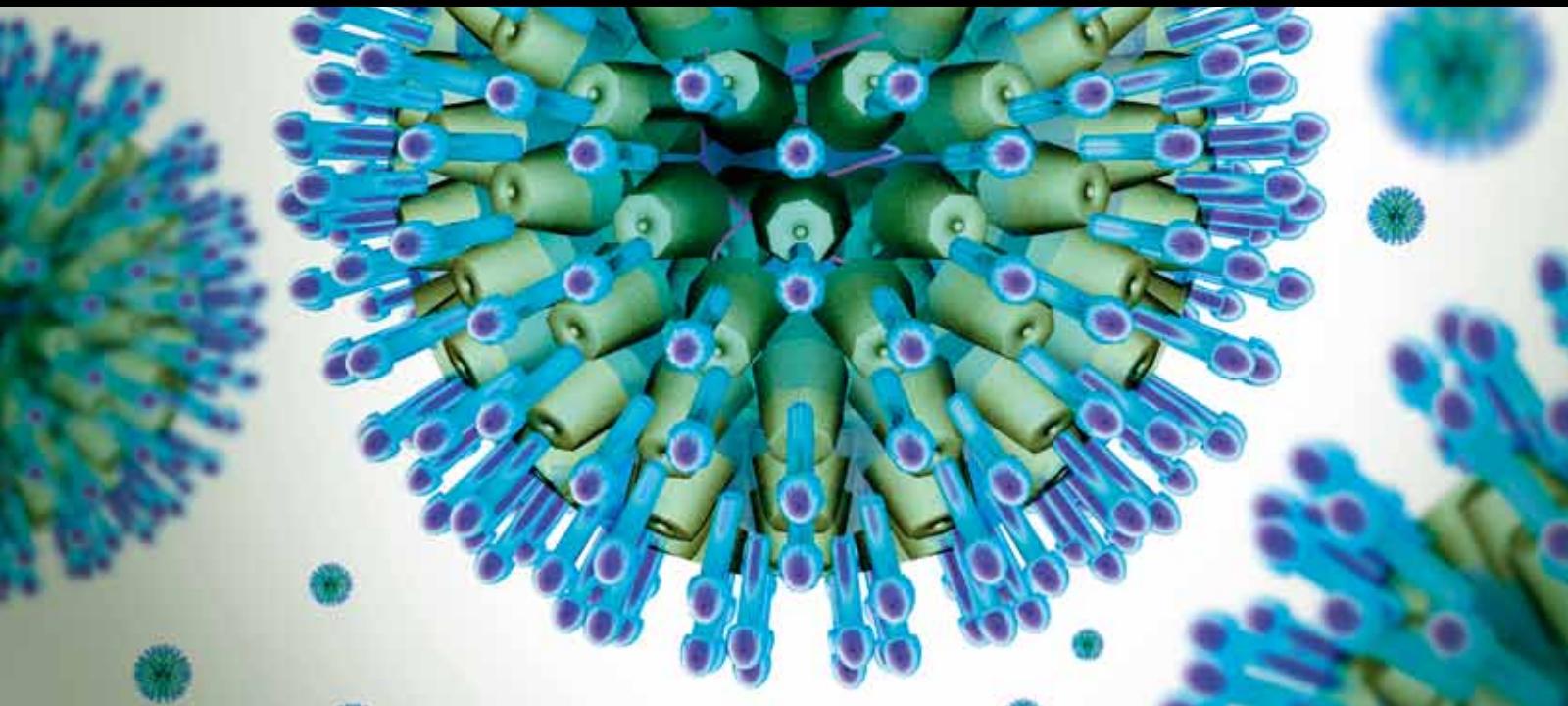


# The Infections of Lower Genital Tract

Guest Editors: Francesco De Seta, Secondo Guaschino, Bryan Larsen,  
Gilbert Donders, and Gonzaga Andabati



# **The Infections of Lower Genital Tract**

Infectious Diseases in Obstetrics and Gynecology

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## Editorial

# The Infections of Lower Genital Tract

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Infections occurring in the lower female genital tract represent a complex topic from several different viewpoints. First, there is a body of scientific knowledge and clinical consequences associated with these infections. Second, the complexity is increased by the biological diversity of infectious organisms involved including bacteria, fungi, virus, and protozoan life forms. A third layer of complexity exists in the interaction between the host and infectious organisms through the inflammatory responses to infections and through the elaboration of host factors such as secreted antibodies and defensin molecules inhibitory to intravaginal microorganisms. Finally, the vagina is host to an indigenous microbiota which is credited with contributing to vaginal health, but also the flora interacts with exogenous microorganisms involved in pathogenesis of vaginal infections.

It is striking that despite the fact that the vaginal flora has been a topic of interest to physicians and scientists since the last decade of the 19th century, there is still much to be learned and newer research techniques are continuing to elucidate our understanding of the vaginal flora, infectious agents involved in pathogenic interactions with the host, and the effects of indigenous and exogenous organisms on the physiology of the vagina. Because of continuing discoveries, the offering of the present special issue is an appropriate venue for presentation of current thinking about vaginal infections. The editors are pleased to present the articles published in this issue.

As mentioned above, the vaginal infections derive from a biologically diverse collection of microbes. Thus, the reader will find that the organisms represented in this issue include bacteria (Group B *Streptococcus* in the A. Lambiase et al. article, *Treponema pallidum* in the M. de Santis et al. contribution, and *Lactobacilli* and bacterial vaginosis associated bacteria in the works of J. M. Bohbot and J. M. Cardot, C. Mitchell et al.). Viral pathogens are represented by Herpes Simplex in the G. Straface et al. article, HIV in the C. Vallone et al. contribution, and HPV in the M. Guadalupe et al. paper. *Candida* species are addressed by A. Palmeira-de-Oliviera and coworkers, while the continuum of biological forms is rounded out by the R. Sehgal et al. paper which focuses on *Trichomonas*.

Another aspect of vaginal infections is the diversity and disparity of epidemiologic details depending on both geography and ethnicity of the populations studied. Several articles in this issue provide information about populations derived from various locations across the globe. It is always useful to become informed about common issues along with differences in prevalence and manifestations at locations other than one's own practice site.

In addition to efforts of physicians and scientists to define and characterize vaginal infections in terms of microbiology and pathophysiology, there is always the overarching question of how we can best deal with these clinical conditions through available or new therapies. The prevailing logic

of using antibiotics to deal with these issues has been both logically and widely applied since the discovery of penicillin in the middle of the last century. But as indicated by M. de Santis et al. review that despite highly effective penicillin applied to treating syphilis, the disease has not been eradicated and congenital syphilis in particular remains a continuing problem. Certainly history has reminded us that the ability of microorganisms to develop resistance to antimicrobial drugs has altered the ability to treat some infections. A. Lambiase and coworkers have explored the occurrence of resistance to macrolides and clindamycin among GBS isolates in Italy. Likewise antiviral drugs may interfere with neonatal infection with Herpes Simplex during pregnancy (G. Straface et al.). In addition to traditional antibiotics, investigators are continuing to discover unanticipated antimicrobial attributes of drugs as illustrated by the work of A. Palmeria-de-Oliviera et al. who studied the anti-*Candida* activity of nitroglycerine and lidocaine. As with any compound with antimicrobial activity in vitro, the translation of the initial findings to clinical use will be a continuing challenge.

Knowing that antibiotics, despite phenomenal historical successes, have not been able to provide a full and final answer to vaginal infections, investigators have turned their attention to exploiting host defense mechanisms in a variety of ways and employing various mechanisms of action. For example, based on the concept that normal flora organisms promote vaginal health, the use of probiotic organisms continues to be studied in relation to bacterial vaginosis (see J. M. Bohbot and J. M. Cardot). In addition to probiotics, known host defense factors have been studied as potential therapeutic drugs. A review of the possible value of lactoferrin as a therapy for the very prevalent *Trichomonas vaginalis* was examined by R. Sehgal and coworkers.

This issue should serve as a reminder that many questions remain about the content of the healthy vagina and the specific organisms capable of producing symptomatic infection. The challenge for the future is to continue to examine both the science behind vulvovaginal infectious pathology and to devise clinically relevant therapeutic approaches to the problems that continue to make women uncomfortable, undermine the quality of life, and in some cases threaten their overall health and the health of their fetuses and infants. We owe it to our wives, mothers, and daughters to continue to bring our intellectual efforts to bear on developing creative approaches to understanding and addressing vaginal infections of all types and in all populations no matter where they are in the world.

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## Review Article

# Trichomoniasis and Lactoferrin: Future Prospects

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*Trichomonas vaginalis* is a parasitic protozoan which infects the urogenital tract and requires iron as an essential nutrient. Iron is known to upregulate various adhesins required for cytoadherence and other factors involved in pathogenesis. At mucosal surfaces, iron is chelated by lactoferrin resulting in low levels of free iron. However, pathogens have evolved mechanisms for an increased uptake of iron. The present review highlights the role of iron in survival of *Trichomonas* during fluctuating concentrations of iron at mucosal surfaces during the menstrual cycle. Future prospects in terms of new drug and vaccine targets related to iron and its receptors have also been described.

## 1. Introduction

*Trichomonas vaginalis* is a flagellated, parasitic protozoan which causes trichomoniasis by infecting urogenital tract. Trichomoniasis is one of the most common causes of non-viral genitourinary sexually transmitted infection (STI) in humans with a worldwide prevalence of 170 million cases annually. According to WHO estimates, it accounts for almost half of all curable STIs [1]. Despite high prevalence, it is one of the poorly studied parasites with respect to virulence properties, pathogenesis, and immunopathogenesis. The present paper highlights the importance of various targets in pathogenesis where iron and its related transporting proteins play an important role. The role of lactoferrin (Lf) has been studied in various infections including parasitic infections, and in the present paper we will discuss the role of iron and lactoferrin in trichomoniasis.

## 2. Brief Description of the Parasite

*T. vaginalis* was discovered by Alfred Donné in 1836 and is an amitochondrial, microaerotolerant flagellate. *Trichomonas vaginalis* varies in shape and size. In a pure culture, typical shape of *T. vaginalis* is pyriform but amoeboid shapes have also been documented in parasites adhering to vaginal tissue. The average size of *T. vaginalis* is about 9 by 7  $\mu\text{m}$ . It has

five flagella; 4 are present anteriorly and the other flagellum is incorporated within the undulating membrane. It lacks a cystic stage and exists as a trophozoite. The trophozoite divides by binary fission and gives rise to progeny in the lumen or on the mucosal surfaces of urogenital tract of humans. Internal organelles include nucleus and axostyle which spans through the cell from anterior end to posterior end. Hydrogenosomes are the energy producing organelles, which appear as chromatic granules under light microscope [2].

## 3. Transmission and Clinical Features

Trophozoites of *Trichomonas* are transmitted from person to person through sexual contact. *T. vaginalis* trophozoites colonize the epithelial surface of the human urogenital tract in which they obtain nutrients, multiply, and face a constant challenge from host immune surveillance. The trophozoites divide by longitudinal binary fission after attaching themselves to mucosal surfaces of the urogenital tract. Incubation period varies from 4 to 28 days [3]. It can survive for long term in the acidic environment of vagina and the disease itself may be chronic. The clinical spectrum in women ranges from asymptomatic carrier state in approximately 50% of infected women, while symptomatic patients may suffer from mild-to-severe inflammation with a foul-smelling

discharge and severe irritation. Infection during pregnancy may be associated with premature rupture of membranes, preterm delivery, and low-birth-weight babies. In men, the clinical spectrum varies from asymptomatic carrier state to acute state characterised by purulent urethritis, dysuria, or mild pruritus [4].

#### **4. Iron an Essential Nutrient**

Iron is an essential nutrient required for the survival of both humans and pathogenic protozoa. Iron is essential for wide range of biological process, including oxygen transport, DNA synthesis, and electron transport [5]. Some protozoa such as amitochondriate protists (e.g., *Trichomonas*, *Giardia*, and *Entamoeba*) require a high extracellular iron concentration (50–200 μM) for their growth as compared to other prokaryotic or eukaryotic cells (0.4–4 μM) [6]. Such a high concentration of iron is required for functional energy metabolism system which relies heavily on Fe-S proteins [7]. To protect against invading pathogens mammals have evolved the scavenging mechanisms to limit the availability of iron near the vicinity of the pathogen. Ferric iron is chelated by lactoferrin which is an extracellular glycoprotein of the host immune system. Pathogens have also developed several mechanisms to obtain iron from the host hololactoferrin (holo-Lf) [6, 8]. Thus, it is the delicate balance between the invading pathogen and host immune response which ultimately determine the clinical outcome.

#### **5. Iron as Ferric or Ferrous Ions**

Iron in the earth exists as ferric ( $\text{Fe}^{3+}$ ) or ferrous ( $\text{Fe}^{2+}$ ) state. It can accept or donate the electrons and catalyzes the important biochemical reactions such as the Fenton reaction: (a)  $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$ , (b)  $\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}^\cdot + \text{H}^+$ . It catalyses the conversion of hydrogen peroxide to toxic free radicals [9, 10]. Thus, it cannot be available in the free state in tissues or cells: it is available only bound to proteins, limiting its ability to cause the damage. Intracellular iron is bound to ferritin or it is present within hemoglobin as a major iron content of the body. Extracellular iron is bound to transferrin which helps in transferring the iron to all cells and at mucosal surfaces it is bound to lactoferrin (Lf) [6, 8].

#### **6. Lactoferrin (Lf)**

Lactoferrin (Lf) is a mammalian non-heme iron-binding glycoprotein which can bind two ferric ions with very high affinity even at an acidic pH of 2.5–4 [11, 12]. A glycan moiety provides the protection from proteolysis and also helps in decreasing its immunogenicity [13]. Lf exists in two different conformations which are governed by the iron binding status and these are apo-Lf (without iron) and holo-Lf (with iron) [14]. Apo-Lf has an open conformation which can bind to iron, and holo-Lf exhibits a closed conformation which is saturated with iron. Lf is a highly conserved glycoprotein having a homology of approximately 70% between humans

and mice and greater than 70% between humans and cattle with respect to amino acid sequence [15].

Lf is found in various mucosal secretions, including saliva, tears, vaginal fluids, semen, nasal and bronchial secretions, gastrointestinal fluids, urine, and most abundantly in milk and colostrum [16, 17]. It is also found in blood, amniotic fluid, and in secondary neutrophils granules, where it plays an important physiological role [18].

#### **7. Functions of Lactoferrin (Lf)**

Lf is a multifunctional protein with a wide range of biological activities including regulation of iron absorption, immune response, antioxidant, anticarcinogenic, anti-inflammatory properties, and antimicrobial activity [12, 19–21]. Recently, Lf is emerged as a pivotal component of iron and inflammatory homeostasis capable of overcoming pregnancy-associated anemia, while decreasing serum IL-6 levels [22–24].

#### **8. Anti-Inflammatory Properties of Lf**

*In vivo* and *in vitro* evidence characterizes Lf as a potent anti-inflammatory compound able to reverse/attenuate inflammatory response triggered by Toll-like receptor (TLR) engagement in antigen-presenting cells. Recently, Puddu et al. [25] have shown that monocyte-derived dendritic cells (MD-DCs) generated in the presence of bovine Lf (bLf) failed to undergo activation by upmodulating CD83, costimulatory and major histocompatibility complex molecules. Consistent with an impaired maturation, bLf-MD-DC primed T lymphocytes exhibit a unresponsiveness characterized by impaired expression of IFN- $\gamma$  and IL-2. These immunosuppressive effects correlate with an increased expression of molecules with negative regulatory functions. Berlutti et al. [26] have shown that Lf downregulates proinflammatory cytokines in intestinal epithelial cells infected with invasive and noninvasive *Escherichia coli* strains. Valenti et al. [27] have also shown similar anti-inflammatory activity of Lf in cystic fibrosis bronchial cells invaded by *Burkholderia cenocepacia*. Thus, Lf may be used as a therapeutic agent as it prevents the inflammation-related damage. Little information is available about the molecular details of interaction of Lf with the cells to mediate its effects. Recently, a study by Suzuki et al. [28] has shown that the N1 domain of human Lf is required for internalization by Caco-2 cells and targeting to the nucleus to mediate its effects.

#### **9. Microbiostatic and Microbicidal Effects**

Apo-Lf is secreted as microbicidal product by secondary granules of neutrophils. Thus, apo-Lf sequesters the iron at the site of infection, resulting in deprivation of iron to microbes. Hence, microbes must compete with host Lf to meet their iron requirement. Due to its iron-chelating property, it is known as microbiostatic [29]. Apo-Lf is a highly cationic in nature and thus, readily binds to anionic

charges on bacterial surfaces, provided by lipopolysaccharides, porins, and teichoic acids. Such binding results in destabilizing the bacterial membrane [30]. Apo-Lf also exhibits a synergistic effect with IgA, lysozyme, antibiotics, and drugs which helps in eradication of microorganisms [20, 31].

## 10. Lactoferrin Friend or Foe

When apo-Lf acquires iron, it becomes saturated with iron and forms holo-Lf which is an important source of iron for microbes. Thus, iron not only abolishes Lf's innate immune effect but also acts a source of nutrition to microbes. Microbes have developed the various strategies to acquire the iron from holo-Lf which helps them in replication causing acute or chronic infections and damage to host. Thus, for microbes iron-loaded Lf is a friend but becomes a foe when iron is not present [29, 32].

## 11. Mechanisms Adopted by Parasites to Acquire Iron from Holo-Lf

Basically there are four different mechanisms by which parasites acquire iron as a source of nutrient from holo-Lf and these are as follows [33].

- (1) *Lf Binding Receptor.* Expression of lactoferrin binding receptors or proteins (Lbps) which directly bind to holo-Lf (*Trichomonas vaginalis*).
- (2) *Enzymatic Degradation.* Secretion of proteases to cleave holo-Lf to release iron as a source of nutrient (*Tritrichomonas fetus* and *Entamoeba histolytica*).
- (3) *Reducing Enzymes.* Reductases help in reducing the ferric state to ferrous state which is more useful to the parasite (*Leishmania* spp.).
- (4) *Xenosiderophores.* This mechanism is used by bacteria and fungi to obtain iron by producing and secreting siderophores, which have high affinity for iron. They can remove iron from holo-Lf, which in turn is captured by specific receptors, and after providing iron to the pathogen, siderophore again becomes available to carry fresh iron from the environment [34]. *Tritrichomonas fetus* is able to use a wide range of foreign siderophores *in vitro* (ferrioxamine B, coprogen, ferrichrome, enterobactin, and pyoverdine) [35].

