or H_2O. The purity and concentration of the DNA were determined by spectrometry at 260 and 280 nm. Samples were preserved at -80 °C until use.

4.4 PCR

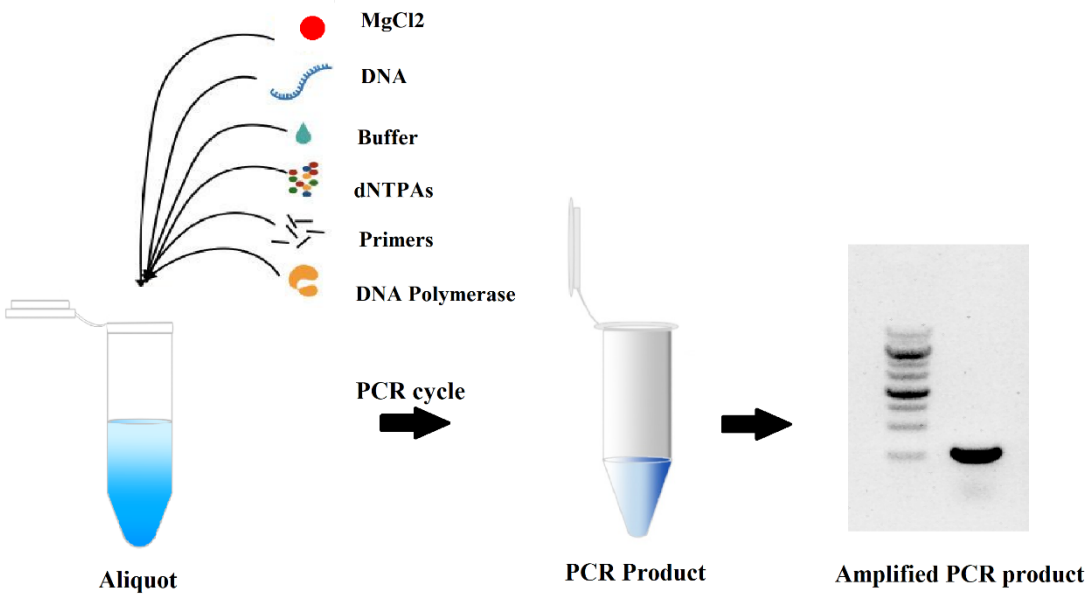


Figure 6. Stages of the PCR and its amplified products.

The DNA region corresponding to the ITS1-ITS4 segment of the 18S rRNA gene was amplified using the PCR technique with the following primers: ITS1 (5'-GCATCGATGAAGAACGCA GC-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') (Table 3). PCR was carried out as describes in table 2. Components and volumes used for the PCR of *Candida* species analyzed are the following 13.3µl of H_20, 10X buffer 5µl, 10x deoxyribonucleotide triphosphates (dNTPs) 2.5 µl, 10 µM Primers 2 µl, and 0.2 µl of Phusion DNA Polymerase (Thermo scientific) and finally template DNA 1µl (Table 2 and Figure 6). Furthermore, the cycles were carried out in the Thermal cycler TM (Applied Biosystems). The PCR procedure was as follows: an initial denaturation step at 94 °C for 5 min was continued by 35 cycles of denaturation at 94 °C for 30s, primer annealing at 50 °C for 45s, and finally extension at 72 °C for 1 min, with a final extension step of 7 min at 72 °C. The amplified DNA fragments were resolved by 1% (w/v) agarose gel electrophoresis in 1X TAE buffer (40 mM Tris 20mM Acetic acid, 1 mM EDTA), with a constant voltage of

100 volts for 30 min. Ethidium bromide (0.5 µg /ml) was used to stain the gel, and 1kb ladder marker (Sinops) as a molecular weight marker. Finally, the products were visualized under UV light and photographed. Strains of *C. albicans*, *C. glabrata*, *C. tropicalis*. *C. parapsilosis* obtained from a local hospital characterized by a Vytek 2 apparatus was used as controls for quality control and protocol optimization.

Table 2. *Components and volumes used for the PCR of Candida spp.*

PCR components	Volume per reaction (µl)
H_2O	13.3
10X Buffer reaction	5
10X dNTPs	2.5
50uM $MgCl_2$	1
10uM Primers (F and R)	2
Phusion DNA Polymerase	0.4
Template DNA	1
Total volume	25

Table 3. *Primers used in the study.*

Oligo name	Sequence (5' to 3')	Length	*Fungus*	Target gene	Amplicon (bp)
ITS1 (Forward)	*TCCGTAGGTGAACCTGCGG*	19	*Candida*	Fungal ITS	290
ITS4 (Reverse)	*TCCTCCGCTTATTGATATGC*	20	*Candida*	Fungal ITS	

4.5 Digestion of PCR products

4.5.1 Precipitation of DNA

PCR products were mixed with water to a final volume of 200 µl. Then, 20 µl of sodium acetate and 450 µl of 100% ethanol were added to precipitate and concentrate DNA. After this, samples were vortexed for 1 min and stored at -20 °C for 30 min. At this point, DNA was spun for 20 min at -4 °C. The pellet was washed with 70% Ethanol (500 µl) at room temperature. Any ethanol residues were removed by evaporation in a laminar flow hood.