## 12. Iron Acquisition by *Trichomonas* Is Receptor Mediated

*T. vaginalis* acquire iron either from holo-Lf or hemoglobin [36, 37]. It recognizes human holo-Lf by two surface proteins which are 178 and 75 kDa. Approximately 90,000 receptors have been documented to be present on each trichomonad. *T. vaginalis* only recognizes holo-Lf, as it is not able to recognize apo-LF or holotransferrin (holo-Tf). A study

by Lehker and Alderete [38] has shown the dynamics of lactoferrin-binding proteins and receptors to changing iron concentration. Lf-binding activity has been shown to be increased by 1.6-fold, under iron-depleted conditions as compared to iron rich conditions. Increase in Lf-binding activity has also been documented when trichomonads with depleted intracellular iron pools were assayed. It has also been observed that a number of holo-Lf-binding receptors increase 2.5 times when trichomonads were cultivated in iron-depleted conditions compared to iron rich-conditions. The number and affinity of holo-Lf receptors in response to changing external and internal iron concentrations clearly shows relevance to *in vivo* situations. Lf concentration in human vaginal mucosa constantly fluctuates throughout the menstrual cycle, varying from 9 µg/mL during mid cycle to 200 µg/mL [39]. Thus, increased affinity during decreased Lf concentration may be a successful mechanism to obtain iron during such conditions.

## 13. Role of Iron on Growth of *T. vaginalis* In Vitro

Yuan and Xue [40] have shown the effect of varying concentration of iron in the TYM (trypticase-yeast extract-maltose) medium for trophozoites of *T. vaginalis*. The generation times were found to be shorter when trophozoites were grown in iron-rich conditions (100–400 µmol/L iron ion). Minimal lethal concentration (MLC) of metronidazole, tested by serial dilution method, was found to be significantly lower in iron-rich media as compared to control group.

## 14. Role of Iron in Cytoadherence

Levels of cytoadherence to HeLa cells have been found to be modulated by different iron levels. Trichomonads isolated from iron-rich media have been shown to mediate higher level of cytoadherence. The extent and expression of adherence property is directly proportional to the concentration of iron added to the medium. This increase in cytoadherence is mediated by increase in expression of genes coding for adhesins. Under iron-rich conditions, increased synthesis of adhesins mediate better cytoadherence. Actinomycin D and alpha-amanitin have been shown to prevent the expression of adhesin molecules resulting in decreased cytoadherence. Thus, iron upregulates the expression of adhesins and the level of cytoadherence, representing an important initial step in pathogenesis. The mucus layer of the vaginal tract is the first barrier encountered by the *T. vaginalis*. *Trichomonas* interacts specifically with the mucin, the predominant component of the mucus. Then the organism comes in contact with the vaginal epithelium cells (VECs) which are under the influence of various hormonal changes induced by the menstrual cycle. So, to colonize the vaginal epithelium *T. vaginalis* has evolved multiple mechanisms. Lipophosphoglycan (LPG) is a major adherence factor but other proteins which help in cytoadherence are adhesion proteins (AP), fibronectin (FN)-binding protein, laminin-binding protein, α-actinin, enolase, phosphoglucomutase,

GTP-binding protein (GTP-BP) [41–43]. There occurs an upregulation of four major iron-regulated adhesion proteins (AP65, AP51, AP33, and AP23), GAPDH, and several hypothetical proteins in a specific receptor-ligand fashion. These APs have sequence homology to metabolic enzymes and the majority are positively regulated by iron at the level of transcription and translation. The ap65-1 gene encodes a 65 kDa malic enzyme involved in cytoadherence [43]. The transcription of this gene is critically regulated by its promoter region where presence of multiple closely spaced DNA regulatory elements regulate iron-induced transcription [44].

Another 120 kDa adhesion protein (AP120) is also induced under iron-rich conditions and has sequence homology with pyruvate: ferredoxin oxidoreductase A (PFO A), a hydrogenosomal enzyme that is absent in humans [7, 45]. The main function of the hydrogenosome, an organelle typical of trichomonads, and converting malate or pyruvate to H<sub>2</sub>, CO<sub>2</sub>, and acetate by a pathway associated with ATP synthesis. This pathway relies on activity of iron-sulphur proteins such as pyruvate: ferredoxin oxidoreductase (PFOR), hydrogenase, and ferredoxin. Studies have shown that like AP120, PFO is localized to the parasite surface and participates in cytoadherence [46]. Thus, *T. vaginalis* PFO is an example of a surface-associated cell-binding protein which lacks enzyme activity and is involved in cytoadherence. Additionally, PFO behaves like AP120 in parasites, when grown under iron-rich conditions.

These adhesion proteins help parasite attachment and their role in pathogenesis has been confirmed by coculture experiments. It has been shown in these experiments that antibodies to adhesion proteins (APs) reduce the parasite adhesion and subsequent cytopathic effects (CE) on host cells. FN-binding proteins bind to multiple FN domains including the cell-binding domain (CBD), N-terminal domain (NTD), and gelatine-binding domain (GBD). During these processes, iron along with calcium and phosphatase is essentially required for differential gene expression which helps in survival, growth, and colonization of parasite in the vaginal hostile environment [42, 47].

*T. vaginalis* glutaraldehyde-3-phosphate dehydrogenase (GAPDH) has been identified as a fibronectin-binding protein that is localized at the parasite surface. The expression and surface localization of GAPDH are positively regulated by iron [41, 48]. Thus, *T. vaginalis* is part of a growing list of microbial pathogens that contain surface-associated enzymes that have alternate, nonenzymic functions. However, the mechanisms by which these enzymes are localized at the *Trichomonas* surface and the pathways in which they act are poorly understood.

The responses of *T. vaginalis* to iron limitation or iron excess have been well established. There is ~80% lower rates of protein synthesis and ≥3-fold decrease in cell densities when organisms are grown in iron-limited culture system as compared to iron-rich conditions. These parasites also exhibit generation times of approximately 10 hours, 2.5-fold longer than organisms grown in the usual complex medium [49].

## 15. Effect of Iron on the Virulence of *T. vaginalis*

Iron is an essential element which has a significant effect on the virulence of *T. vaginalis*. Ryu et al. [50] evaluated the role of iron in relation to the virulence of *T. vaginalis* in mice. Trophozoites cultivated from normal Diamond's trypticase-yeast extract-maltose (TYM) media and iron-supplemented TYM media produced subcutaneous abscesses, whereas trophozoites cultivated in iron-deficient media failed to produce any pathology. Iron also affects the level of adherence and the cytotoxicity of trichomonads to HeLa cells, which are significantly reduced when trophozoites were cultivated in iron-deficient media.

## 16. Iron Upregulates Proteinases Involved in Complement Resistance

It has been seen that *T. vaginalis* is readily lysed by the activation of alternative complement pathway. However, parasites become resistant to complement when grown in iron-rich media. The resistance to complement has been shown to be dependent on iron concentration, and other divalent ions other than iron do not modulate the complement activity. Lactoferrin is known to provide the iron source to the trophozoites and it also renders the low-iron parasites resistant to complement lysis. Pretreatment of high-iron, complement-resistant parasites with proteinase inhibitors prevented the degradation of C3 on the trichomonal surface and results in degradation of parasite by complement activity. Thus, proteinases are the enzymes which provide resistance to complement degradation when iron sources are available [51].

*T. vaginalis* faces the profound change in the environment during menstrual cycle. The vaginal microenvironment undergoes dramatic change during menstruation as it is flooded with serum proteins, erythrocytes, and other macromolecules. During this stage, *T. vaginalis* encounters the complement which is an important effector system against *T. vaginalis* infection. Activation of alternative complement system does not require antibodies and it can be activated by magnesium ions, factors B and D, properdin, and C3 for activation. It has been observed that menstrual blood complement is trichomonacidal, mediated by activation of alternative pathway. However, trichomonads are known to undergo adaptive changes *in vivo*, which enable the parasites to avoid lysis by complement as a survival strategy [51]. Most important being the iron which regulates the gene expression of surface immunogens and adhesions as described above.

Even after menstruation, infection persists as some parasites evade the immune mechanism of alternative complement pathway by secreting various proteinases. These proteinases not only help the parasites acquire the nutrients through lysis of erythrocytes but also help in recognition and binding to host cells. Thus, trichomonad proteinases have dual role in host pathology and parasite survival.

## 17. Iron Modulates Major Surface Immunogen P270

P270 is an immunogenic protein which causes phenotypic variation of *T. vaginalis* infected with double-stranded RNA virus (designated as TVV: *T. vaginalis* virus) [52, 53] on the basis of its differential cytoplasmic versus surface expression. Two types of isolates have been documented during infection with *T. vaginalis*. Type I isolates comprise homogeneous nonfluorescent (negative phenotype) trichomonads which synthesize and express P270 in the cytoplasm. In contrast, type II isolates comprise both fluorescent and nonfluorescent subpopulations (positive and negative phenotypes). Growth of virus-positive organisms in high-iron medium induces expression of trichomonad adhesins but yields parasites without surface P270. P270 has been found to be highly phosphorylated in high-iron parasites and modulating the low surface expression of P270. Thus, iron plays a role in modulating surface localization of P270 in virus-harbouring parasites [54].

## 18. Irony about Iron Regulation of CP65 Cysteine Proteinase

It has been seen that iron is essential for the virulence of the parasite and *Trichomonas* has evolved different strategies for acquiring iron. In *T. vaginalis*, iron up-regulates amounts of adhesins and levels of cytoadherence [55, 56]. Iron also up-regulates the cysteine proteinases (CPs) involved in complement resistance and several parasite functions as described above. However, a study by Alvarez-Sánchez et al. [57] has shown that iron specifically down-regulates proteolytic activity, expression, and transcription of CP65, negatively affecting trichomonal cytotoxicity *in vitro*. Several CPs participate in the virulence of *Trichomonas vaginalis* and 65 kDa CP, CP65, is one involved in cytotoxicity. A similar effect has also been observed with CP39, involved in cytotoxicity [58]. Similarly, iron also downregulates the other genes such as *tvcp12* and *fip-1,2* which encodes for papain-like CP and fibronectin-like proteins, respectively [59, 60]. Thus, it is very surprising that similar iron concentrations exert opposite effects on expression of adhesions and some CPs. This irony has been explained by the fact that iron concentration fluctuates in the vagina during menstrual cycle. Lactoferrin concentration is also known to vary during the different phases of menstrual cycle; that is, it is present in highest concentration after the menstruation and decreases progressively during the cycle with minimum levels present immediately before menstruation. *Trichomonas* has evolved the two different mechanisms to maintain its pathogenicity at different phases of menstrual cycle which helps in its survival. It has been postulated that just before the menstruation when iron concentration is lowest, *T. vaginalis* may enhance the level of cytotoxicity by increasing the CP65 activity. Simultaneously, low iron levels may reduce the expression of adhesions leading to decreased level of cytoadherence, allowing the parasite to move around in search for new iron sources. The opposite effect might be occurring during

the highest level of iron concentrations (during and after menstruation); there occurs an up-regulation of adhesin expression, providing an opportunity for the parasites to adhere firmly to prevent the flushing by menstrual blood flow [57].

## 19. Future Prospects

Drug resistance among the protozoa is increasing at an alarming rate, with relatively few options left for the treatment. In 1962, resistance among *T. vaginalis* was first reported [61] and is at risk of developing increased resistance to recommended therapeutics due to empirical use of drugs. The 5-nitroimidazole family of drugs (metronidazole and tinidazole) are the only class of drugs approved for the treatment of trichomoniasis [62] and P. Upcroft and J. A. Upcroft [63] have reported up to 10% of infections which are not responding to treatment in the United States. Resistant organisms are cosmopolitan in distribution and are of considerable concern as *Trichomonas* infections are linked to human immunodeficiency virus (HIV) transmission. Further scientific research is required to fill the gaps in knowledge about understanding the pathogenesis, so that newer drug targets can be evaluated in order to overcome the increasing burden of drug resistance.

## 20. *T. vaginalis* Receptor as a Vaccine Candidate

Lehker and Alderete [38] examined the sera of patients by immunoblotting to detect the proteins from parasites grown in low- and high-iron media, recognized by antibodies. Twelve trichomonad immunogens from parasites grown in iron-rich medium and 7 from those grown in low-iron medium were found. Sera from patients with vaginitis recognized the purified Lf receptor protein. Thus, *T. vaginalis* receptor could be tested as a vaccine candidate.

## 21. Drug Delivery

Lf may be used as a carrier for drug delivery as it specifically binds to receptor at the surface of *Trichomonas*. Moreover, it is concentrated at places where infection is present at the mucosal surface.

## 22. Inhibition of Lf and Receptor Binding

Inhibition of Lf binding sites at *Trichomonas* surface may deprive the parasites from iron availability and can be used as an attractive therapeutic strategy.

Thus, search for newer drug targets and vaccine candidates in relation to lactoferrin, its receptors, and *Trichomonas* is required in coming years to win the fight against this parasite.

## 23. Conclusion

Iron is an essential nutrient required by *T. vaginalis* to maintain its virulence in changing vaginal environment due to

cyclic menstruation. *Trichomonas* has evolved the receptor-mediated uptake of iron through holo-Lf and its concentration and affinity vary according to the availability of iron in the external environment. Usually, iron is known to upregulate various adhesins and other factors which increase which help in maintaining the virulence. However, it has been seen that cystein proteinases (CP65 and CP39) are downregulated when the external iron concentration is high. It is important for the survival of parasite as when abundant iron is available, that is, during menstrual flow, there is an increase in cytoadherence, preventing flushing of parasites during blood flow of menstruation. On the other hand, just before menstruation, iron concentration is low; thus cytoadhesion is also decreased and CP65 and CP39 are activated which help moving the parasite in search of iron and maintain its virulence. Furthermore, drugs can be formulated which can target the holo-Lf receptor or various immunogens can be tested which can provide immunity or holo-Lf can be used a carrier for drug delivery. However, studies are required in animal models to check the efficacy and adverse effects of new drugs, targeting Lf or its receptor. At present there is no drug available which targets the Lf or its receptor for the treatment of trichomoniasis. Finally to conclude apo-Lf is lethal to parasite, but when it acquires iron (holo-Lf), it is used as a iron source by the parasite.

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## Review Article

# Syphilis Infection during Pregnancy: Fetal Risks and Clinical Management

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Congenital syphilis is still a cause of perinatal morbidity and mortality. Untreated maternal infection leads to adverse pregnancy outcomes, including early fetal loss, stillbirth, prematurity, low birth weight, neonatal and infant death, and congenital disease among newborns. Clinical manifestations of congenital syphilis are influenced by gestational age, stage of maternal syphilis, maternal treatment, and immunological response of the fetus. It has been traditionally classified in early congenital syphilis and late congenital syphilis. Diagnosis of maternal infection is based on clinical findings, serological tests, and direct identification of treponemes in clinical specimens. Adequate treatment of maternal infection is effective for preventing maternal transmission to the fetus and for treating fetal infection. Prenatal diagnosis of congenital syphilis includes noninvasive and invasive diagnosis. Serological screening during pregnancy and during preconception period should be performed to reduce the incidence of congenital syphilis.

## 1. Introduction

Syphilis is a sexually transmitted disease (STD) caused by the bacterium *Treponema pallidum*, but little is known about its mechanism of action or what determines virulence of infection [1]. Untreated syphilis in pregnancy leads to adverse outcomes among more than half of the women with active disease, including early fetal loss, stillbirth, prematurity, low birth weight, neonatal and infant death, and congenital disease among newborn babies [2]. In 2010, a total of 13,774 cases of primary and secondary syphilis were reported to Centers for Disease Control and Prevention [3]. According to World Health Organization (WHO), 12 million people were infected each year [4]. It was estimated that the lifetime medical cost per case of syphilis is \$572 (in year 2006 dollars) and they could be much higher if CS and HIV infections occurred [5]. Screening and early detection can reduce these costs because treatment for early stage syphilis is less expensive than treatment for later stage disease: \$41.26 (in year 2001 dollars) compared to \$2,061.70 for late syphilis [6].

Moreover, CDC recommends that all persons who have syphilis should be tested for HIV infection [7]. Genital sores caused by syphilis can bleed easily and make it easier to transmit HIV infection, with a 2- to 5-fold increased risk of acquiring HIV [8]. Changes in the population incidence of primary and secondary syphilis among women are usually followed by similar changes in the incidence of congenital syphilis (CS) [9]. CDC reported that the rates of both female and CS increased during 2005–2008 in the United States of America (USA), and have since declined. The rate of syphilis among women was 1.1 cases per 100,000 women in 2010, and the rate of CS was 8.7 cases per 100,000 live births in 2010 [10]. According to the most recent (2008) estimates from WHO, about 1.9 million pregnant women had active syphilis [11]. In Italy, the incidence rate of syphilis was 0.86 per 100,000 population in 2008 [12], and CS is strictly related to immigration, mostly from Eastern Europe. In 2007 an Italian prospective study on 19,548 pregnant women showed that the overall syphilis seroprevalence was 0.44% but it was 4.3% in women from Eastern Europe and 5.8% in women

from Central-South America. This high prevalence could be explained by the fact that immigrant women do not always receive adequate prenatal care owing to lack of knowledge of free healthcare services [13].

Without screening and treatment, about 70% of infected women will have an adverse pregnancy outcome [14].

## 2. Clinical Manifestations

Clinical manifestations of acquired syphilis are not apparently altered by pregnancy. Syphilis is passed from person to person through direct contact with a syphilitic sore, called chancre. Transmission of the organism occurs during vaginal, anal, or oral sex. Sores of primary syphilis occur about 3 weeks after contact, mainly on the external genitals, vagina, cervix, anus, or in the rectum. They are often unrecognized in women because they can be asymptomatic. Syphilitic sore is firm, round, small, and painless and lasts 3 to 6 weeks. It should be distinguished by Genital Herpes, which causes small, painful blisters filled with clear or straw-colored fluid. When blisters break, they leave shallow ulcers that are very painful and eventually crust over and slowly heal over 7–14 days or more [2].

Syphilitic sore can increase the risk of HIV transmission by disrupting mucosal and epithelial barriers [15] and is followed several weeks or months later by widespread cutaneous, mucosal, and sometimes systemic indications of the dissemination of the spirochetes of secondary syphilis. This phase can last up to a year and syphilis is particularly contagious at this stage. Even without treatment both primary and secondary lesions resolve and the infection enters a latent stage. Despite the lack of clinical manifestations, the infection can still be transmitted to the fetus [2]. Tertiary syphilis may occur in a third of untreated people, approximately three to 15 years after the initial infection. It is characterized by infiltrative tumors of skin, bones, or liver (gumma) (15%), central nervous system disorders (neurosyphilis) (6.5%), and cardiovascular problems (10%). People with tertiary syphilis are not infectious [16].

## 3. Fetal Infection

Spirochetes can cross the placenta and infect the fetus from about 14 weeks' gestation, and the risk of fetal infection increases with gestational age [17]. The manifestations of CS are influenced by gestational age, stage of maternal syphilis, maternal treatment, and immunological response of the fetus [18]. CS can lead to spontaneous abortion, usually after the first trimester, or late-term stillbirth in 30 to 40 percent of cases or premature or term delivery of live infants who may have obvious signs of infection or be fully asymptomatic (approximately two-thirds of liveborn cases) [19]. Placental infection and the reduction in blood flow to the fetus are the most common causes of fetal death. An untreated woman has about 70% of chance of fetal infection during the first 4 years of disease. In 35% of cases, infected fetuses are born alive with CS. Low birth weight can be the only sign of infection. In fact about 60% of liveborns are asymptomatic at birth [20, 21]. CS has been traditionally classified in early congenital

syphilis (ECS) and late congenital syphilis (LCS). In ECS signs appear in the first 2 years of life while in LCS signs appear over the first 2 decades. Clinical manifestations of ECS are the result of active infection and inflammation while clinical manifestations of LCS are malformation or stigmata that represent the scars induced by initial lesions of ECS or can be the result of chronic inflammation [2]. After fetal infection occurs, any organ system can be affected because of the widespread spirochetal dissemination.

## 4. Early Congenital Syphilis

Hepatomegaly is present in nearly all infants with CS, while splenomegaly is present in half of cases. Jaundice has been recorded in 33% of cases, as a consequence of syphilitic hepatitis or of hemolytic anemia [2, 22]. Elevated serum transaminase and alkaline phosphatase concentrations and direct hyperbilirubinemia can occur; the prothrombin time may be prolonged [23–25]. Generalized lymphadenopathy has been described in 50% of patients. Large epitrochlear nodes are typical of CS [22].

Hematological manifestations, such as anemia, thrombocytopenia, leukopenia, and leukocytosis are common findings in CS [2]. Hydrops fetalis may also be a manifestation. In presence of a negative coomb's test in a hydropic infant with hemolytic anemia CS should be considered [26].

Mucocutaneous involvement occurs in as many as 70% of infected infants and may be present at birth or develop during the first few weeks of life. The most common cutaneous manifestation consists of small copper-red maculopapular lesions, and the hands and feet often are most severely affected. Desquamation and crusting occur over the course of 1 to 3 weeks [2, 22]. Rhinitis may be an early symptom which appears after the first week of life and usually before the end of the third month. Mucus discharge is often is blood-tinged and secondary bacterial infections can occur. "Saddle nose" deformity is one of the later stigmata of the disease, and can occur when ulceration of nasal mucosa involves the nasal cartilage. All mucocutaneous lesions and discharges contain abundant spirochetes and are highly infectious. After the first 2 or 3 months of age, the perioral and the perineal area may be affected by wart-like or flat lesions called condyloma lata that can lead to deep fissures and can result in fine scars called rhagades [2, 27]. Petechial lesions may be seen if severe thrombocytopenia is present [22].

Bone involvement is very frequent in untreated ECS. The metaphyseal and diaphyseal portions of long bones are usually affected by periostitis and cortical demineralization, while osteochondritis involves the joints, especially knees, ankles, wrists, and elbows. Osteochondritis and periostitis may be painful and manifested by the pseudoparalysis of a limb due to pain (pseudoparalysis of Parrot), which affects more frequently the upper extremities [28].

A nephrotic syndrome can appear at 2 or 3 months of age, and it can lead to generalized edema [2].

Congenital neurosyphilis may be asymptomatic. More than 25 WBC/mm<sup>3</sup> and protein greater than 150 mg/dL (170 mg/dL in premature infants) in cerebrospinal fluid (CSF) are considered suggestive of neurosyphilis although

normal CSF indices do not exclude neurosyphilis. A reactive CSF Venereal Disease Research Laboratory (VDRL) test generally indicates the presence of neurosyphilis. CSF abnormalities are present in approximately 8 percent of asymptomatic infants born to mothers with untreated early syphilis [2, 29, 30].

Ocular manifestations are rare and include chorioretinitis, glaucoma, uveitis, cataract, salt and pepper fundus, and chancres of the eyelid. Other findings are less common [2].

## 5. Late Congenital Syphilis

LCS is actually very rare and occurs in approximately 40 percent of untreated children [2, 30].

Syphilitic vasculitis around the time of birth can lead to dental abnormalities that occur in teeth that undergo calcification during the first year of life. Hutchinson's teeth are peg-shaped, notched central incisors while mulberry molars are multicuspid first molars. The deciduous teeth have an increased risk of dental caries [30, 31].

Interstitial keratitis is the typical ocular manifestation, usually diagnosed between 5 and 20 years of age. It can lead to secondary glaucoma or corneal clouding [2].

Eight nerve deafness occurs in 3% of cases and is secondary to luetic involvement of the temporal bone. Eight nerve involvement can be unilateral or bilateral, and it may be responsive to corticosteroids. Although it is usually diagnosed between 30 and 40 years of age, it often occurs in the first decades [32].

The constellation of Hutchinson's teeth, interstitial keratitis and eight nerve deafness is called Hutchinson's triad [2], described by Sir Jonathan Hutchinson (1828–1913) from England. Fortunately, it actually represents a rare finding.

Syphilis rhinitis can impair the maxilla growth, resulting in an abnormal configuration in the middle section of the face, while nasal cartilage destruction resulting from inflammation can cause the perforation of the nasal septum and lead to saddle nose [2, 33].

Rhagades around the body orifices can result from the fissuration of early linear scares [31].

Neurological manifestations of LCS include mental retardation, hydrocephalus, convulsive disorders, cranial nerve abnormalities (including blindness and deafness), and juvenile general paresis [2, 27].

Bony involvement is less frequent than in ECS and it includes the sequelae of prolonged periostitis of the skull (resulting in frontal bossing), of the tibia (resulting in saber shin) and of the sternoclavicular portion of the clavicle (resulting in a deformity called Higouménakis sign).

Clutton's joints are symmetric, painless, sterile, synovitis usually localized to the knees and characterized by local tenderness and limitation of motion [2, 27, 31].

## 6. Diagnosis

Maternal syphilis can be suspected based on clinical findings and confirmed by direct identification of treponemes in clinical specimens and by positive serologic findings or can be accidentally diagnosed through screening serological tests.

Dark field microscopy is the most specific technique for diagnosing syphilis when an active chancre or condyloma latum is present [34]. Other possible methods include direct fluorescent antibody (DFA) testing and the rabbit infectivity test (not used in clinical practice) [2]. Serological tests for syphilis can be classified in nontreponemal (NTTs) and treponemal (TTs) tests. NTTs are usually used for screening and monitoring therapy, while TTs are used to confirm the diagnosis. Nontreponemal tests detect antibodies to cardiolipin, a component of membranes and mammalian tissue. The two commonly used nontreponemal tests are the Venereal Disease Research Laboratory (VDRL) and the Rapid Plasma Reagins (RPR) tests. False-positive reactions can occur because of pregnancy, autoimmune disorders, and infections [35]. NTTs are usually positive in 75% of cases of primary syphilis. Secondary syphilis is always characterized by a reactive VDRL, with a titer greater than 1/16 [2]. The titer of antibodies reflects disease activity: fourfold decrease suggests adequate therapy, while fourfold increase indicates active disease. NTTs usually become negative one year after receiving adequate treatment of primary syphilis and within two years with secondary syphilis. In a small percentage of patients low positive titers persist despite receiving adequate therapy [30]. TTs detect an interaction between serum immunoglobulins and surface antigens of *Treponema pallidum*. They include the fluorescent treponemal antibody absorption (FTA-ABS) test, the treponemal-specific microhemagglutination test (MHATP) and *Treponema pallidum* particle agglutination test (TP-PA). These tests are positive in 75% (TP-PA) to 85% (FTA-ABS) of patients with primary syphilis and in 100% of patients with secondary syphilis. False-positive tests can occur in patients with Lyme disease, leptospirosis, and diseases caused by other pathogenic *Treponema* spp. [2]. TTs usually remain positive for life. Polymerase-chain-reaction-(PCR)-based tests and immunoglobulin M immunoblotting tests have been developed, but they are not largely used in clinical practice. Although no *Treponema pallidum* detection tests are commercially available, some laboratories provide locally developed PCR tests for the detection of *Treponema pallidum* [2].

Prenatal diagnosis of CS includes noninvasive and invasive diagnosis. Ultrasonographic fetal examination for signs of CS is recommended prior to therapy after 20 weeks' gestation. Fetal syphilis is the presumed diagnosis when the sonographic findings of fetal hydrops, abnormally large abdomen (hepatosplenomegaly), hydramnios, and thick placenta are found in the presence of maternal syphilis [36–38]. Invasive diagnosis includes amniocentesis and percutaneous umbilical blood sampling. Dark field examination, rabbit infectivity testing, and polymerase chain reaction for detection of *Treponema pallidum* can be performed on amniotic fluid. Hematologic and chemical testing can be performed on fetal blood and fetal antitreponemal IgM can be detected. Abnormal liver transaminases, anemia, and thrombocytopenia are signs of fetal infection. If fetal infection is suspected, antepartum fetal heart rate testing is indicated before treatment. In some cases of fetal hydrops, fetuses can have late decelerations or nonreactive nonstress testing that led to fetal distress soon after maternal treatment [36]. Evaluation of infants for

suspected CS should include careful physical examination, nontreponemal serologic tests of infant serum, specimens for testing for the presence of spirochetes from mucocutaneous lesions (if these are present), complete blood count, CSF analysis (in all infants with physical findings compatible with CS quantitative nontreponemal titer >4-fold higher than the current maternal titer, or direct evidence of *Treponema pallidum* in clinical specimens), long bone radiographs (unless the diagnosis has been confirmed otherwise), adequate clinical tests in case of specific signs or symptoms, and pathologic examination of the placenta or umbilical cord [39].

## 7. Treatment

Adequate treatment of maternal infection is effective for preventing maternal transmission to the fetus and for treating fetal infection [40]. Penicillin G, administered parenterally, is the preferred drug for treating of syphilis. The effectiveness of penicillin was established through clinical experience and randomized controlled clinical trials. It provides weeks of treponemicidal levels of penicillin in the blood, but it does not efficiently cross the blood brain barrier. Aqueous crystalline penicillin G is the drug of choice for neurosyphilis treatment [7]. Treatment failure was described in few case reports, particularly in patients with HIV infection, but there is no documented penicillin resistance in *T. pallidum* [41]. CDC recommends that pregnant women should be treated with the penicillin regimen appropriate for their stage of infection [7]. Evidence is insufficient to determine optimal, recommended penicillin regimens [42]. In primary, secondary, and early latent syphilis, benzathine penicillin G 2.4 million units IM in a single dose is recommended [7]. Additional therapy can be beneficial for pregnant women in some settings. Some authors suggest that a second dose of benzathine penicillin 2.4 million units IM administered 1 week after the initial dose for women who have primary, secondary, or early latent syphilis [36]. In late latent syphilis or latent syphilis of unknown duration, benzathine penicillin G 7.2 million units total should be administered, as 3 doses of 2.4 million units IM each at 1 week intervals. In case of neurosyphilis, aqueous crystalline penicillin G 18–24 million units per day, administered as 3–4 million units IV every 4 hours or continuous infusion, for 10–14 days represents the suggested treatment [7]. Pregnant women who have a history of penicillin allergy should be desensitized and treated with penicillin [7, 36]. In case of HIV positive patients, placental inflammation from congenital infection might increase the risk for perinatal transmission of the virus. No sufficient data are available to recommend a specific regimen for HIV-infected pregnant women [7].

The Jarisch-Herxheimer reaction can occur in some patients 2 to 12 hours after receiving therapy for active syphilis. It is characterized by fever, headache, myalgia, and malaise, and it is caused by the release of treponemal endotoxin-like compounds during penicillin-mediated lysis [2, 43, 44]. The Jarisch-Herxheimer reaction can increase the risk of premature labor and/or fetal distress during the second half of pregnancy [45, 46]. Serologic titers should be repeated at 28–32 weeks' gestation and at delivery and should

be checked monthly in women at high risk for reinfection or in high-risk geographic areas [7]. Maternal treatment can be inadequate if delivery occurs within 30 days of therapy, or if the maternal antibody titer at delivery is fourfold higher than the pretreatment titer [36].

## 8. Conclusions

Syphilis infection during pregnancy still represents a worldwide public health problem. The American College of Obstetricians and Gynecologists and the American Academy of Pediatrics recommend prenatal syphilis screening at the first prenatal visit and again at 32–36 weeks, if the woman is at risk for syphilis [47]. CDC recommends that all women should be screened serologically for syphilis at the first prenatal visit and, for patients at high risk, during the third trimester and at delivery [7]. Moreover, any woman who delivers a stillborn infant after 20 weeks' gestation should be tested for syphilis [36]. The Italian Guidelines of Istituto Superiore di Santità for Physiological Pregnancy (2011) stated that serological screening for syphilis should be offered to all pregnant women during the first and the third trimester of pregnancy [48]. Preconception serological tests for syphilis could represent the key to reduce the incidence of CS. Moreover, preconception counseling could play an important role, evaluating the woman and her partner for exposure to sexually transmitted diseases, identifying high-risk behaviors, and providing health promotion messages and education.

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## Research Article

# Prevalence of Human Papillomavirus in Women from Mexico City

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**Introduction.** Cervical cancer is the most common cancer among Mexican women. The goal of the present study was to determine the prevalence and distribution of HPV types in women from Mexico City. **Methods.** Our study was conducted in the Clinica de Especialidades de la Mujer de la Secretaría de la Defensa Nacional, Mexico. Random samples were taken from 929 healthy women requesting a cervical Papanicolaou examination. Detection and genotyping of HPV were performed by multiplex PCR, with the HPV4A ACE Screening kit (Seegene). **Results.** 85 of nine hundred twenty-nine women (9.1%) were infected with HPV. Of HPV-positive women, 99% and 1% had high- and low-risk HPV genotypes, respectively. The prevalence of the 16 high-risk (HR) HPV types that were screened was 43% : 42% (18) were HPV positive and 14% (16) were HPV positive, which includes coinfection. Multiple infections with different viral genotypes were detected in 10% of the positive cases. Abnormal cervical cytological results were found in only 15.3% of HPV-positive women, while 84.7% had normal cytological results. **Conclusions.** We found a similar prevalence of HPV to previous studies in Mexico. The heterogeneity of the HPV genotype distribution in Mexico is evident in this study, which found a high frequency of HPV HR genotypes, the majority of which were HPV 18.

## 1. Introduction

Cervical cancer is the second most common cancer among women worldwide [1] and the most common cancer among women in Mexico [2].

Cervical cancer is caused by oncogenic HPV infection. More than 100 HPV genotypes have been described, of which approximately 40 are responsible for genital infection [3]. HPV is classified as low or high risk based on its association with premalignant and malignant lesions, respectively. Low-risk HPV types include 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81. Of these types, 6 and 11 cause 90% of the external anogenital wart cases and low-grade changes in cervical cells.

[4, 5]. The high-risk types include 16, 18, 31, 33, 35, 45, 52, and 58, of which 16 and 18 cause approximately 70% of all invasive cervical cancer cases [6].

Some authors have suggested that geographical differences in HPV distribution may have an impact on the effectiveness of the HPV vaccine in different populations [7].

In 2005, the International Agency for Research on Cancer HPV [8] reported the worldwide distribution of HPV types in women with normal cervical cytology. The most frequent type is HPV 16, followed by HPV 42, 58, 31, 18, 56, 81, 35, 33, and 45. In Mexico, HPV types 16, 18, 31, 33, 39, 45, 53, 58, and 59 have frequently been found in normal cervical samples [9–15].

Regional data on the prevalence and type distribution of HPV are essential for estimating the impact of vaccines on cervical cancer and developing screening programs.

The goal of the present study was to determine the prevalence and distribution of HPV types in women from Mexico City.

## 2. Materials and Methods

**2.1. Study Population.** This study was conducted in the Clínica de Especialidades de la Mujer de la Secretaría de la Defensa Nacional in Mexico City, Mexico. Random samples were taken from healthy women requesting a cervical Papanicolaou examination. A total of 929 women, aged between 18 and 76 years, were recruited for the analysis. Written informed consent was obtained from each participant. The protocol and informed consent documents were approved by the Human Research Ethical Committee of the Clínica de Especialidades de la Mujer de la Secretaría de la Defensa Nacional.

**2.2. Specimen Collection.** Two cervical samples were obtained from each patient using a cytobrush for cytological analysis and HPV detection. Cervical smears were used for cytomorphological examination using conventional Papanicolaou (Pap). For HPV detection, cytobrushes with cervical scrapes were placed in phosphate buffered saline (PBS) and stored at  $-70^{\circ}\text{C}$  until analysis.