4.5.2 RFLP analysis

In an RFLP ("restriction fragment length polymorphism") DNA is digested into small fragments by the enzymes. So, it is used to identify the most medically important *Candida* species (Mirhendi et al, 2005). The precipitated DNA, which was obtained after amplification, was digested with the restriction enzyme *MspI* (Thermo scientific). The enzyme recognizes and cleaves at the CCGG sequence. Digestion reactions were accomplished in a total volume of 25 µl containing 2.5 µl of 10X buffer, 10 µl of the PCR products of each sample and 11.5 µl of distilled water, and 1 µl of restriction enzyme (10 U/µl). Incubation took place at 37 °C for 1.5 h.

The restriction fragments were then separated by 2% agarose gel electrophoresis with 0.5 µg/ml ethidium bromide in 0.5x TAE buffer, for 45 min, at 100 V. Ten microlitres of the digestion products and 5 µl of loading dye were mixed and loaded into the gel, together with an aliquot of 1Kb DNA marker (Synapse Inc). Following this, band patterns were visualized using an ultraviolet transilluminator and photographed.

4.6 Antifungal sensitivity tests

The disk diffusion test is the most used technique to determine antimicrobial susceptibility, which provides quantitative results through the lecture of inhibition halos that define whether the strain is susceptible or resistant to the antifungal. Samples that produced fungal growth on solid YPD plates were used to analyze their sensitivity to the following antifungals: fluconazole (25µg), voriconazole (1µg), and clotrimazole (2%) because these were the most commonly prescribed to patients with vulvovaginal fungal infections in the Imbabura Province.

A single strain colony was inoculated into 1ml of YPD liquid medium, and the culture was incubated at 37 °C for 8 to 12 hours, shaking at 2 g-force. That usually resulted in dense cultures containing approximately 10^8 cells/ml. A dilution of the culture was made to measure its absorbance in a Nanodrop apparatus (Thermo Fisher Scientific). Dilutions were then made to a final concentration of 10^6 cells/ml. An aliquot (50 µl) of the appropriate dilution was transferred to a YPD plate with ampicillin and spread evenly. Upon drying, two disks containing antifungals (fluconazole and voriconazole) were placed onto the plate. A spot of clotrimazole cream was applied in an area similar to that of the disks. Plates were incubated for 24 hours at 37°C.

In addition, it was adopted a standardized method to produce reliable results like EUCAST. This is based on direct colony suspension based on 0.5 McFarland turbidity

standard, approximately corresponding to 10^6 CFU/ml for *C. albicans*, using MHC as a medium (EUCAST, 2020). A sterilized swab was used to inoculate the plate, rotating it in three directions, spreading the inoculum rapidly over the entire agar surface, ensuring that there were no gaps between streaks. Then, within 15 minutes, the antifungals were placed on the plate as previously mentioned. Plates were incubated during 24 hours at 37 °C.

According to "Clinical and Laboratory Standards Institute" (NCCLS) criteria, the minimum inhibitory concentration (MIC) value of antifungals drugs for the *Candida* spp. were evaluated as susceptible, intermediate, and resistant (NCCLS, 2004). Additionally, inhibition diameters were measured.

4.7 Additional experiments for the identification of *Candida* species

4.7.1 Comparative study of susceptibility to antifungals using cells that grew in RPMI, YPD and TJ media

A single colony was inoculated in the three media: RPMI, YPD, and Tomato Juice (TJ), and these cultures were incubated at 30 °C for 8 to 12 hours, shaking at 2 g-force. Then, the absorbance was measured in a Nanodrop apparatus (Thermo Fisher Scientific). In order to know the concentration of cells. Dilutions were made for the three replicates. This was based on direct colony suspension based on 0.5 McFarland turbidity standard, approximately corresponding to 10^6 CFU/ml for *C. albicans*, using the cells that grew in the RPMI, YPD, and TJ as media (EUCAST, 2020). Strains were propagated on TJ liquid medium at 37 °C for 7 hours, as described (Shiyamalee & Panagoda, 2020). The rest of the process was the same as mentioned above (antifungal susceptibility tests).

4.7.2 Human serum induces filamentation

Firstly, blood was collected from one healthy donor in a blood collection tube. Soon after, cells were pelleted by centrifugation, and the serum was stored at 4 °C until use. 10 µl of an overnight YPD culture of the fungus was inoculated in 0.9 ml of YPD or TJ medium, to which was added 0.1 ml of serum, and grown at 37 °C for two hours with shaking. The same process was done for TJ medium without serum. It is important to mention that the pH of TJ is 5.7. After two hours, cells were washed at 435 g-force to concentrate, and then cell morphologies were examined microscopically.

Finally, this work´s results were shared with the collaborating medical personnel to contribute to patients' proper treatment.