**2.3. Cytology.** Pap smears were interpreted by the head of the Cytopathology Laboratory Pathology Unit at the Clínica de Especialidades de la Mujer de la Secretaría de la Defensa Nacional. All Pap smears were described with the Bethesda System terminology.

**2.4. HPV Detection and Typing.** DNA extraction was performed with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. HPV detection and genotyping were performed by Multiplex PCR using the HPV4A ACE Screening kit (Seegene) according to the manufacturer's protocol. The kit is a qualitative, *in vitro* test for the identification of HPV 16 and 18 and for screening of 16 high-risk HPV (HR) types 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 and the low-risk HPV types 6 and 11.

**2.5. Statistical Analysis.** The mean age and the frequency of risk factors were calculated. Significant differences between groups with or without HPV were determined by the Mann-Whitney rank sum test and the Fisher's exact test at  $P < 0.05$ . Statistical analyses were performed using the SigmaStat program, version 2.03.

## 3. Results

**3.1. Characteristics of the Population.** The mean age of women in the study was 40.5 years old. The majority of participants reported one lifetime sexual partner (97%) and

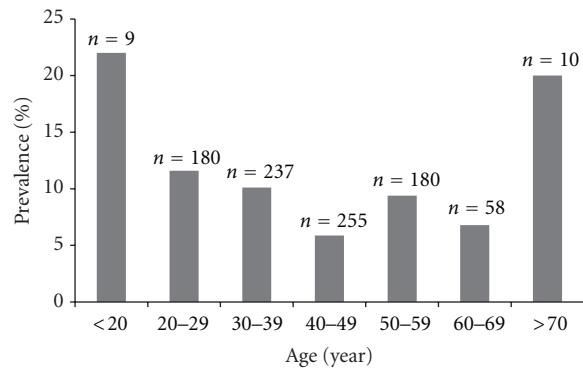


FIGURE 1: Age-specific prevalence of human papillomavirus (HPV) infection among women.

only approximately 3% of women reported more than one partner in their sexual history. The age of first intercourse was younger than 18 years for approximately 8.6% of women in the study. Histories of sexually transmitted diseases were rare (3.9%). Four percent of the women in the study were current smokers.

**3.2. Cytology.** Conventional Pap smears identified normal cytological results or inflammation in 916 of 929 samples (98.6%). Only 13 (1.4%) cases displayed abnormal cytology: 10 (1.1%) had low-grade squamous intraepithelial lesions (LSIL), 2 (0.2%) had atypical squamous cells of undetermined significance (ASC-US), and 1 (0.1%) had squamous cell carcinoma (CC). No high-grade squamous intraepithelial lesions (HSILs) were detected.

**3.3. HPV Detection and Genotyping.** 85 of 929 women (9.1%) were infected by HPV. The age-specific prevalence of HPV is summarized in Figure 1, which shows the highest prevalences, 22% and 20%, in women aged less than 20 years and 70–76 years, respectively. Women aged 40–49 years old had the lowest prevalence of HPV of 6%.

HPV incidence was significantly higher among women with a history of more than one sexual partner or who were current smokers. There were no differences in the frequency of HPV infection in women who had intercourse younger than 18 years old or those with histories of sexually transmitted diseases (Table 1).

Of the HPV-positive women, 99% had high-risk HPV genotypes and 1% had low-risk HPV genotypes. HPV HR was detected in 43% of women screened in this study: 42% (18) were HPV positive, and 14% (16) were HPV positive, which includes those with both infections. Multiple infections with different viral genotypes were detected in 10% of the positive cases. Abnormal cervical cytology was detected in only 15.3% of HPV-positive women, and normal cytology was found in the other 84.7% of those cases, as summarized in Table 2.

## 4. Discussion

In this study, we identified a 9.1% prevalence of HPV: 84.7% of women had normal cytology and 15.3% of

TABLE 1: Risk factors for HPV infection between HPV-negative and HPV-positive women.

Characteristics	HPV-negative n (%)	HPV-positive n (%)	P
Number of women	844 (90.9)	85 (9.1)	
Mean age (years)	41.4	39.6	0.158
Intercourse younger than 18 years old	72 (8.5)	8 (9.4)	0.349
History of having more than one sexual partner	22 (2.6)	5 (5.8)	<0.001
Smokers	31 (3.7)	4 (4.7)	0.016
History of sexually transmitted diseases	34 (4.0)	2 (2.3)	0.157

TABLE 2: Prevalence of HPV genotypes.

Cytological diagnosis	HPV-HR*	HPV genotypes				
		HPV-18	HPV-16	HPV-HR/HPV-18**	HPV-HR/HPV-16***	HPV- 6 and HPV-11
n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Total (n = 85)	37 (43)	30 (35)	8 (10)	6 (7)	3 (4)	1 (1)
Normal	28 (33)	30 (35)	7 (9)	5 (6)	2 (3)	0
ASC-US	1 (1)	0	0	1 (1)	0	0
LSIL	7 (8)	0	1 (1)	0	1 (1)	1 (1)
HSIL	0	0	0	0	0	0
CC	1 (1)	0	0	0	0	0

ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; CC, squamous cell carcinoma; HSIL, high-grade squamous intraepithelial lesions.

\*HR (16 high-risk HPV types): 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82.

\*\*Detection of multiple infections for HPV 18 and HR.

\*\*\*Detection of multiple infections for HPV 16 and HR.

women had abnormal cytology. The worldwide prevalence of HPV in women with normal cytology has been previously reported [8]. Other HPV studies in Mexico using an open population of women with normal cervical cytology reported an incidence of HPV between 4.8 and 43.6% [9, 13]. A possible explanation for the difference in the reported HPV prevalences includes variables related to HPV acquisition, such as age, age of first sexual intercourse, number of lifetime sexual partners, socioeconomic status, education level, parity, marital status, number of pregnancies, use of hormonal contraceptives, smoking, and interregional variation [2, 9, 15].

Similar to previous age-specific studies, both in Mexico and other regions of the world, we found two age groups with higher HPV prevalences, women less than 20 years old and women 70–76 years old. The younger age group (<20 years old) with a higher incidence of HPV infection may be an indicator of sexual transmission, as it coincides with the initiation of sexual activity. The older age group (70–79 years old) of HPV-positive women may have been exposed to high rates of HPV transmission when they were young or may have reactivated latent HPV infections by factors associated with older age [14].

The heterogeneity of HPV genotype distribution in Mexico is evident in this study, with the major frequency of HPV HR at 43%, followed by HPV 18 at 42% (including coinfection). The genotype distribution could be explained by the dynamic population in Mexico City.

Similar to previous studies, statistically significant differences in HPV incidence were found among women with

a history of more than one sexual partner or who were smokers. No significant differences in HPV frequency were found in women who had intercourse younger than 18 years old or had histories of sexually transmitted diseases. These results could have important implications for future screening procedures to assist the prevention of cervical cancer in Mexico.

The differences in HPV prevalence and distribution identified in this study have a potential impact on the effectiveness of HPV vaccinations, which may be investigated in future studies.

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## Research Article

# Identification of *Candida* Species Associated with Vulvovaginal Candidiasis by Multiplex PCR

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**Background.** Vulvovaginal candidiasis is a common infection. The aim of this study was to identify the species of vaginal *Candida* isolates by using multiplex PCR technique. **Methods.** 191 isolates from patients admitted to Mahdieh hospital were identified. The vaginal swab specimens were cultured on Sabouraud Dextrose Agar. The ITS1 region between the 18S and 5.8S rRNA genes and a specific DNA fragment within the ITS2 region were amplified. The multiplex PCR products were separated by electrophoresis in 2% agarose gel, visualized by staining with ethidium bromide, and photographed. Descriptive statistics, Chi-square test, and Spearman correlation were used to summarize the findings. **Results.** *C. albicans* and *C. glabrata* were the most common species isolated from the specimens. A mix of *C. glabrata* and *C. albicans* was the most common mixed infection isolated from the samples. The analysis revealed a significant positive association between older age and infection with *C. glabrata* isolates (Spearman's rho = 0.89, P = 0.015). **Conclusion.** Multiplex PCR is a fast, yet reliable method to identify *Candida* species. *C. albicans* and then *C. glabrata* are the two most common causes of vulvovaginal candidiasis. The number of mixed fungal infections is higher among Iranian population compared to international reports.

## 1. Introduction

*Candida* species are the second most common cause of vulvovaginitis worldwide [1]. The prevalence of vulvovaginal candidiasis (VVC) is increasing due to the extensive utilization of broad-spectrum antibiotics as well as increased cases of immunocompromised patients [2, 3]. Nearly 75% of women over 25 years of age, reported to have at least one episode of physician approved VVC during their lifetime and 5% experienced recurrent type; which is defined by getting infected for at least 4 times in a one-year period [4]. However, 20–50% of women have *Candida* species in their vaginal flora without showing any clinical symptoms [4, 5]. *C. albicans* is the most common and clinically relevant species that accounts for 85–90% of VVC [4]. However, there has been a

significant trend towards the emergence of other species such as *C. glabrata*, *C. krusei*, and *C. parapsilosis* which ironically show more resistance to the first line antifungal treatments [6]. Hence, the differentiation of diverse species of *Candida* in the laboratories seems necessary. Traditionally, the identification and classification of *Candida* species were done by time consuming and unreliable methods such as serotyping [7], colony morphotyping [8], conventional culture techniques, and morphological and biochemical analysis [9]. Nonetheless, the improvements in molecular assay technology for identifying *Candida* species, such as randomly amplified polymorphic DNA analysis (RAPD), has overcome these limitations during the last couple of years. However, methods such as single and direct PCR or multiplex PCR have not been used extensively despite being highly sensitive

and specific with a shorter turn-around time [10–13]. Multiplex PCR is a rapid diagnostic assay which combines many specific species primers in one PCR tube. Hence, it could be used to identify more than one species in a specimen simultaneously [14]. Very little is known regarding the epidemiology of VVC in Iran. The aim of this study was to identify different species of *Candida* in an Iranian patient population by using different methods. Multiplex PCR method was also evaluated as a rapid and reliable method to identify *Candida* species by comparing the results with the traditional methods such as germ tube formation in serum, chlamydospore production on Corn Meal Agar (CMA), and carbohydrate absorption.

## 2. Materials and Methods

Participants of this study were women with signs and symptoms of VVC, who were admitted to the gynecology clinic in Mahdieh Educational Hospital in Tehran, Iran during a 2-year period from March 2006 to 2008. Participants who experienced the signs of VVC less than four times (including their latest admission to the clinic) in the previous year were categorized in nonrecurrent VVC (NRVVC), and those who experienced the vaginal itch and secretions equal or more than four times in the previous year were considered recurrent VVC (RVVC) after being confirmed by finding three positive cultures of VVC within a year in their medical records. Specimens were collected from the first 100 consecutive patients with NRVVC and likewise for the first 100 consecutive patients with RVVC. Each patient was tested only once at her first visit to the clinic during the two-year period of the study. Out of a total 200 collected specimens, 25 were excluded from the study due to contamination or having no signs of fungal growth. Hence, the final number of specimens was 175. To further evaluate the risk factors for VVC, we also asked our participants to complete a questionnaire regarding their demographic and behavioral characteristics. This study was approved by Iran's National Research Ethics Committee. All of the patients signed a written consent form before participating in the study and patients' confidentiality was strictly protected. Furthermore, no complication was seen after taking the vaginal samples.

Vaginal sampling of the participants performed by using a sterile swab by the principle researcher and was cultured simultaneously onto sabouraud dextrose agar medium. *Candida* species colonies were also identified by germ tube formation in serum, chlamydospore production on CMA, and carbohydrate absorption using the API 20 C-AUX kit (bioM'erieux, Paris, France), results of which have already been published elsewhere [15]. Colonies were placed in transport medium at room temperature before being processed for PCR analysis.

DNA extraction and purification were performed using a Genomic DNA Extraction kit (AccuPrep Bioneer Corporation) based on the guidelines. Multiplex PCR was performed using the PCR premix kit (AccuPower Bioneer Corporation) with a total reaction volume of a 50  $\mu$ L consists of 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each),

4  $\mu$ M primers (0.16  $\mu$ M each), and Taq DNA polymerase (1 U). The primers used in this reaction were synthesized at TAGC (Berlin, Germany) including universal primers (ITS1 [5'-TCCGTAGGTGAAACCTGCGG-3'] and ITS2 [5'-GCTGCGTTCTTCATCGATGC-3']) [12] and *C. albicans*-specific primers (CA3 [5'-GGTTTGCTTGAAAGACGG-TAG-3'] and CA4 [5'-AGTTGAAGATATACTGGTAG-3']). A conserved portion of the 18S rDNA region, the adjacent ITS1, and a portion of the 28S rDNA region were amplified, using the ITS1 and ITS2 primers. A portion of the ITS2 region of *C. albicans* was amplified by including CA3 and CA4 in the PCR mixture. PCR amplification process was carried out with an Eppendorf thermal cycler under the following conditions: initial denaturation (94°C, 3 min); 35 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min); final extension (72°C, 5 min). PCR products were analyzed by electrophoresis through a 2% agarose gel (Roche) containing ethidium bromide (Sigma), and UV visualization were performed according to the protocols provided (UVdoc, GAS9000, England). The length of the bands was measured by UVIsoft software. Positive controls were included in each PCR experiment and consisted of one strain of each *C. albicans* ATCC14053 (218 or 219, and 110 bp), *C. glabrata* CBS2175 (482 or 483 bp), *C. parapsilosis* CBS2195 (229 bp), *C. tropicalis* CBS94 (218 bp), and *C. krusei* CBS573 (182 bp). Pyrogen-free water was used as negative control. Two DNA bands were identified for *C. albicans* while one DNA band was corresponded to other *Candida* species. An assessment of the different species of RVVC and NRVVC was also made. Descriptive statistics, chi square test, and Spearman's correlation were used to analyze the data.

## 3. Results

The mean age ( $\pm$ SD) of the 175 participants was 32.4 ( $\pm$ 8.2) years. There was no significant difference between the mean age of women with RVVC and those with NRVVC ( $P < 0.9$ ). There was a significant association between the age of participants and detection rates of *C. albicans* ( $P < 0.05$ ). The results of the analysis indicate that older participants were less likely to be infected with *C. albicans*. However, as depicted in Figure 1, they were most prone to get infected with *C. glabrata*.

Figure 2 shows the results of the multiplex PCR method.

Among vaginal samples, 89.7% contained only one species of *Candida* and 10.3% contained more than one species of *Candida*. The prevalence of different species of *Candida* was as follow: *C. albicans* (65.1%), *C. glabrata* (13.1%), *C. tropicalis* (6.2%), *C. krusei* (4%), *C. guilliermondii* (0.6%), *C. parapsilosis* (0.6%), mixed infection of *C. glabrata* and *C. albicans* (5.7%), *C. parapsilosis* and *C. albicans* (1.1%), *C. krusei* and *C. albicans* (0.6%), *C. albicans* and *C. tropicalis* (0.6%), *C. glabrata* and *C. tropicalis* (0.6%), *C. krusei* and *C. tropicalis* (0.6%), *C. krusei* and *C. glabrata* (0.6%), and a combination of *C. glabrata*, *C. krusei*, and *C. albicans* (0.6%). Only one species of *Candida* was identified in 90.2% of participants with NRVVC while 9.8% of them were infected with more than one species. Also,

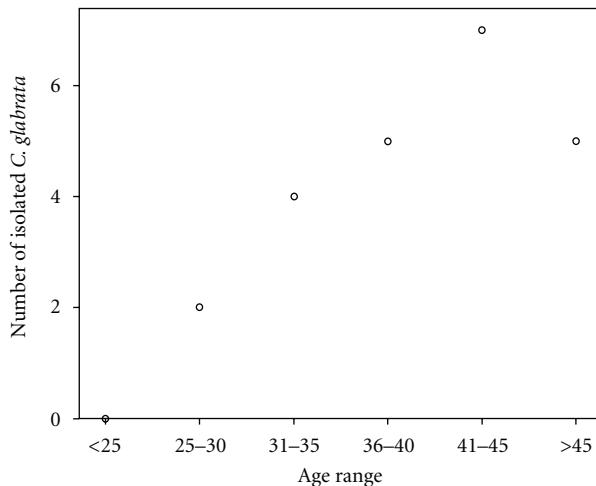


FIGURE 1: Correlation of participants' age range and isolated *C. glabrata*.

89.7% of those with RVVC were infected with one species compared to 10.8% who were infected with more. There was no statistically significant difference between these groups ( $P < 0.3$ ). Chi-square test showed no significant difference between the prevalence of *Candida* species among recurrent and nonrecurrent groups ( $P < 0.5$ ). A more detailed comparison of demographical and behavioral characteristics of the participants has already been published elsewhere [15]. The results of the multiplex PCR method perfectly matched the results of the germ tube test and chlamydospore production on CMA and API 20C-AUX kit.