5 Results

5.1 Incidence of vulvovaginal infections

Of the 134 samples studied from women with symptoms of candidiasis at the Atuntaqui Hospital and Health Center of Urcuquí. Only, 42 samples (31,34%) were positives for *Candida* species, and the remainder (68,6%) did not present candidiasis (Figure 7). And out of this 68,6%, most of them were resistant bacteria to ampicillin. Indeed, fungus colonies are creamy-white and smooth while bacterial colonies are shiny and small (Figure 15).

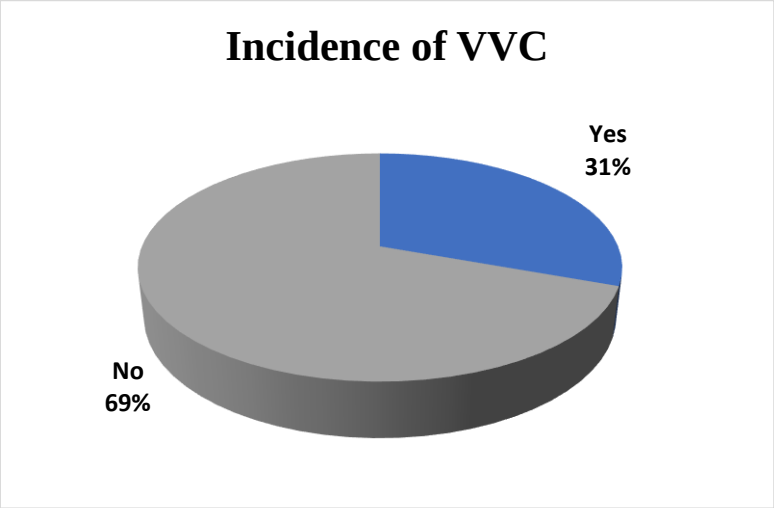


Figure 7. Incidence of VVC.

This study showed a distribution of *Candida* species following the various age groups of women with candidiasis. *Candida* isolates in the age group 18- 23 (35.29%) was the highest followed by 24-29 (23.53%), then 30-35 (20.59%), 36-41 (14.71%) and finally 42-46 (2.94%) (Figure 8). We observed that 42-46 (2.94%) had the lowest incidence of women with candidiasis. The result generally showed a decreasing tendency as you move down of peak group 18- 23 years old (the group with the highest percentage of *Candida* spp.).

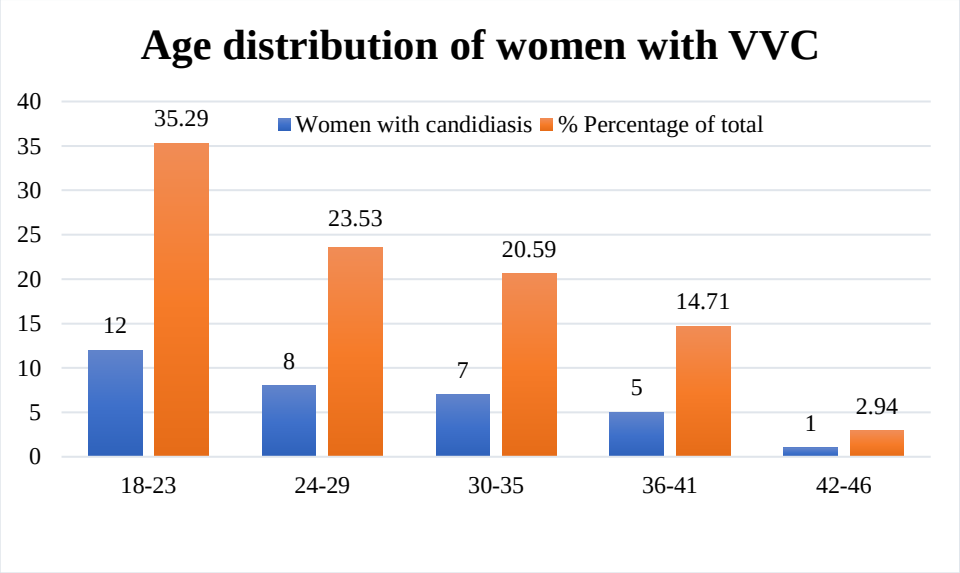


Figure 8. Age distribution of women with VVC.

5.2 PCR and RFLP assay

The DNA concentration and the respective absorbance rates at 260/280 and 260/230 nm are shown in table 7 that are attached in the annexes. Fungal specific primers (ITS1 and ITS4) could amplify all isolates´ ITS region, resulting in a PCR product of approximately 500 bp (Figure 9). The PCR product was digested with *Msp1*, as mentioned in the Methods section. Figure 10 shows the product of the digestions as bands corresponding to four *Candida* species more clinically important. Digestion of PCR products by *Msp1* generated by two bands for *C. albicans*, *C. glabrata*, *C. tropicalis* of approximately 300 ~200 bp; however, there were no recognition sites for this enzyme in *C. parapsilosis* sequence, obtaining only one band of 500 bp. In some exceptional cases, when we used 0.4 µl of the restriction enzyme (10 U/µl), we obtained one band of 500 bp and two bands of 200 bp and 300 bp (Figure 9, lane 2 and 7). These results suggest that the amount of enzyme used was not capable enough to cut the PCR product, as in the other (lanes), in which we used 1 µl of *Msp*1 (10 U/µl) (Figure 10, lane 1,3,4,5,6,8). In order to have consistency, the results of the identification at the species level (Figure 10B) were compared with those reported in similar studies (Figure 10A). As a consequence, identical patterns of *Candida* species were obtained.

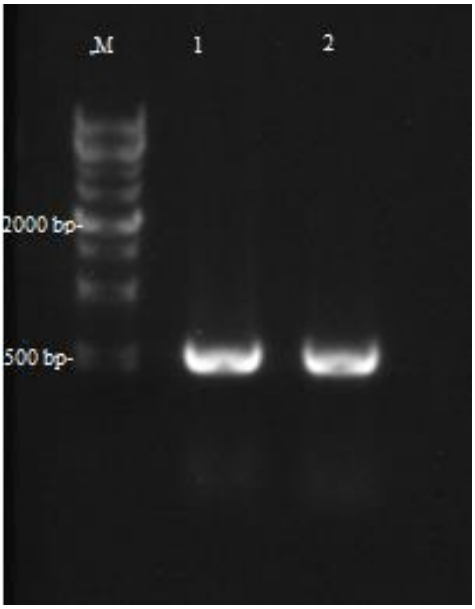
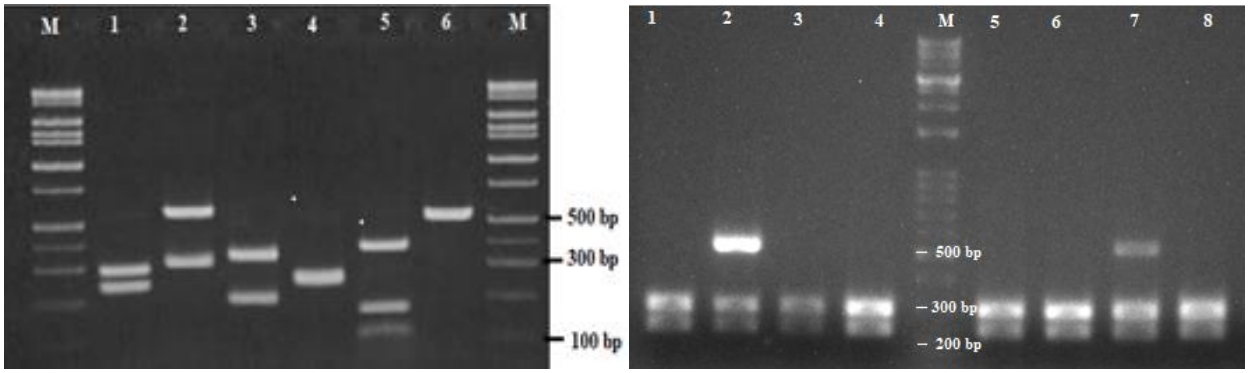


Figure 9. PCR product of *Candida* species. Lanes 1 and 2: PCR products obtained using ITS1 and ITS4 primers. Lanes M: Molecular size marker: 1kb ladder (Sinops).



A) B)

Figure 10. Digestion of PCR product of *Candida* species with the enzyme *Msp1*. This figure shows the digestion of the PCR products. A) Lanes 1-6: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, and *C. parapsilosis*. Lane M: molecular size marker. B) Lanes 1-4, 5-8: *C. albicans* and Lane M: molecular size marker. Image A taken from *"A one enzyme PCR-RFLP Assay for Identification of Six Medically Important Candida Species,* by Mirhendi et al., 2006*, Med Mycol."*

5.3 Identification of *Candida* species

Of the 30 samples characterized by RFLP assay, *C. albicans* was the specie most isolated with 96.7% (n = 29), and the "non-albicans" species represented 3.3%, of which *C. parapsilosis* with 3.3% (n=1) and *C. glabrata, C. tropicalis* represent the 0% (Table 4 and Figure 11).