#### 4. Discussion

*C. albicans* is still the most common yeast infection worldwide. Hence, the reliable and rapid identification method of this species is a fundamental goal of microbiology laboratories. The multiplex PCR method is a highly sensitive and specific technique based on the results of the previous studies [16]. Despite their demonstrated reliability, molecular methods have not been routinely used to identify *Candida* species. Liguori et al. compared different chromogenic and biological methods to PCR for *C. albicans* identification. They pointed out high incubation time, lack of experienced personnel, lower sensitivity and specificity, and lower discrimination power as disadvantages of other methods and suggested using them for screening and preliminary assays, while introduced the multiplex PCR as a precise and simple to implement method with no requirement of toxic and expensive chemical reagents [17]. This cross-sectional study used a multiplex PCR method previously used by Chang et al. [12] to identify the *Candida* species in a sample of Iranian population. It has been previously shown that 30.7% of isolated *C. glabrata* are resistant to common antifungal therapies compared to 0.6% of *C. albicans* [6, 18]. Hence, including methods which can identify the nonalbicans species is important and useful in choosing the appropriate treatment. The results of this study showed that *C. glabrata* is more common in women at older age while other species are

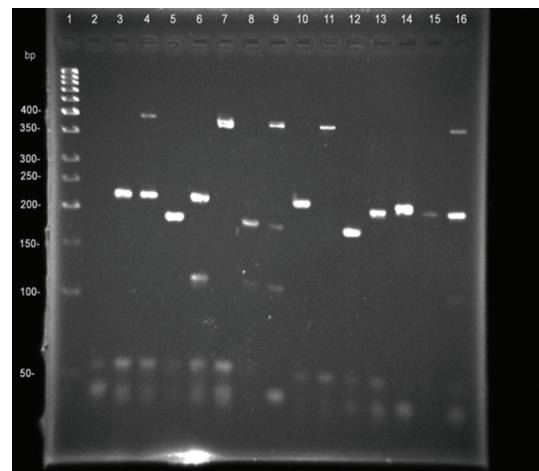


FIGURE 2: Multiplex PCR using primers ITS1, ITS2, CA3, and CA4. Lane 1: 50-bp DNA ladder. Lane 2: negative control. Lanes 3 to 8, 11 to 13 and 15 to 16: eleven clinical isolates. Lane 9: mixed reference strains *C. krusei* CBS573, *C. albicans* ATCC14053, and *C. glabrata* CBS2175, Lane 10: reference strain *C. tropicalis* CBS94, and Lane 14: reference strain *C. parapsilosis* CBS2195.

more common among the young population. This finding is in agreement with previous studies [19–22] and could be due to the development of antifungal resistance, the immune response, or hormonal changes among women of older age [19]. However, the result could be due to an unanticipated bias, and further studies seem essential to solely address this observation. The results of this study showed that 10.3% of participants were infected with more than one species of *Candida* which is a higher rate compared to the other studies conducted in China [23], United States [24], and Jordan [25]. The first and second most common isolated species in this study were *C. albicans* and *C. glabrata* which was in agreement with similar studies conducted worldwide [23, 25–31]. The prevalence of other species had the same pattern in our study as seen in earlier findings [25, 32–37]. Finally, of interest is using the Genomic DNA Extraction kit (AccuPrep Pioneer Corporation) as an effective and rapid method for extracting the DNA in 35 minutes. This eliminated the use of phenol-chloroform which is a cumbersome and tedious step of other PCR methods, resulting in significant improvements in the processing speed (the whole process could be completed in less than 6 hours). Another advantage of Multiplex PCR method is its ability to identify more than one species in a single specimen. Despite its strengths, our study was subject to some limitations such as low number of participants due to the lack of budget and personnel and collecting samples from patients admitted to one hospital which can affect the results of this study. Therefore, the results of our study should be used with caution and further research is recommended.

#### 5. Conclusion

The results of this study showed that *C. albicans* is the most common *Candida* species in VVC among women followed

by *C. glabrata* which has a higher prevalence among older women. A multiplex PCR method was used to identify the *Candida* species which seemed to be a reliable, rapid, and cost effective technique since it only requires PCR components and a commercial DNA extraction kit.

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## Clinical Study

# Vaginal Impact of the Oral Administration of Total Freeze-Dried Culture of LCR 35 in Healthy Women

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The use of probiotics in the prevention or treatment of some vaginal infections has been the subject of numerous studies. To assess the presence of *Lactobacillus casei rhamnosus* (LCR35) in the vagina after an oral administration, an open randomised pilot study was conducted on 20 healthy women of child-bearing age. *Materials and Methods.* 2 groups of 10 women were given a 28-day oral course, that is, at least 108 CFU/day (group 1) or 2 × 108 CFU/day (group 2) of LCR35. Nugent score and vaginal screening for LCR35 were undertaken before and after 28 days of treatment. *Results.* The mean Nugent score decreased in group 1 ( $-0.2$ ) as well as in group 2 ( $-0.3$ ). 10% of women in group 1 versus 40% of women in group 2 were carrying LCR35 at the end of the trial. *Conclusion.* LCR35, at the minimal dose of  $2 \times 108$  CFU/day, can return the Nugent score to normal in healthy women of child-bearing age, by means of a well-tolerated vaginal temporary presence. Phase III clinical trials will specify the preventive or curative impact of this orally administered strain on a range of vaginal disorders such as bacterial vaginosis or vulvovaginal candidiasis.

## 1. Introduction

Lactobacilli play a fundamental role in the ecological balance of the vagina. Many vaginal infections result from the disappearance or the quantitative or qualitative decrease in lactobacilli naturally present in the vagina. This is particularly true with bacterial vaginosis (BV), one of the most common vaginal infections estimated to have a prevalence between 15% and 30% [1].

Over the last few years, studies have been conducted to assess the usefulness of probiotics to treat or prevent vaginal infections such as BV or vaginal candidiasis. The clinical results differ widely because of the range of probiotics used, the routes of administration of probiotics (oral or vaginal), the duration of treatment and the cohorts studied. Among the points requiring clarification are the type of lactobacilli used in the probiotic preparations and the route of administration (oral or vaginal).

*Lactobacillus casei rhamnosus* LCR35 (LCR35) has shown *in vitro* that it has the required characteristics for vaginal colonization [2]:

- (i) proven fast adherence (one hour) to the vaginal wall, and
- (ii) prevention of growth of potential pathogens such as *Prevotella bivia*, *Gardnerella vaginalis*, and *Candida albicans* from the 4th hour after incubation [2].

About the route of administration, an oral preparation seems to be interesting since studies have shown that the rectum was the natural reservoir for commensal vaginal lactobacilli [3, 4].

An open pilot study was conducted in healthy women to assess the efficacy and safety of this route of administration for LCR35.

## 2. Material and Methods

An open randomised trial was conducted on 20 healthy women to assess the vaginal impact of the daily oral administration of two different doses of LCR35 for 28 days.

The protocol was submitted to the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS) and the

Ethics Committee (Comité de Protection des Personnes, CPP Ile de France VII) who, respectively, issued an authorisation and a favourable opinion.

**2.1. Study Cohort and Trial Design.** This single-centre trial (Institute Alfred Fournier, Paris) included 20 healthy women who were randomized into two groups of ten women each.

At the screening visit, inclusion criteria were women between 18 and 45 years old, premenopausal, nonpregnant, using effective contraception (i.e., all kind of contraception (hormonal, IUD, chemical, condoms) except “natural contraception” like coitus interrompus), not taking any anti-infectious treatment and not immunodeficient.

All patients were asked for genital symptoms of infections (abnormal discharge, pruritus, burning, or dyspareunia etc.). Symptomatic patients were excluded.

The simple practical technique of self-sampling was chosen because studies demonstrated that self-collected samples had the same microbiological diversity as physician-collected samples [5, 6]. All the women took a sample from their own vagina at the inclusion visit using two sterile cotton swabs mounted on a wooden handle (Deltalab) and introduced over a distance of 2 to 3 cms into the vagina. One swab was used to determine the Nugent score [7]. The other swab was placed in a survival medium (FT-MRS) and frozen for *LCR35* screening.

During the screening visit, all the women received an information leaflet on the study and all signed an informed consent form.

Three to seven days later, women were included, randomised in two groups, and given the treatment.

- (i) Group 1 (10 women): 1 gel capsule of *LCR35* per os per day (i.e., a minimum of  $10^8$  CFU) for 28 days.
- (ii) Group 2 (10 women): 2 gel capsules of *LCR35* per os per day (i.e., a minimum of  $2 \times 10^8$  CFU) for 28 days.

The period of 28 days for the control visit has been chosen to sample vaginal secretions at the same time of the menstrual cycle.

## 2.2. Microbiological Methods

**2.2.1. Nugent Score.** After spreading the vaginal secretions on a slide and colouring with the Gram technique, the score was used to classify the vaginal microbiota in three categories: normal (0 to 3), intermediate (4 to 6), and bacterial vaginosis (7 to 10).

**2.2.2. *LCR35* Identification.** At days D7 and D30, immediately after sampling, vaginal swabs were homogenised in FT-MRS, then the solution was divided into 6 cryotubes (containing 33% final glycerol). The divided fractions were then stored at  $-80^{\circ}\text{C}$  before analysis [(1) to (3)].

**(1) Culture.** After thawing at  $37^{\circ}\text{C}$ , a fraction was used to establish a diluted series at 1/10th (up to a dilution of  $-7$ ). Cultures were done on MRS, Rogosa and DP media.

The Rogosa, and DP media were incubated in anaerobic conditions for 72 h at  $37^{\circ}\text{C}$  and the MRS medium was incubated in both aerobic and anaerobic conditions for 72 h at  $37^{\circ}\text{C}$ . The cultures were done in duplicate for the purposes of statistical analysis.

**(2) REP-PCR.** After culture on MRS for 72 h at  $37^{\circ}\text{C}$  in aerobic conditions, 10 colonies per sample were taken and the REP-PCR profile of each one was performed. The colony was lysed by heat shock directly in the thermocycler on the basis of previous tests carried out in our laboratory (data not shown). The DNA of each of the 10 colonies was amplified in a reagent medium containing MgCl<sub>2</sub> 1X (MPbiomedicals), 0.2 mM of DNTP (MPbiomedicals), 0.5  $\mu\text{M}$  of primer (GTG)<sub>5</sub>, 1.5 mM of MgCl<sub>2</sub> (MPbiomedicals), 2.5 units of Taq polymerase (MPbiomedicals) with sterile water added to make 50  $\mu\text{L}$ . The amplification cycle is as follows: 5 min/94°C; (30 s/94°C; 1 min/45°C; 2 min/72°C)  $\times$  29 cycles; 7 min/72°C; Hold 10°C. The result of amplification is visible on 1.5% agarose gel, supplemented with 10% B.E.T, following migration for 52 min at 75 volts.

**(3) Specific PCR.** The total DNA of a fraction from a sample is extracted according to supplier recommendations with the QIAamp MiniKit for stool (QIAGEN). Amplification of a specific region of strain *LCR35* was done using hyb 21 primers [8]. The adjustment of this technique enabled us to obtain an amplification of *LCR35* at a hybridization temperature of 56°C, in a mix made up of 3 mM of MgCl<sub>2</sub>, 2U of Taq polymerase (QBiogen), 0.2 mM of each dNTP, 0.5 mM of primers and 1X of Taq buffer (Qbiogen). The DNA is amplified using successive cycles of: (94°C, 5 min, (94°C, 30 s; 56°C, 30 s; 72°C, 1 min/kb)  $\times$  25–35, 72°C, 7 min). The amplified fragments were then deposited on 2% agarose gel before migration at 100 V for 30 min.

## 2.3. Efficacy and Safety Criteria

**2.3.1. Vaginal Criteria.** The vaginal impact of the oral treatment was evaluated on the following.

- (i) Assessment of the Nugent scores on inclusion and at the end of the trial.
- (ii) PCR screening for *LCR35* in the vagina at the end of treatment.

**2.3.2. Safety.** Patients were told to note any adverse event (local or general) during the study and to consult their physician if necessary. At the control visit, the physician asked the patients about genital or general symptoms and taken medications during the study.

## 3. Results

The mean age of women included was  $27.2 \pm 6.8$  years. No significant difference was noted as regards demographic characteristics (age, weight, height, BMI, urinary pregnancy test) between groups 1 and 2.

TABLE 1: Changes in Nugent scores.

Nugent score	Group 1 ( <i>n</i> = 10)		Group 2 ( <i>n</i> = 10)	
	V1	V3	V1	V3
Normal (0 to 3)	8 (80%)	9 (90%)	10 (100%)	10 (100%)
Intermediate (4 to 6)	1 (10%)	1 (10%)	—	—
Bacterial vaginosis (7 to 10)	1 (10%)	—	—	—

All the women in group 1 took their treatment in accordance with the protocol. 1 patient in group 2 missed 1 day of treatment but nevertheless took all the capsules.

### 3.1. Vaginal Criteria (Table 1)

**3.1.1. Group 1 (at Least  $10^8$  CFU of LCR35 per Os per Day).** Before treatment, the mean Nugent score for group 1 was 1.8: 8 women had normal microbiota, 1 had intermediate microbiota, and 1 had BV (asymptomatic patient).

After treatment, the mean score for group 1 was 1.6: 9 women had normal microbiota, 1 had intermediate microbiota. Note that the patient with microbiological BV on inclusion had normal microbiota by the end of the trial. At the end of the trial, *LCR35* had been revealed in the vaginal microbiota of one out of 10 women (10%) in this group.

**3.1.2. Group 2 (at Least  $2 \times 10^8$  CFU of LCR35 per os per Day).** Before treatment, the mean Nugent score was 1 (10 women with normal microbiota).

After treatment, the mean Nugent score was 0.7 (10 women with normal microbiota).

At the end of the trial, *LCR35* had been revealed in the vaginal microbiota of four out of 10 women (40%) in this group.

Nugent score decreased in both groups but slightly more significantly in group 2 despite a lower score at inclusion (resp., -0.3 versus -0.2).

At the end of the trial, *LCR35* was revealed in the vaginal microbiota of 5 out of 20 women (25%).

**3.2. Safety.** No side effects and no withdrawal of treatment were reported either by patients or by physician during the trial involving 20 women.

## 4. Discussion

One of the exclusion criteria was postmenopausal women, which led to an age group between 18 and 45 years old. After menopause, the lack of oestrogen causes a change in the vaginal ecosystem, with a significant decrease in the number of lactobacilli [9, 10]. The inclusion of postmenopausal women would therefore have introduced bias into the trial.

The exclusion of women with clinical signs of vaginal infection is justified for ethical reasons. It would have been controversial not to start specific treatment of the infection and this, again, would have introduced a bias into the trial.

On the other hand, the patient with asymptomatic BV was not excluded because there was no risk of complications (as this patient was not pregnant). Treatment of the infection could therefore be postponed, especially in the absence of any clinical symptoms.

The two bacteriological samples were taken at an interval of four weeks to ensure that the women were at the same point in their cycle or in the taking of contraception, that is, under the same conditions of hormonal impregnation as studies [11, 12] demonstrated slight changes in lactobacillus microbiota during the menstrual cycle.

The Nugent score has been used for this trial because, even if it is an imperfect test, it stays the benchmark score [5] used to evaluate the quality of the vaginal microbiota and the load of lactobacilli in a semiquantitative manner. It is based on a direct examination after colouring of the vaginal secretions. The examination has been standardised but remains observer-dependent. During this trial, all the Nugent tests were conducted in the same microbiology laboratory (Institute Fournier, Paris) by the same observer.

At the end of treatment, *LCR35* was identified, by REP-PCR, four times more often (resp., 40% versus 10% of women) among women in group 2 (at least 2 capsules dosed at  $10^8$  CFU of *LCR35*/day) than among the women in group 1 (at least 1 capsule dosed at  $10^8$  CFU of *LCR35*/day). The daily dose of *LCR35* therefore played a significant role in the vaginal uptake of the probiotic.

19 of the 20 treated women had a normal Nugent score at the end of the study (Table 1); one woman had an intermediate score. Note that the patient with asymptomatic BV at inclusion (Nugent score  $\geq 7$ ) returned to a normal Nugent score at the end of the study. Thus overall, the treatment improved the Nugent scores, particularly at the dose of  $2 \times 10^8$  CFU of *LCR35*/day.

Few studies have been conducted on the vaginal uptake of lactobacilli following an oral dose of probiotics. Reid et al. [13] randomised 42 healthy women (mean age 31 years) into 3 groups:  $8.10^8$  or  $6.10^9$  or  $2 \times 8.10^8$  of a combination of *L. rhamnosus* GR-1 + *L. fermentum* RC-14 for 28 days. A change to normal vaginal microbiota (appreciated semiquantitatively by the Nugent score) was significant only at a dose of  $1.6 \cdot 10^9$  GR-1/RC-14, showing the potential ability of oral probiotics to restore or maintain a normal vaginal ecosystem.

The same author published in 2003 [14] an other study about 64 healthy women who received either an oral dose of *L. rhamnosus* + *L. reuteri* or an oral dose of placebo for 60 days. A microbiological examination showed a significant rise in the level of vaginal lactobacilli at D28 and D60 in the probiotic group compared to the placebo group. However, in that study, there was no PCR identification of the strains of vaginal lactobacilli before or after treatment.

Morelli [15] administrated *L. rhamnosus* GR-I + *L. fermentum* RC-14 for 14 days *per os* to a group of 10 women. A vaginal colonisation was identified by REP-PCR between 0% and 60% depending on the observation period after the oral administration.

The results of our study of *LCR35* therefore agree with the results of other studies using other strains of lactobacilli.

Finally, the literature on probiotics reports an improvement in general immunity [16–18] when taking probiotics, mainly resulting from indirect effects:

- (i) increased migration of immune cells (macrophages, lymphocytes, etc.);
- (ii) increased phagocytosis;
- (iii) Production of proinflammatory cytokines, and so forth.

The same type of result was shown in children with *LCR35* [19].

## 5. Conclusion

This randomised trial is the first formal demonstration of the positive impact of the oral administration of *LCR35* on vaginal microbiota. Treatment with a daily dose of 2 gel capsules *LCR35 per os* produced an overall decrease in the Nugent score in healthy women and, therefore, the maintenance of the quality of their vaginal microbiota. A return to normal was even observed in one case of asymptomatic BV. Our work also demonstrated, by using REP-PCR, *LCR35*'s ability to be temporarily present in the vagina after 28 days oral treatment without generating any adverse events. This means that the probiotic is able to survive through the gastric tract before being temporarily present in the vaginal area. Note that the vaginal impact of *LCR35* appears to be dose-dependent since, after treatment, we observed that 40% of women who received at least 2 capsules dosed at  $10^8$  CFU/day of *LCR35* were carrying *LCR35*, compared to only 10% of women in the group that received at least one capsule dosed at  $10^8$  CFU/day.

This shows that, at a dose of  $2 \times 10^8$  CFU/day, *LCR35* is able to restore or maintain a normal vaginal microbiota in healthy premenopausal women, with a high level of safety and compliance with treatment.

New clinical trials are now required to specify the preventive or curative impact of this strain, administered orally, in the treatment of various vaginal diseases such as BV or vulvovaginal candidiasis.