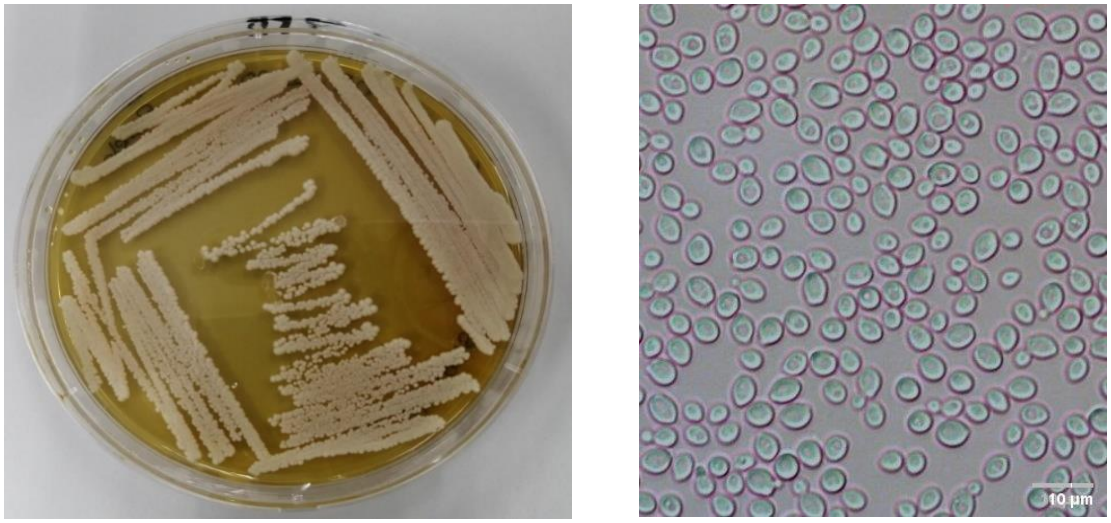


Figure 11. Isolated *C. albicans.* Left, growth on YPD plate; Right, visualization under the microscope of *C. albicans* cells. Bar 10 µm.

Table 4. *Distribution of Candida species in women with candidiasis.*

Species	N° of isolates	Percentage
C. albicans	29	96.7
C. glabrata	0	0
C. tropicalis	0	0
C. parapsilosis	1	3.3
TOTAL	30	100

5.4 Antifungal susceptibility

The relative abundance of fungi resistant or susceptible to the various antifungals (Figure 12) was determined, as indicated in the section of materials and methods. The susceptibility and resistance for *Candida* species are interpreted as a percentage in Table 5. *C. albicans* was more susceptible to clotrimazole (CLO), voriconazole (VOR), and fluconazole (FLU) with 80%, 80% and 53.4% respectively. We observed that *C. albicans* was resistant to fluconazole (FLU) with 23.4%, followed of voriconazole with 10%. And, there are not resistance to clotrimazole. In the same way, *C. parapsilosis* was more susceptible to clotrimazole than voriconazole and fluconazole.

Table 5. *Antifungal susceptibility and resistance breakpoints for Candida spp. in vitro.*

Antifungal	Species	Inhibition zone diameter (mm).		
		S (%)	SDD (%)	R (%)
Voriconazole 1µg	*C. albicans* (n=29)	80 (> 17)	10 (14-16)	10 (< 13)
Fluconazole 25µg		53.3 (> 19)	23.4 (15-18)	23.4 (< 14)
Clotrimazole 2%		80 (> 20)	20 (12-19)	0 (< 14)
Voriconazole 1µg	*C. parapsilosis* (n=1)	-	100	-
Fluconazole 25µg		-	100	-
Clotrimazole 2%		100	-	-

Nota. Interpretation of antifungal susceptibility according to CLSI standards. Susceptible (S), susceptible dose dependent (SDD), and resistant (R).

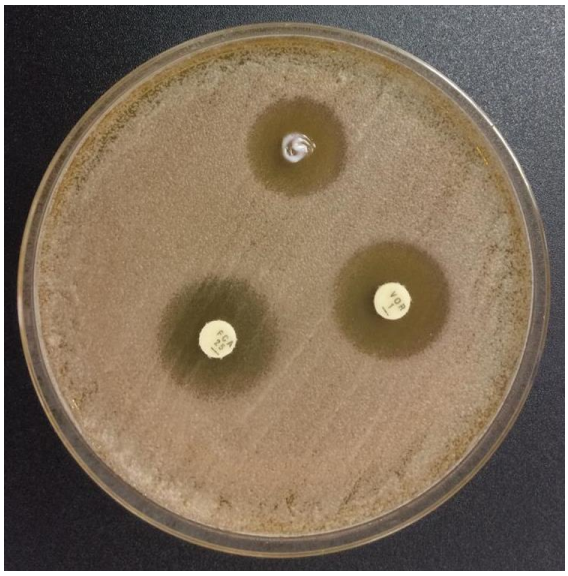


Figure 12. Antifungal drug susceptibility test. Right: voriconazole; left: fluconazole; top center: clotrimazole cream.

5.5 Additional experiments for the identification of *Candida* species

5.5.1 Comparative study of antifungals susceptibility using cells grown in RPMI, YPD and TJ media.

As part of our studies, we decided to compare the growth of *C. albicans* cells in three different media: RPMI, YPD, and TJ at 30 °C. The growth of cells in the three media differed

significantly. There was more cell growth in the YPD medium, followed by TJ and RPMI medium (Table 6). In one of the replicates, cells grew better in RPMI than in TJ.

Table 6. *Concentration of cells in RPMI, YPD, TJ media.*

RPMI (cells/ml)	**YPD (cells/ml)**	**TJ (cells/ml)**
0.104x10^8	0.539x10^8	0.278x10^8
0.234x10^8	0.757x10^8	0.321x10^8
0.104x10^8	0.931x10^8	0.06x10^8

Furthermore, resistance to voriconazole (VOR), fluconazole (FLU), and clotrimazole (CLO) were 19 mm, 19 mm, 17 mm. There were no significant differences in susceptibility to the antimycotic compounds along the three replicates when using cells grown in different media. The concentration of cells that were spread on the YPD agar plate were the same. (Figure 13).

Figure 13. Antifungal susceptibility in YPD agar, using the same concentration of *C. albicans* cells that grown in RPMI, YPD and TJ media.

5.5.2 Filamentation assay.

The vulvovaginal environment is a sophisticated space that favors the morphological transition from yeast cells to hyphal cells. In this study, serum and a temperature of 37 °C induced filamentation. In this assay, we observed many *C. albicans* hyphae in the groups YPD and TJ with serum. The results indicate that serum has a direct effect inducing filamentation in *C. albicans.* The serum also caused clumping of the cells, which indicates the expression of adhesin proteins in the hyphal cell walls (Figure 14). Interestingly, we

observed a significant filamentation in TJ in the absence of serum. Since, TJ does not induce filamentation at 30 °C, but it seems that the shift in temperature to 37 °C is sufficient to induce formation of hyphae in this medium.

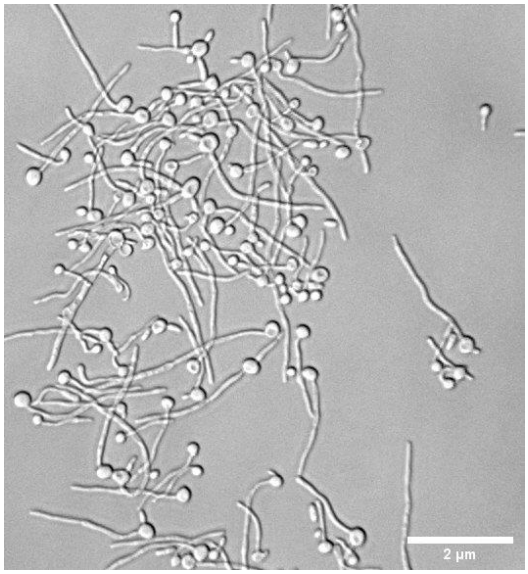


Figure 14. Filamentation of *C. albicans.*
(from F. J. Álvarez library and adapted). Bar scale. 2µm

6 Discussion

Vulvovaginal candidiasis is not a reported disease and diagnosis only is based on signs and symptoms without the support of laboratory analysis. As a result, the pathogenic fungi at the specie level and their antifungal susceptibility profile are not determined. VVC is the second common cause of vaginal infections in women (Buscemi, Arechavala, & Negroni, 2004), following by BV. In our study population, the prevalence of fungal infections was 42 patients representing 31,34%, concerning 68,6% that did not present candidiasis and may be suffering from BV or other vaginal infections. This is in agreement with current studies in which 31% (Kalia et al., 2015), 41.4% (Bitew, 2018), or less than 50% corresponded to VVC, and the remained to BV or another infection. This result suggests that symptoms of vaginal infections are similar to candidiasis, that is to say, the signs mentioned above are not unique to VVC. Besides, there was no information on how the patients exhibited the symptoms. According to Bitew, VVC is associated with pre-menopausal states, patients using birth control methods, taking antibiotics at least for 2 weeks, high glucose levels and sex parents (2018) (Kalia et al., 2015). In addition, hormonal changes and any disturbance in the immune system induce the overgrowing of *C. albicans*, causing candidiasis.

Moreover, most women with candidiasis in this study belonged to 18- 23 (35.29%) and 24- 29 (23.53%) years old. In similar studies, the prevalence of VVC is high in women aged 18 years old, then this decreases with age (Blostein et al., 2017). The hormonal changes produced by menstruation, number of sexual partners, and pregnancy promote the overgrown of *C. albicans.* The high levels of estrogens and progesterone promote the glycerol production that serves as a carbon source, thus benefiting *C. albicans* to the vaginal epithelium (Tsega, 2019). However, some studies said that age range is not statistically significant to develop VVC (Bitew, 2018).

Molecular techniques are among the most sophisticated tools developed for gene amplification and genetic mapping to detect new diseases. In the present study, using the universal primers, ITS1 and ITS4, we obtained an amplified PCR product of 500 bp of rDNA region from the genomic DNA of *Candida* strains. The PCR product was cut with the endonuclease *MspI*. This enzyme produces two RFLP bands of 200 bp and 300 bp for *C. albicans*, *C. tropicalis*, and *C. glabrata*, and one band of 500 bp for *C. parapsilosis*, which is in agreement with other studies (Mohammadi et al., 2015) (Maduro et al., 2012). Finally, this technique allows identifying *Candida* isolates at the species level, with no observed misidentification cases.