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## Research Article

# Behavioral Predictors of Colonization with *Lactobacillus crispatus* or *Lactobacillus jensenii* after Treatment for Bacterial Vaginosis: A Cohort Study

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**Objective:** Evaluate predictors of vaginal colonization with lactobacilli after treatment for bacterial vaginosis (BV). **Methods.** Vaginal fluid specimens from women with BV underwent qPCR for *Lactobacillus crispatus*, *L. jensenii*, and *L. iners* pre- and posttreatment. **Results.** Few women with BV were colonized with *L. crispatus* (4/44, 9%) or *L. jensenii* (1/44, 2%), though all had *L. iners*. One month posttreatment 12/44 (27%) had *L. crispatus*, 12/44 (27%) *L. jensenii*, and 43/44 (98%) *L. iners*. Presence of *L. jensenii* posttreatment was associated with cure (Risk Ratio (RR) 1.67; 95% CI 1.09–2.56); *L. crispatus* showed a similar trend (RR 1.41; 95% CI 0.89–2.24,  $P = 0.14$ ). Receptive oral sex was associated with 2.2-log<sub>10</sub> lower concentration of *L. crispatus* (95% CI –4.38, –0.02), and digital-vaginal sex with 2.6-log<sub>10</sub> lower concentration (95% CI –4.87, –.33). **Conclusion.** One month after BV treatment, few women established colonization with *L. crispatus* or *L. jensenii*. Few behaviors were associated with colonization.

## 1. Introduction

Bacterial vaginosis (BV) is the most common cause of vaginal discharge in reproductive age women [1], is present in approximately 29% of women in the United States [2], and is characterized by vaginal colonization with anaerobic bacterial species along with loss of lactobacilli. The clinical sequelae of BV are significant—a nearly two-fold-increased risk of HIV-1 acquisition [3, 4], preterm delivery [5, 6], and pelvic inflammatory disease (PID) [7]—and affect millions of women worldwide each year, making BV a significant health problem. Treatment with antibiotics has a cure rate of 50–80% [8, 9] but recurrence within 1 to 3 months is common (30–52%) [10–12].

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ -) producing species of vaginal lactobacilli are associated with decreased rates of BV [13, 14], and better reproductive health outcomes [15, 16] compared

to non- $\text{H}_2\text{O}_2$ -producing species. *Lactobacillus crispatus* is the most common vaginal  $\text{H}_2\text{O}_2$ -producing *Lactobacillus* species [17, 18]. *L. jensenii* is another frequently isolated  $\text{H}_2\text{O}_2$ -producing species [18, 19]. Some hypothesize that the recurrence rate of BV is high because these protective lactobacilli do not recolonize the vagina after antibiotic treatment aimed at eradicating BV-associated anaerobes, and so leave an ecologic void that is quickly refilled by opportunistic organisms. In one study, only 40% of women were recolonized with any  $\text{H}_2\text{O}_2$ -producing species of lactobacilli 30 days after oral metronidazole treatment and 57% were recolonized 30 days after vaginal clindamycin [20]. Most women are colonized with a single dominant species of *Lactobacillus* [21], but it is unclear if this is because there is competition between species for the vaginal niche. A majority of women studied in the US are colonized with *Lactobacillus iners* [22, 23], a fastidious species that does not commonly produce  $\text{H}_2\text{O}_2$ .

and that has been associated with increased risk of abnormal vaginal microbiota in pregnant women [24]. Little is known about the effect of *L. iners* on a woman's ability to colonize with beneficial H<sub>2</sub>O<sub>2</sub>-producing species.

Presence of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli [14, 25, 26], specifically *L. crispatus* [24], has been associated with decreased risk of abnormal vaginal microbiota and BV; thus, recolonization with these species after treatment for BV is likely an important marker of vaginal health. We undertook this nested cohort study to evaluate the effect of sexual behavior on vaginal recolonization with two hydrogen peroxide producing *Lactobacillus* species, *L. crispatus* and *L. jensenii*, one month after treatment for BV.

## 2. Methods

**2.1. Study Population and Design.** We conducted an analysis of women diagnosed with and treated for BV while enrolled in an observational cohort study in Seattle, WA. As previously described, participants were recruited through advertisements, media, and community referral, and had to be  $\geq 16$  years old and report having had sex with at least one woman in the previous year, a group with relatively high BV prevalence [27]. Study visits were scheduled every three months for a year, with additional visits for vaginal symptoms and/or 4 weeks after treatment for BV. At each visit, participants completed a computer-assisted self-interview (CASI) that collected information about demographics, sexual practices, medical, and reproductive history. The study was approved by the University of Washington Institutional Review Board and all participants provided informed consent at enrollment. Participants underwent pelvic examination with collection of vaginal swabs for saline microscopy, Gram stain, and bacterial culture. A separate foam swab was collected by rolling along the vaginal wall and was then frozen at  $-80^{\circ}\text{C}$  for use in molecular assays. Women diagnosed with BV by Amsel's clinical criteria [28] were treated with vaginal metronidazole gel, 37.5 mg nightly for 5 nights, and assessed at a followup visit after 4 weeks. Vaginal fluid Gram stains were scored using the criteria outlined by Nugent et al. [29], however, treatment success was defined solely as absence of BV by Amsel's criteria.

We included all participants who were diagnosed with BV during the study and whose followup visits occurred 3 to 8 weeks after treatment. Only the first BV-positive visit was included for participants who were diagnosed with BV more than once. The study was conducted between October 2003 and December 2006, but between 3/2/2004 and 12/8/2005, only women with vaginal pH > 4.5 at the followup visit had samples taken follow-up (due to limitations in study funding). Because of this differential assessment and the resulting potential bias, all women whose followup visits fell within this time period were excluded. Participants whose samples did not have enough material to complete all PCR assays were also excluded.

**2.2. Molecular Assays.** Frozen vaginal swabs from the BV-positive visit and a followup visit within 3–8 weeks were processed as previously described [30]. All extracted DNA was

tested in a quantitative PCR assay using primers targeting the human 18S rRNA gene to validate that successful DNA extraction occurred. An internal amplification control PCR using exogenous DNA from a jellyfish gene was used to test for presence of PCR inhibitors [31].

Vaginal fluid samples were then subjected to taxon-directed 16S rRNA gene quantitative PCR assays for the detection and quantification of *L. crispatus*, *L. jensenii*, and *L. iners* [30, 32]. Each assay has previously been validated and proven sensitive (to a level of 1–10 DNA copies/reaction) and specific (does not detect other bacteria at a concentration of 10<sup>6</sup> copies/reaction). The assays use a TaqMan format, and are run on an ABI 7500 Thermocycler (Applied Biosystems, Foster City, CA) or Eppendorf Mastercycler ep Realplex thermal cycler (Eppendorf, Westbury, NY).

**2.3. Statistical Analysis.** The primary outcome of interest was presence or absence of *L. crispatus* or *L. jensenii* after treatment for BV. In secondary analyses, we assessed the relationship between sexual behaviors and quantities of bacteria, expressed as 16S rDNA gene copies/vaginal swab and log transformed. Univariate log binomial regression was used to assess the relationship between presence or absence of either *L. crispatus* or *L. jensenii* and (a) different behaviors, and (b) presence and quantity of *L. iners*. Univariate linear regression was used to assess the relationship between sexual behaviors and quantity of *L. crispatus* or *L. jensenii* in the subset of women who were colonized. Given the relatively small number of women, we did not perform multivariate analyses.

## 3. Results

A total of 336 women were enrolled in the observational cohort. Of these, 136 (40%) were diagnosed with BV during the study: 96 at enrollment, and 40 at a routine study visit or a nonscheduled visit with symptomatic BV. Eleven women never returned for followup, 58 women had followup visits that fell during the period of exclusion, and 23 did not have adequate sample remaining for all of the assays and were excluded, leaving 44 women available for this analysis.

**3.1. Baseline Characteristics.** The 44 women had a mean age of  $25 \pm 3$  years and were primarily white (35/44; 80%). Half of the visits occurred during the proliferative phase of the menstrual cycle, and half in the luteal phase. The majority of women had only female partners (31/44; 70%), while smaller percentages had male only (4/44; 9%), partners of both genders (4/44; 9%) or no sexual partner in the last 3 months (5/44; 11%). All women had BV by Amsel's criteria, and 98% (43/44) also had BV by Nugent's score, which was significantly different than women excluded from this substudy, of whom only 85% (78/92) had BV by Nugent's score ( $P = .02$ ). This was the only characteristic that differed between women in the substudy and those that were excluded. Women in the substudy were as likely to complete antibiotic treatment for BV as women in the larger cohort (89% versus 90%;  $P = .95$ ).

At diagnosis, 29/44 (66%) women reported having had receptive oral-vaginal contact in the previous 90 days. Slightly more reported digital-vaginal sex (82%), while fewer

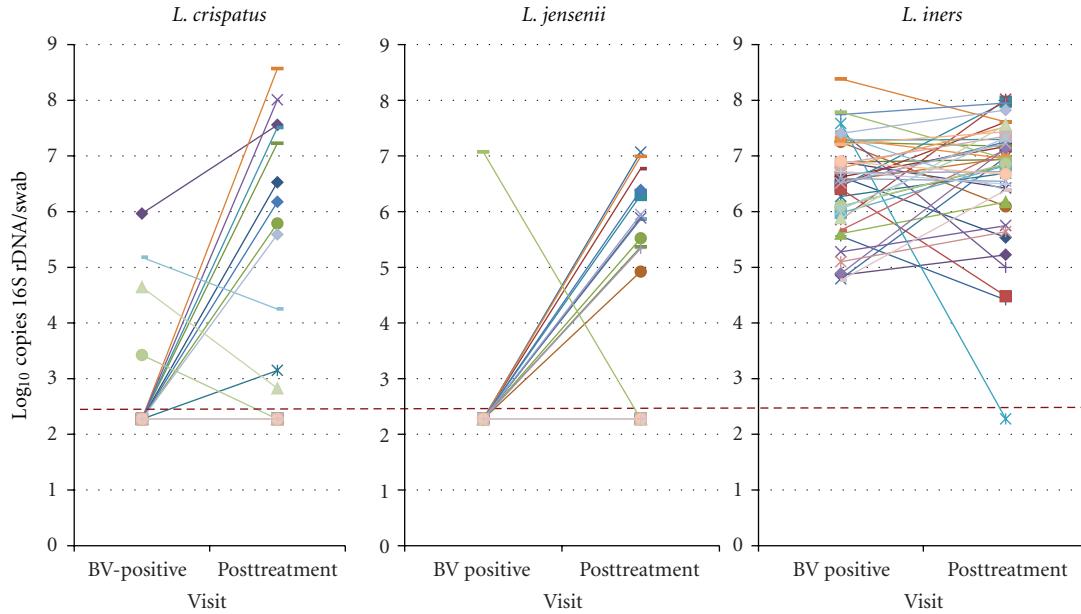


FIGURE 1: Change in concentrations of three different species of lactobacilli, as measured by species-specific quantitative PCR, 4 weeks after treatment for bacterial vaginosis with vaginal metronidazole. Each line represents an individual patient and her concentration of each bacterium before and after treatment. The dotted line represents the lower limit of detection of the qPCR assay.

reported toy-vaginal sex (36%) during that same time. Only 8 (18%) women reported sexual contact with a male partner in the 3 months prior to BV diagnosis, 7 of whom reported having penile-vaginal sex during that time. All 44 women were colonized with *L. iners* at BV diagnosis, while few were colonized with *L. crispatus* (4/44, 9%) or *L. jensenii* (1/44, 2%).

**3.2. Posttreatment Characteristics.** Nearly all women (43/44; 98%) were colonized with *L. iners* after treatment. Only 12/44 (27%) were colonized with *L. crispatus* and 12/44 (27%) with *L. jensenii*. Of those, six women were colonized with both species, and six each with only one of the two species. Posttreatment, 18 women (41%) still met Amsel's criteria for BV and were considered to have failed treatment, all of whom also had a Nugent score  $\geq 7$ . Among these women, only 3 (16.7%) were colonized with *L. crispatus* and 2 (11.1%) with *L. jensenii*. Of the 26 women who achieved cure, a slightly higher percentage (but still a minority) were colonized with *L. crispatus* (9/26; 35%) and *L. jensenii* (10/26; 38%). Presence of *L. crispatus* at diagnosis or followup trended towards association with cure (Risk Ratio 1.41; 95% CI .89, 2.24;  $P = .14$ ), but this was not statistically significant. Women colonized with *L. jensenii* after treatment had significantly higher rates of treatment success (RR 1.67; 95% CI 1.09, 2.56;  $P = .02$ ).

Of the four women colonized with *L. crispatus* at BV diagnosis, one achieved cure and had higher concentrations after treatment, while 3 failed treatment, and had lower ( $n = 2$ ) or undetectable ( $n = 1$ ) concentrations. The one woman colonized with *L. jensenii* at BV diagnosis no longer had detectable colonization after treatment, and also

failed treatment (Figure 1). Among colonized women, mean  $\text{Log}_{10}$  concentration of *L. crispatus* after treatment was  $6.1 \pm 1.9$  gene copies/mL and for *L. jensenii* was  $6.0 \pm .7$  gene copies/mL. All 44 women were colonized with high quantities of *L. iners* at the BV diagnosis visit (mean  $\text{Log}_{10}$  copies  $6.5 \pm .9$ ), and the quantity did not change significantly at the followup visit (mean  $\text{Log}_{10}$  copies  $6.7 \pm 1.1$ ;  $P = .40$ ).

Between the visit at which BV was diagnosed and treatment provided, and subsequent followup (median 33 days, IQR 28–37), 21/44 women (48%) reported oral-vaginal sex, 26 (59%) digital-vaginal sex, 8 (18%) penile-vaginal sex, and 9 (20%) toy-vaginal sex. Among all 44 women, no interim sexual behaviors were associated with presence or absence of either *L. jensenii* or *L. crispatus* at the followup visit or with treatment failure (Table 1). Among women who were colonized with *L. jensenii* or *L. crispatus* at the followup visit, we examined whether behaviors reported in the interim period between treatment and followup at 32 days were associated with quantity of bacteria detected at the followup visit (Table 2). In the subset of 12 women establishing colonization by followup, report of digital-vaginal sex was significantly associated with  $2.6\text{-}\text{Log}_{10}$  lower concentrations of *L. crispatus* (95% CI  $-4.87, -.33$ ). Report of receptive oral sex was associated with  $2.2\text{-}\text{Log}_{10}$  lower concentrations of *L. crispatus* (95% CI  $-4.38, -.02$ ). No behaviors were associated with quantity of *L. jensenii* detected at that visit.

#### 4. Discussion

In this cohort of women reporting sex with women, rates of vaginal colonization with two species of commensal  $\text{H}_2\text{O}_2$ -producing lactobacilli four weeks after treatment

TABLE 1: Univariate association between reported sexual behaviors during treatment and followup and presence of *L. crispatus* or *L. jensenii* at the posttreatment visit.

Sexual behavior during followup period	N	Presence of <i>L. crispatus</i> (n = 44)	Presence of <i>L. jensenii</i> (n = 44)	BV diagnosis by Amsel's at followup (n = 18)
		Prevalence ratio	Prevalence ratio	Prevalence ratio
Number of partners	12	Reference	Reference	Reference
0				
1	26	1.62 (.39, 6.65)	1.12 (.34, 3.46)	1.1 (.50, 2.44)
2+	6	3.0 (.67, 13.4)	1.33 (.30, 5.96)	.4 (.06, 2.70)
Oral vaginal sex	21	2.19 (.77, 6.22)	1.53 (.57, 4.1)	1.10 (.54, 2.23)
Digital-vaginal sex	26	2.08 (.65, 6.63)	2.08 (.65, 6.63)	.87 (.43, 1.76)
Toy-vaginal sex	9	1.30 (.44, 3.82)	.78 (.21, 2.94)	1.11 (.48, 2.56)
Penile-vaginal sex	8	.9 (.24, 3.34)	.9 (.24, 3.34)	.9 (.34, 2.38)
Use of vaginal lubricant	11	2.42 (.66, 8.93)	.30 (.04, 2.21)	.73 (.30, 1.78)

TABLE 2: Association between reported sexual behaviors during treatment and followup and quantity of *L. crispatus* or *L. jensenii* in women who were colonized at the posttreatment visit.

Sexual behavior during followup period	N	<i>L. crispatus</i>		<i>L. jensenii</i>	
		Log <sub>10</sub> difference in 16S rRNA copies/mL	N	Log <sub>10</sub> difference in 16S rRNA gene copies/mL	N
Number of partners					
0	2	Reference	3	Reference	
1	7	-1.66 (-4.87, 1.55)	7	-.03 (-1.21, 1.16)	
2+	3	-3.13 (-6.52, .25)	2	-.16 (-1.73, 1.40)	
Oral vaginal sex	8	-2.20 (-4.38, -.02)	7	-.11 (-1.05, .83)	
Digital-vaginal sex	9	-2.60 (-4.87, -.33)	9	-.06 (-1.13, 1.02)	
Toy-vaginal sex	3	-1.65 (-4.32, 1.03)	2	-.15 (-1.39, 1.09)	
Penile-vaginal sex	2	.69 (-2.67, 4.04)	2	-.14 (-1.39, 1.10)	
Use of vaginal lubricant	4*	-2.92 (-6.09, .24)	1*	-.53 (-2.07, 1.0)	

\*Missing data for 5 women.

for bacterial vaginosis were low. Though colonization was infrequent, women who were able to establish colonization achieved high concentrations of each of these bacteria.

For clinicians and affected women, the high rate of BV recurrence after antibiotic treatment is exceedingly frustrating [10–12]. Several groups have evaluated whether adding probiotic compounds containing lactobacilli to treatment improves outcomes, but results have been mixed [33–36]. In a study of healthy women treated with vaginal probiotic capsules containing *L. crispatus*, participants who reported penile-vaginal sex between treatment and followup were less likely to establish colonization with the probiotic strain [37]. We hypothesized that sexual activity in the month after treatment may inhibit vaginal colonization with beneficial lactobacilli, possibly through reinoculation with BV-associated bacteria from vulvar or rectal reservoirs, which might increase risk for BV recurrence.