C. albicans was the most frequently identified yeast in our study with 96.7% of the samples, which coincides with different authors who report between 58%, 76% (Buscemi et al., 2004) to 97% (Ignjatović et al., 2020). The "non-*albicans*" species like *C. parapsilosis* represented 3.3%, while *C. tropicalis* and *C. glabrata* were not found in this study. However, most of the studies have reported lower prevalence than our results, showing 65% to 80% for *C. albicans* (Bitew, 2018). A broader study with more samples should be carried out to know the prevalence of *Candida* species in women with VVC in the Imbabura province. The physiological and immunological changes may explain this prevalence of *C. albicans* during pregnancy, antibiotics use, and some other factors that alter the vaginal microbiota.

In relation, to the in *vitro* susceptibility study, most of the *C. albicans* strains were susceptible to clotrimazole (80%,) followed by voriconazole (80%) and fluconazole (53.3%). Moreover, *C. albicans* showed 0%, 10%, and 23.3% resistance to these drugs, respectively. In the same way, the isolate of *C. parapsilosis* were much more susceptible to clotrimazole. However, this result is not significant. These results have similarity to those found by other investigators and justify the frequent therapeutic failures of fluconazole in vaginal candidiasis infections (Zhai et al., 2018). Additionally, in a study, 25% *C. albicans* isolates from vulvovaginitis clinical patients were fluconazole resistant (Marchaim., 2012). Resulting, in

acquisition of mutations in *ERG11* and *ERG3* and overexpression of efflux pumps that increase antifungal resistance (Berkow & Lockhart, 2017).

In this study, human serum induces filamentation at 37°C; cells started forming hyphae in response to serum nutrients after 2 hours of incubation. This result coincides with other studies. And, it is a qualitative microbiological method more used to detect *C. albicans* (Neppelenbroek et al., 2014). Serum albumin is an important component that contributes to filamentation (Feng, Summers, Guo, & Fink, 1999). On the other hand, cells that grow in TJ without serum also filamented after two hours of incubation. This study corroborates the recent findings of researchers who reported that TJ alone induces germ tube test formation in *C. albicans* (Shiyamalee & Panagoda, 2020). It is important to note that in their article, they did not use our same recipe. Our recipe has V8 juice as a main ingredient of the TJ medium. This result suggests that the nutrients and sugar of V8 induce filamentation in *C. albicans*.

Interesting, this happens with a pH of 5.7, that of the TJ, because it is not common for a medium to be able to induce filamentation in the absence of serum and at a pH lower than neutral. Our result revealed that tomato juice is an effective method for the differentiation of *Candida* species. Besides, it is rapid, economical, and less hazardous than human serum for differentiation of *C. albicans* from non-*albicans* species. Filamentation is a way to differentiate *C. albicans* of non-*albicans* species*;* however some studies reported that *C. tropicalis* have more similarity with *C. albicans* and also form hyphae (Zhang et al., 2016).

Our comparative study of culture media used cells grown in RPMI, YPD, and TJ media. The results indicate that YPD is the best culture media that favors the planctonic (not filamentous) growth of the fungus. And also, tomato juice is an optimal media and cheap (Shiyamalee & Panagoda, 2020); however, it takes a longer time to prepare the medium because we need to adjust the pH and sometimes filtrate it. RPMI is the media most used for susceptibility testing based on CLSI and EUCAST standards (Arendrup et al., 2010) but, it is more expensive. YPD is a complete medium for yeast, besides being easy to prepare.

Finally, the early treatment of candidiasis decreases the possibility of *C. albicans* acquiring resistance to the antifungals clinically most used. Furthermore, molecular techniques are appropriate and reliable methods for the precise identification of fungal pathogens at the species level, which would be useful in clinical laboratories and lead to proper infection control.

7 Conclusions

C. albicans is a commensal organism that affects us when there is some disturbance in our immune system or when the vaginal microbiota is altered because of many risk factors. Of the 134 samples collected from women presenting signs and symptoms of candidiasis at Atuntaqui Hospital and in the Urcuquí Health Center in the Imbabura province, only about 34% presented candidiasis with 64% that did not present it. The age group most affected by VVC was between 18 and 23 (35.29%) and between 24 to 29 (23.53%) years old. Out of positive cultures, 30 were identified at the species level, and of them, *C. albicans* was the species more prevalent following *C. parapsilosis*, *C. glabrata*, and *C. tropicalis* represent 96.7%, 3.3%, and 0%, respectively.

Although clotrimazole appeared to be active against most isolates of *C. albicans*, some samples were resistant to fluconazole. This may have been due to excessive use, self-medication, or incomplete intake of fluconazole. YPD and TJ were found to be optimal media for fungal growth. Moreover, TJ alone is an effective technique to differentiate *C. albicans* from non-*albicans* species. TJ induce filamentation in *C. albicans*. The TJ medium takes a long time to prepare because we need to adjust the pH; we need to filtrate because it is susceptible to bacterial contamination.