In the parent study of nearly 350 women from which this nested case control study was derived, we demonstrated that women cured of BV had higher rates of colonization by *L. crispatus* after treatment (42%) than women with persistent BV (26%;  $P = .0003$ ); data on *L. jensenii* were not available

[30]. A different study obtained vaginal swabs for culture and found that by 4 weeks after treatment with vaginal metronidazole 59% of women were colonized with hydrogen peroxide producing lactobacilli [20]. Other studies used Nugent score to characterize shifts of the vaginal bacteria, and reported that as many as 66% of treated women had at least some lactobacilli at 21–30 days after treatment [38], though H<sub>2</sub>O<sub>2</sub> production was not measured. Our group previously measured posttreatment quantity of *L. crispatus* in a cohort of pregnant women using PCR and found that only 9/53 (17%) of women had detectable levels 4–6 weeks after treatment [39].

Few studies have evaluated behavioral predictors of colonization with lactobacilli. In women with BV, those who report more sexual partners are less likely to be colonized with H<sub>2</sub>O<sub>2</sub>-producing lactobacilli [40]. In our cohort, women colonized with *L. crispatus* who reported digital-vaginal and/or oral-vaginal sex had lower quantities of this bacterium. Although it did not reach statistical significance, we saw a paradoxical opposite trend in the risk related to these behaviors for vaginal colonization with *L. crispatus* or *L. jensenii*, suggesting that women with more partners, or

reporting more frequent oral-vaginal or digital-vaginal sex, were more likely to be colonized. One possible explanation is that women colonized by *L. crispatus* and *L. jensenii* more likely achieved cure of BV, thus reducing the likelihood of vaginal symptoms that might deter them from engaging in sex. This observation highlights the difficulty in studying the complex relationships between sexual behaviors and the dynamic nature of vaginal microbiology—temporal associations are difficult to ascertain unless both outcomes are measured frequently (ideally, daily).

The main limitation of this study is the small sample size, which reduced our power to detect potential associations between behaviors and colonization with specific lactobacilli. A significant number of participants with BV did not have a posttreatment sample, which limited our ability to examine the entire study group. Participants selected for this substudy were similar to the larger cohort except for having higher Nugent scores at diagnosis, which may partially explain their high rate of treatment failure. This cohort is composed primarily of women who have sex exclusively with women, and our results may differ from those obtained in a cohort of primarily heterosexual women. However, this allowed us to study the effect of several different types of sexual behavior on the vaginal microbiota. The population had well-characterized information about sexual activity during the treatment period, and a very high rate of followup (92%). Our quantitative PCR analysis allowed detection of small quantities of bacteria and analysis of changes in quantity of bacteria after treatment with respect to sexual behaviors.

## 5. Conclusions

Vaginal colonization with H<sub>2</sub>O<sub>2</sub>-producing lactobacilli 4 weeks after treatment for BV was uncommon, suggesting that there is a window of vulnerability during which women may be more susceptible to reinfection or recurrence. While no sexual behaviors were found to impact presence of colonization, quantity of *L. crispatus* was decreased in women reporting digital-vaginal and oral-vaginal contact. Quantity of *L. jensenii* was not affected by any reported sexual behaviors. This suggests that some species of commensal lactobacilli may be more sensitive to the effect of sexual activity on the vaginal environment.

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## Research Article

# In Vitro Resistance to Macrolides and Clindamycin by Group B Streptococcus Isolated from Pregnant and Nonpregnant Women

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**Background.** Despite the introduction of screening bases intrapartum prophylaxis, *Streptococcus agalactiae* is still an important etiological agent of perinatal infections. The increasing rate of resistance and the differences in resistance pattern among countries suggest that a program of surveillance at the institutional level is important in determining optimal prophylaxis. In contrast, knowledge on GBS epidemiology in Italy is limited, and no data are available in the Southern region of the country. We sought to determine the occurrence of resistance to macrolides and clindamycin of GBS isolates in pregnant and nonpregnant women.

**Methods.** Between 2005 and 2008, 1346 vaginal and 810 rectovaginal swabs were obtained from pregnant and non-pregnant women.

**Results.** The occurrence of macrolides and clindamycin resistance was 16.5% in 2005 increasing up to 69.9% in 2008. A high percentage of isolates was resistant to tetracycline through all the study period with no statistically significant annual. **Conclusions.** In our cohort, an increase of *in vitro* resistance of GBS to macrolides and clindamycin is clearly evident. The discordance with reports from different countries emphasize the crucial role of microbiological methods in setting possible therapeutic strategies.

## 1. Introduction

Among Gram-positive bacteria, *Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS) is considered a common commensal of the female urogenital tract and rectum [1] whose importance is referred to severe neonatal pathologies by perinatal transmission from women to newborns. Neonatal infections by GBS are usually distinguished in early onset (occurring in the first 7 days of life) and late onset (occurring between 7 days of life and 3 months of age); this temporal distinction reflects differences in the spectra of infection [2, 3].

In studies carried out in the 1970s, GBS emerged as the leading cause of neonatal morbidity and mortality, with a frequency of 2–3 cases per 1000 live births and case-fatality ratios of 50% [3, 4]. The vaginal colonization prevalence among pregnant women varies in European

countries between 10 and 20%, and the incidence of neonatal infections ranges from 0.5 to 2 per 1000 live births [5–8].

Between 1996 and 1997, the American College of Obstetricians and Gynecologists, the Centers for Disease Control and Prevention, and the American Academy of Pediatrics produced recommendations for prevention of perinatal GBS disease. These guidelines recommended the use of culture-based screening for GBS colonization between 35 and 37 weeks gestation and the antibiotic prophylaxis of all colonized women [9, 10].

The intrapartum antibiotic prophylaxis (IAP) for GBS carriers indicates the use of penicillin (or ampicillin). For penicillin-allergic women without a history of anaphylaxis, angioedema, respiratory distress, or urticaria, cefazolin is the preferred agent. Vancomycin and clindamycin are recommended for penicillin-allergic women at high risk for anaphylaxis. The introduction of IAP for GBS carriers has

been associated with a substantial decline in the incidence of early-onset neonatal infections [11].

The purpose of the present study was to assess the antibiotic susceptibility patterns of GBS isolates obtained from a heterogeneous female population (pregnant and nonpregnant) in a region of Southern Italy and to evaluate whether statistically significant changes in GBS antibiotic resistance regarding macrolides and clindamycin occurred in the years in order to generate local data for the development of rational interventions for prevention of GBS infection in our country.

## 2. Materials and Methods

**2.1. Study Population.** In the period from January 2005 up to December 2008, a total of 2156 biological samples (1346 vaginal swabs from nonpregnant women and 810 rectovaginal swabs from pregnant women at 35–37 weeks of gestation) were collected in the Microbiology Laboratory of University Hospital “Federico II”, Naples, Italy. All women gave their consent to take part in the study. Therapeutic protocols were not modified for women enrolled in the study.

**2.2. Processing of Samples, Culture of Microorganisms, and Identification Analysis.** All swabs were maintained in the Stuart transport medium and transported to the Microbiology Laboratory. Swabs were plated on several agar media, including Columbia colistin-nalidixic acid (CNA) agar with addition of 5% of sheep blood, MacConkey agar, Sabouraud agar, and chocolate agar and incubated at 37°C overnight in aerobic or microaerobic conditions and were examined microscopically to evaluate the preservation of *Lactobacillus* microbial status.

Bacteria were identified by conventional methods (Gram stain, catalase test) and automated system (Vitek II, bio-Mérieux, France). The identification of the Lancefield antigen was obtained by Streptococcal Grouping Kit (Oxoid, Hampshire, England).

**2.3. Antimicrobial Susceptibility Testing Method.** To check the sensitivity to antimicrobial agents, an automated microdilution method (Vitek II) was utilized. The susceptibility criteria were in accordance with the National Committee for Clinical Laboratory Standards Interpretative Criteria [12]. Antibiotics tested were as follows: amoxicillin/clavulanic acid, ampicillin, cefaclor, cefotaxime, ceftriaxone, clindamycin, erythromycin, penicillin, teicoplanin, tetracycline, trimethoprim/sulfamethoxazole, vancomycin, levofloxacin, azithromycin, clarithromycin, quinupristin/dalfopristin, and linezolid.

**2.4. Statistical Analysis.** Statistical analysis, including comparison of proportions and chi-squared test, was applied throughout the study. A  $P < 0.05$  was considered statistically significant.

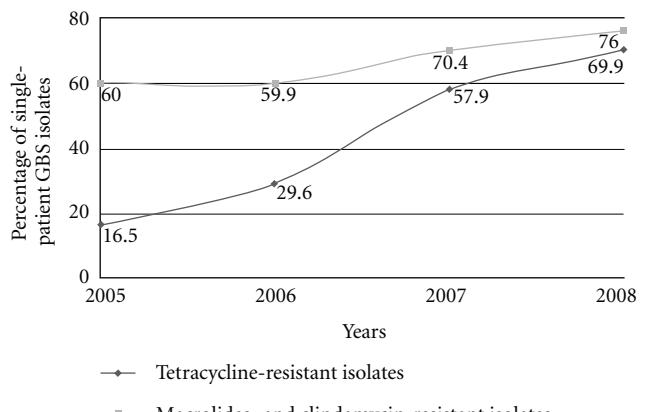


FIGURE 1: Annual increment (%) of resistant isolates during the study period.

## 3. Results

In the study period, a total of 879 GBS from all samples (2156 between vaginal and rectal-vaginal swabs) were isolated. The distributions of swabs, positive cultures, and patients in the period of study are indicated in Table 1.

The distribution of single-patient resistant GBS isolates is showed in Table 2 as well as the susceptibility pattern over the study period. The antibiotic susceptibility profiles indicate that isolates showed sensitivity to beta-lactams, glycopeptides, quinolones, quinupristin-dalfopristin, trimethoprim-sulfamethoxazole, and linezolid.

The number of isolates resistant to tetracycline was high through all the study period, indicating not statistically significant fluctuations. Instead, the increment of resistance to macrolides and clindamycin was statistically significant through the study period ( $X^2$  for trend = 8.100,  $P = 0.004$ ). The annual increment in percentage of macrolides- and clindamycin-resistant isolates during the study period is indicated in Figure 1.

The GBS isolates resistant to tetracycline showed a MIC value  $\geq 16 \mu\text{g/mL}$  through all the study period. The MICs obtained for macrolides and clindamycin range from  $\leq 0.25 \mu\text{g/mL}$  to  $\geq 8 \mu\text{g/mL}$ .

## 4. Discussion

In our experience, in accordance with CDC 2010 guidelines, penicillin and ampicillin are still the first choice for IAP, followed by first-generation cephalosporins as cefazolin in penicillin allergic women. In fact all GBS isolates were susceptible to these antibiotics. The prevalence of isolates resistant to macrolides and clindamycin is considerably high (55%) and has increased significantly from 16.5% to 70% during the study period ( $P < 0.05$ ).

Although very few women GBS positive give birth to babies who are infected with GBS, antenatal screening is routinely performed to reduce the rate of early-onset infections in newborns. However, there is still controversy about its prevention since antenatal screening and treatment

TABLE 1: Distribution of swab type, positive cultures, and number of infected patients.

Year	Total swabs N (%)		Total positive cultures N (%)	
	Vaginal-rectal	Vaginal	Vaginal-rectal	Vaginal
2005	76 (7.7%)	153 (13%)	23 (6.4%)	62 (11.9%)
2006	192 (19.5%)	212 (18.1%)	51 (14.3%)	97 (18.6%)
2007	345 (35.1%)	357 (30.4%)	134 (37.5%)	146 (28%)
2008	370 (37.6%)	451 (38.4%)	149 (41.7%)	217 (41.6%)
	Total 2156			Total 879

TABLE 2: Distribution (number and percentage) of resistant GBS strains during the 4-year study period.

	2005		2006		2007		2008	
	N°	%	N°	%	N°	%	N°	%
AMC	0	—	0	—	0	—	0	—
AMP	0	—	0	—	0	—	0	—
CEC	0	—	0	—	0	—	0	—
CTX	0	—	0	—	0	—	0	—
CRO	0	—	0	—	0	—	0	—
CLI	14	16.5	48	29.6	162	57.9	256	69.9
ERY	14	16.5	48	29.6	162	57.9	256	69.9
PEN	0	—	0	—	0	—	0	—
TEC	0	—	0	—	0	—	0	—
TET	51	60	97	59.9	197	70.4	278	76
SXT	0	—	0	—	0	—	0	—
VAN	0	—	0	—	0	—	0	—
LVX	0	—	0	—	0	—	0	—
AZM	14	16.5	48	29.6	162	57.9	256	69.9
CLR	14	16.5	48	29.6	162	57.9	256	69.9
Q-D	0	—	0	—	0	—	0	—
LZD	0	—	0	—	0	—	0	—

AMC = amoxicillin-clavulanic acid; AMP = ampicillin; CEC = cefaclor; CTX = cefotaxime; CRO = ceftriaxone; CLI = clindamycin; ERY = erythromycin; PEN = penicillin; TEC = teicoplanin; TET = tetracycline; SXT = trimethoprim-sulfamethoxazole; VAN = vancomycin; LVX = levofloxacin; AZM = azithromycin; CLR = clarithromycin; Q-D = quinupristin-dalfopristin; LZD = linezolid.

may carry disadvantages for the mother and the baby. The usual recommendation for prevention of GBS transmission from colonized women to their infants during labour is to administer intravenous penicillin or ampicillin every 4 h for the duration of labour [11].

On the maternal side, IAP's risks are allergic reactions. Even if there are some anecdotal reports of maternal mortality due to anaphylaxis, usually allergic reactions are not severe and mainly with maculopapular rashes [11, 13, 14].

On the fetal/neonatal side, there is no risk for anaphylaxis resulting from IAP, but there is a growing concern about the development of antibiotic resistance among GBS isolates and other pathogens. The increased resistance may have two effects: exposure of neonates to antibiotic-resistant pathogens with development of intractable sepsis and reduction of the chance to prevent maternal-fetal transmission by GBS.

Two recent published surveys have demonstrated that in England and France neonatal infections are still mainly

caused by GBS, and the current policy of GBS maternal prophylaxis is not associated with an excessive risk of pathogen resistance [15, 16]. The incidence of early-onset sepsis (EOS) ranged among 0.9 to 1.9/1000 live births, and GBS (58–62%) and *Escherichia coli* (18–25%) were the most common organisms. About the antibiotic resistance, the majority of pathogens (95%) causing EOS were susceptible to commonly used empiric first-line antibiotic combinations.

About the risk of reduced efficacy of IAP for GBS, data are reassuring. Worldwide, there have been only a few reports of penicillin resistance [17, 18] or elevated MIC [19, 20] secondary to the alterations in penicillin-binding proteins (PBP). In the majority of isolates with alteration of PBP, the measured MICs were just at the threshold of susceptibility, but the clinical significance of higher MIC values remains unclear. Elevated MICs to cefazolin also were reported, but as penicillin/ampicillin, the clinical significance of higher MICs to cefazolin among GBS isolates remains unclear [11]. In our experience GBS isolates have not yet developed

any resistance against penicillin and ampicillin and first-generation cephalosporin. This aspect is very reassuring if we consider that these antibiotics are constantly indicated as first choice in women positive for GBS. Their efficacy as IAP was demonstrated for the first time in clinical trials by Boyer and Gotoff [21] in 1986 and by Garland and Fliegner [22] in 1991. On the contrary the efficacy of alternatives to penicillin/ampicillin for allergic women (including cefazolin, clindamycin, erythromycin, and vancomycin) has not been tested in controlled trials. About cephalosporin, it has been supposed that, given the similar activity, pharmacokinetics, and dynamics of cefazolin to penicillin/ampicillin, it could be a second-line antibiotic in penicillin allergic women with low risk of anaphylaxis. As long as allergic women with high risk of anaphylaxis, the guidelines suggest the use of clindamycin/erythromycin or vancomycin although their ability to reach bactericidal levels in the fetal circulation and amniotic fluid are very limited [23–25]. The choice of one or another antibiotics is made on the results of antimicrobial susceptibility testing. These women should receive clindamycin if their GBS isolate is susceptible to clindamycin and erythromycin or if it is resistant to erythromycin but sensitive to clindamycin with negative testing for inducible clindamycin resistance. Otherwise, if susceptibility to both agents is unknown, these women should receive vancomycin. At the moment, erythromycin is no longer considered an alternative for IAP in penicillin-allergic women at high risk for anaphylaxis [11].

Starting from our data, in the next future the problems related with antibiotic resistance will become bigger and bigger and the treatment of allergic women will be a major obstacle. In fact during the study period, the number of colonized women is increased from 85 to 366. If we consider stable the number of allergic women at risk for anaphylaxis, we will have that more and more women will be treated with alternative antibiotics which will be potentially ineffective or will increase the spectrum of resistance.

In reports published, the prevalence of resistance among GBS ranged from 7% to 25% for erythromycin and from 3% to 21% for clindamycin [26, 27]. Resistance to erythromycin was frequently but not always associated with clindamycin resistance. In our series resistance was always to both erythromycin and clindamycin, and the prevalence was considerably higher, ranging from 16.4% to 70% in the study period, showing a statistically significant increment ( $P < 0.05$ ). This finding is very far from previous reports from other nations indicating significant country variations and supporting the usefulness of research about GBS in each population. The increased resistance to macrolides, particularly to erythromycin observed all over the world, can be ascribable to the treatment of *Chlamydia* infections of the lower reproductive tract [28]; however we have no explanation for the higher rate of resistance in our population. An hypothesis is that the variation may be due to differences in techniques as well as characteristics of the population investigated.

The prevalence of GBS-positive women observed in our population is higher even when compared with other Italian studies. In the study of Savoia et al. [29], among 300

pregnant women screened, 73 single-patient GBS isolates were collected and only 3 out of 73 (4.1%) were resistant to erythromycin. Also the lincosamides (lincomycin) were less efficient. Overall the infection prevalence was 18.2% versus 41% observed in our population (879 infected patients out of 2156 patients). In another Italian study by Sensini et al. [6], the prevalence of GBS was even lower (11%). Comparing the numbers, an hypothesis is that the prevalence of infection is growing over the years (11% versus 18.2 versus 41%).