Sampling of vaginal secretions and applying molecular techniques (PCR and RFLP) is an accurate and advisable alternative to establish a good diagnosis for candidiasis in symptomatic patients. To have a higher sample size could contribute to a more precise analysis of the prevalence of *Candida* species in the Imbabura province. However, sample size could not be increased due to the sanitary emergency (COVID-19 pandemic) that resulted in fewer women visiting the gynecologist.

8 Recommendations

With the results obtained in this work, it is recommended the study of vulvovaginal infections should be done carefully, associating the clinical data with the bacteriological findings before starting the treatment.

It is also desirable to acquire more samples to know more exactly the prevalence of *Candida* species and their susceptibility test in the region subject of study.

Finally, it is advisable to inform women about the possible risk factors that promote *Candida* colonization of their reproductive areas. Women should get gynecological check-ups more often and follow the proper medical prescriptions to not develop resistance to the drugs.

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10 Annexes

Table 7. *DNA concentrations and their absorbance rates at 260/280 and 260/230nm.*

Sample ID	Nucleic Acid (ng/µl)	260/280	260/230
3	770,5	2,00	1,98
5	608,6	2,01	1,96
6	232,7	1,88	1,84
7	274,3	1,83	1,79
9	176,5	1,85	1,80
10	120,9	1,68	1,66
13	129,8	2,04	1,66
15	709,2	2,08	2,22
16	510,8	2,14	2,26
20	255,7	2,08	2,17
24	246,0	2,07	1,95
25	320,7	2,10	2,23
32	545,4	2,12	2,16
35	168,2	2,12	1,96
37	87,1	2,08	2,03
38	168,5	2,08	2,23
40	181,8	1,96	2,14
49	76,8	2,10	2,17
50	113,4	2,04	1,98
52	124,6	2,06	2,21
56	82,3	2,03	1,94
60	50,1	2,00	1,83
61	1082,7	1,97	2,11
62	1063,0	2,01	2,16
63	876,2	1,99	2,17
64	763,8	2,04	2,29
65	983,5	2,02	2,21

75	188,5	2,13	2,28
80	113,6	2,11	1,94
86	128,1	2,11	2,08

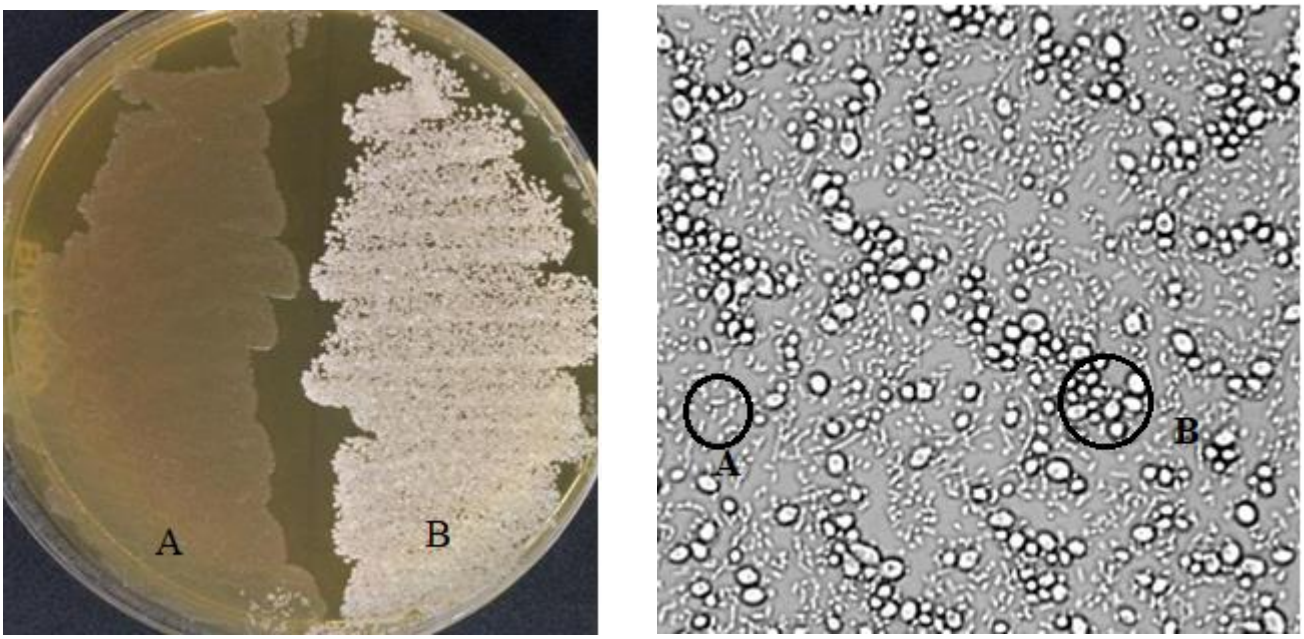

Figure 15. Culture of bacteria and fungus. Left, culture of bacteria (A) and fungus (B) on a plate; Right, visualization under the microscope of bacteria (A) and fungus (B) at 40X magnification.