The main limitations of our study are the difference in surveillance population (pregnant and nonpregnant) and, for pregnant women, the lack of clinical data about the pregnancy and neonatal outcome. This aspect could be a starting point for new research since to date whether *in vitro* resistance of GBS has direct clinical implications remains unclear.

## 5. Conclusion

Antibiotics are used for both GBS prevention and treatment. The introduction of IAP for GBS carriers has been associated with a substantial decline in the incidence of early-onset neonatal infections. However, the potential side effect of the protocol is the risk of development of pathogen resistance to antibiotics. Until now GBS isolates remain susceptible to penicillin and ampicillin and first-generation cephalosporin, but resistance to alternative agents as erythromycin and clindamycin is an increasing concern. In fact these agents are suggested in women with high risk of anaphylaxis although their ability to reach bactericidal levels in the fetal circulation and amniotic fluid is very limited. Comparing reports of the literature, epidemiology of infection, and resistance pattern change substantially among countries suggesting the need of local study to map the prevalence of resistant isolates.

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## Research Article

# In Vitro Anti-Candida Activity of Lidocaine and Nitroglycerin: Alone and Combined

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The aim of this work was to study the anti-*Candida* activity of lidocaine and nitroglycerin alone and in combination. Ten *Candida* strains were included, corresponding to 1 collection type strain (ATCC 10231) and 9 clinical isolates: 4 *C. albicans*, 2 *C. glabrata*, 1 *C. tropicalis*, 1 *C. krusei*, and 1 *C. parapsilosis*. The CLSI reference M27-A3 micromethod was used to determine the anti-*Candida* activity of the drugs alone; minimal inhibitory and lethal concentrations were determined. The classic checkboard technique was used to determine the activity of combined drugs. Lidocaine fungicidal effect was dose-dependent. Nitroglycerin exhibited a higher effect. The drugs combination resulted in a reduction of the inhibitory concentration, corresponding to an additive effect. In conclusion, both drugs exhibited an interesting anti-*Candida* activity. The combination of lidocaine with nitroglycerin was shown to have an additive effect against *Candida* spp., predicting the interest to include, in the future, these drugs in a new delivery system for the treatment of mucocutaneous candidosis.

## 1. Introduction

*Candida* spp. are microorganisms frequently found in the human oral cavity, gastrointestinal tract, and vagina [1–4]. Among mucocutaneous infections, vulvovaginal candidosis (VVC) is the second most frequent vaginal infection, after vaginal bacteriosis and is one of the most common clinical diseases caused by *Candida* spp. It affects 70–75% of women at least once in their lifetime while 40–50% of them will experience a recurrence; 5–8% of adult women develop recurrent vulvovaginal candidosis (RVVC), defined as four or more episodes within a year [3–7]. The main goal of VVC treatment is the control and immediate relief of its signs and symptoms, related to vulvovaginal inflammation, as quickly as possible and the mycological cure to be confirmed some days later; recurrence prevention is also pursued [8]. VVC is usually treated very effectively with azoles, which are present in the most prescribed therapeutic regimens,

unless a suspected or confirmed azole-resistant *Candida* strain is involved. On the other hand, most gynecologists believe that the control of RVVC requires both systemic and local therapy, also involving new antifungal drugs and strategies [3, 9, 10]. Despite RVVC being considered a *Candida* infection that is more dependent on the host characteristics, therapeutics approaches available and able to allow a remission of the symptoms between episodes are antifungal drugs used for a long period of time [8, 9, 11]. Some authors consider that new therapeutic strategies must be considered for RVVC control [3, 9, 10]. In addition to the limited number of available antifungal drugs, the restrictions to its use stress the need for the development and validation of new therapeutic strategies exhibiting distinct mechanisms of action and/or evasion of resistance [12–15].

Lidocaine is used as anesthetic, and its anti-*Candida* activity has been previously reported as a fungicidal drug exhibiting a dose-dependent effect and related with a

primary lesion of the cytoplasmic membrane [12]. Also its ability to inhibit *Candida albicans* germ tube formation was reported [16]. Nitroglycerin has been used to treat haemorrhoidal symptoms under an ointment pharmaceutical formulation, commercially available as Rectogesic [17]. An association of lidocaine with isosorbide-di-nitrate has also been proposed for the treatment of anorectal problems [18] on behalf of the expected benefits of combining their individual properties, namely, anaesthetic and blood supply promoter.

As in VVC, irritation leads to excoriations and fissuring [19]. We investigated the *in vitro* anti-*Candida* activity of lidocaine and nitroglycerin, both alone and in combination, in view of its possible future inclusion in a pharmaceutical formulation for the treatment of mucocutaneous candidosis.

## 2. Materials and Methods

**2.1. Chemicals and Drugs.** Pure and analytic grade compounds were used to prepare the tested solutions used in this work. A 6% lidocaine solution was prepared by solubilization of lidocaine cloridrate (Sigma-Aldrich, Portugal) in sterile water. Stock solution of 1% glycerol trinitrate (Merck, Germany) was used to prepare the work solution, meaning 0.5% nitroglycerin in RPMI 1640 culture medium (Biochrom AG, Berlin). Serial concentrations of the products were obtained by geometric dilution in RPMI.

**2.2. Yeast Strains.** A total of 10 *Candida* strains were used, including 5 *C. albicans*, 2 *C. glabrata*, 1 *C. tropicalis*, 1 *C. krusei*, and 1 *C. parapsilosis*. With exception of the type strains *C. albicans* ATCC 10231 from the American Type Culture Collection, all the other strains tested were isolates from patients with RVVC and showed variable degree of resistance to fluconazole (Table 1). Such isolates had been characterized to species level using API ID 32 (BioMérieux, Vercieux, France), and its susceptibility pattern to classic antifungals (fluconazole and amphotericin B) was determined according to the CLSI M27-A3 micromethod. The strains were kept frozen in Brain-Heart Broth (Difco Laboratories, Detroit, MI, USA) with 5% glycerol at  $-70^{\circ}\text{C}$  until testing. After thawing, the strains were subcultured twice on Sabouraud agar (Difco) to assure optimal growth ( $37^{\circ}\text{C}/24\text{ h}$ ).

**2.3. Anti-*Candida* Activity.** The lidocaine and nitroglycerin anti-*Candida* activity was assessed according to the CLSI reference M27-A3 micromethod protocol [20]. Minimal inhibitory concentration (MIC) values were read visually after 48 h of incubation at  $37^{\circ}\text{C}$ . For each tested concentration, yeast growth was compared with the positive control (growth control). Only the total growth inhibition was considered as MIC. All determinations were performed in duplicate, and only concordant results from three independent experiments were considered.

The modified protocol proposed by Canton et al. [21] was used to determine minimal lethal concentrations (MLCs).

**2.4. Anti-*Candida* Activity of Lidocaine Plus Nitroglycerin.** The classical checkerboard methodology, as described by Vitale et al. [22], was used to determine the MIC resulting from the products association. One *C. albicans* ATCC 10231 was included. Briefly, a two-dimensional microplate with 50  $\mu\text{l}$  of each product was prepared; microplates were incubated during 24 h at  $37^{\circ}\text{C}$ .

MIC for products association was calculated from three independent experiences with concordant results. To evaluate the compound interactions, the fractional inhibitory concentration index (FICI) was calculated as follows: (MIC of Drug A in combination/MIC of Drug A alone) + (MIC of Drug B in combination/MIC of Drug B alone). The interpretation of the FICI corresponds to a synergic effect for values  $\leq 0.5$ : additive effect when  $>0.5$  but  $<4.0$  and antagonism when  $\geq 4.0$  [22].

## 3. Results and Discussion

Lidocaine and nitroglycerin exhibited antifungal activity upon *Candida* spp. MIC varied from 10 mg/mL to 30 mg/mL for lidocaine and from 0.15 mg/mL to 0.30 mg/mL for nitroglycerin (Table 2). The antifungal susceptibility pattern of the selected strains to classical antifungals, namely, fluconazole and amphotericin B, was unrelated to the tested compounds activity, predicting distinct mechanisms of action.

Lidocaine fungicidal effect was confirmed for concentrations corresponding to at least double MIC, varying from 15 mg/mL to 30 mg/ml, showing a dose-dependent effect. *C. krusei* and *C. albicans* ATCC10231 were the most susceptible strains to lidocaine (MIC 10 mg/mL). Other *C. albicans* exhibited an increased MIC, 15 mg/mL, similar to *C. parapsilosis*. On the other hand, *C. glabrata* and *C. tropicalis* were the less susceptible, having their growth inhibited by lidocaine at 20 mg/mL. Our results are in accordance with other authors that confirm the higher *C. albicans* susceptibility to this drug [12]. However, some MIC and MLC differences were noticed (onefold dilution). This is probably related to the fact that those authors choose the macromethod from the same protocol that we used for the micromethod.

Regarding nitroglycerin, the effect was fungicidal at concentrations able to inhibit *Candida* growth, being MIC and MLC coincident. *C. albicans* was the most susceptible species tested (0.15 mg/mL). Other species exhibited higher MIC values (0.30 mg/mL), coincident with MLC for *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* AP 426: lower to MLC for *C. glabrata* AP 425 (0.6 mg/mL) and *C. krusei* (1.25 mg/ml).

The possible antifungal advantage of the two products association was studied by the *checkerboard* procedure upon *C. albicans*. A reduction effect was evident for both MIC products (lidocaine alone—7,5 mg/mL, lidocaine in combination—5 mg/mL; nitroglycerin alone—0,15 mg/mL, nitroglycerin in combination—0,075 mg/mL) expressed by the resulting FICI value (1.17) that corresponds to an additive effect ( $>0.5$  and  $<4.0$ ).

TABLE 1: *Candida* spp. origin and susceptible profile to classic antifungals, namely, fluconazole and amphotericin B. Minimal inhibitory concentrations (MICS) are presented in  $\mu\text{g}/\text{mL}$ .

Species	Origin	Fluconazol MIC ( $\mu\text{g}/\text{mL}$ )	Amphotericin B MIC ( $\mu\text{g}/\text{mL}$ )
<i>C. albicans</i> ATCC 10231	Collection	1	0.5
<i>C. albicans</i> AP440	Vaginal	0.25	0.25
<i>C. albicans</i> AP416	Vaginal	<0.125	1
<i>C. albicans</i> AP437	Vaginal	2	0.5
<i>C. albicans</i> AP439	Vaginal	0.5	1
<i>C. glabrata</i> AP426	Vaginal	32	0.25
<i>C. glabrata</i> AP425	Vaginal	32	0.25
<i>C. krusei</i> AP3	Vaginal	>64	0.5
<i>C. tropicalis</i> AP407	Vaginal	2	1
<i>C. parapsilosis</i> AP11	Vaginal	1	0.5

TABLE 2: *Candida* spp. susceptibility to nitroglycerin and lidocaine. Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) are presented in  $\text{mg}/\text{mL}$ .

Species	Nitroglycerin		Lidocaine	
	MIC	MLC	MIC	MLC
<i>C. albicans</i> ATCC 10231	0.15	0.15	10.0	15.0
<i>C. albicans</i> MC440	0.15	0.15	15.0	20.0
<i>C. albicans</i> MC416	0.15	0.15	15.0	20.0
<i>C. albicans</i> MC437	0.15	0.15	15.0	20.0
<i>C. albicans</i> MC439	0.15	0.15	15.0	20.0
<i>C. glabrata</i> MC426	0.30	0.30	20.0	30.0
<i>C. glabrata</i> MC425	0.30	0.625	20.0	30.0
<i>C. krusei</i> OL103	0.30	1.25	10.0	20.0
<i>C. tropicalis</i> MC407	0.30	0.30	20.0	30.0
<i>C. parapsilosis</i> AP11	0.30	0.30	15.0	25.0

The possible pH variation with the addition of both solutions to culture medium was limited by the tampon effect of the RPMI medium. Additionally, the pH value at the drugs most concentrated conditions was measured and confirmed to be between 6.9 and 7.1, guarantying no pH influence on the results.

#### 4. Conclusions

In this study we tested the anti-*Candida* activity of lidocaine and nitroglycerin alone and in combination in view of their use as topical treatment of acute VVC. Nitroglycerin's vasodilator effect may promote healing while at the same time the anesthetic effect of lidocaine may relief pain, burning, and pruritus. Our results show that adding to the well-known therapeutic effects of these two individual drugs an anti-*Candida* activity may also be relevant. The combination of lidocaine with nitroglycerin showed to have an additive effect against *Candida* spp.

In the future, the development of a new delivery system including both lidocaine and nitroglycerin at concentrations higher than MIC values here reported and that allow compounds to produce local effect limiting possible systemic absorption, stands up as an interesting approach for VVC topical treatment. These *in vitro* studies showing their

significant antifungal activity must now be followed by *in vivo* studies in animal models, to evidence the efficacy of this cheap and believed to be safe new strategy.

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## Review Article

# Pregnancy in HIV-Positive Patients: Effects on Vaginal Flora

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A high proportion of HIV-infected pregnant women present pathogenic organisms in their lower genital tract. This has been associated with the development of postpartum morbility, HIV transmission to the partner and offspring, and other gynaecological conditions, such as cervical dysplasia or cancer. Vaginal flora alterations can range from 47% in Western countries to 89% in Africa in pregnant HIV-positive patients, much higher than about 20% of the general population. Pathogen organism retrieval is high. As peripartum complications due to vaginal infections seem higher in HIV-positive patients, accurate investigation and treatment of such infections are strongly mandatory.

## 1. Introduction

The classification system, drawn by the Centers for Disease Control and Prevention (CDC) of HIV infection, includes several gynaecological conditions such as persistent, frequent, or poorly responsive episodes of vaginal candidiasis, from light to severe cervical intraepithelial neoplasia (CIN), pelvic inflammatory disease (PID), chronic herpes simplex virus ulcers, and invasive cervical cancer. Three of these conditions may have a strong impact on pregnancy, namely, lower genital tract neoplasia, sexually transmitted infections and vaginitis [1].

Vaginal infections during pregnancy in HIV-positive patients have been deeply investigated in some recent studies. Preliminary data indicate an association between vaginal infections, and perinatal morbility.

## 2. Materials and Methods

Pertinent international literature was reviewed; however, due to the scarcity of studies on the matter, a systematic review resulted very difficult. We focused our attention especially on HIV-positive pregnant women. All data concerning vaginal

infections during pregnancy in HIV-positive patients were thoroughly analysed.

Data concerning lower genital tract intercurrent pathogens, risks of infection, perinatal complications of vaginal infections, risks of associated conditions, effects on obstetric management, and results of prevention and therapy are discussed.

We report our preliminary findings on 54 pregnant HIV patients. Vaginal cultures were performed during the first and third trimester of pregnancy.

Cultures included tests for chlamydia, mycoplasma gram positive and negative bacteria, candida. A smear was performed for protozoa identification, notably trichomonas.

## 3. Results and Discussion

**3.1. Pathogenic Lower Genital Tract Organisms in HIV Pregnancy.** A study from a high-risk USA population on 854 HIV-infected women and 434 controls reports that the prevalence of bacterial vaginosis was 47% in the HIV-positive women compared with 44% in the HIV-negative women: this difference was not statistically significant ( $P = 0.36$ ) [2]. After adjustment for other covariates, HIV-positive

women were at higher risk of contracting bacterial vaginosis compared to HIV-seronegative women (odds ratio (OR) 1.31; 95% confidence interval (CI) 1.01–1.70) by Gram's stain but not by clinical criteria (OR 1.16; CI 0.87–1.55). Among HIV-positive women, the use of antiretroviral drugs was associated with a lower prevalence of bacterial vaginosis (adjusted OR 0.54; CI 0.38–0.77). These high rates of bacterial vaginosis are similar to the ones reported in three studies carried out on women attending sexually transmitted disease centers (range 12–61%) but are generally higher compared to healthy pregnant women (range 10–32%).

A study conducted in Cameroon compares first trimester screening cultures from 198 HIV-positive pregnant patients to 1810 controls [1]. All lower genital tract infections, except candidiasis, were more prevalent among HIV-positive compared to HIV-negative women: vaginal candidiasis (36.9% versus 35.4%;  $P = 0.678$ ), Trichomoniasis (21.2% versus 10.6%;  $P < 0.001$ ), gonorrhoea (10.1% versus 2.5%;  $P < 0.001$ ), bacterial vaginosis (21.2% versus 15.2%;  $P = 0.026$ ), syphilis (35.9% versus 10.6%;  $P < 0.001$ ), and *Chlamydia trachomatis* (38.4% versus 7.1%;  $P < 0.001$ ).

Leroy et al. reported a similar prevalence of vaginal candidiasis among HIV-infected pregnant women (22.3%) and noninfected pregnant women (20.1%) [3].

In South Africa a recent study on 418 HIV-infected and 383 uninfected women delivered vaginally reports that 54.8% of women had positive cultures at birth (439/801), more among HIV-infected patients compared to the uninfected ones (60% versus 49.1%,  $P = 0.002$ ) [4].

According to Joao et al., the overall anogenital prevalence of GBS colonization was 49/158 (31.0%) in a cohort of HIV-positive pregnant women during the third trimester of pregnancy [5].

Genital tract infections such as *Neisseria Gonorrhoea*, *Chlamydia trachomatis*, *Candida albicans*, and *Trichomonas vaginalis* infection have been reported to be more common in HIV-infected women (WHO/RHT/98.24 (World Health Organization), UNAIDS/98.44 (United Nations Program on HIV/AIDS)). African studies report a higher incidence of syphilis in HIV-positive women. Concurrent infection with syphilis was shown in 33% of HIV-positive pregnant patients in South Africa, three times higher than the rate in HIV-seronegative women.

**3.2. Reasons for Common Vaginal Infections in HIV Patients.** The prevalence of vaginal candidiasis in HIV-infected women depends on CD4 count. Burns et al. reported a 3-fold increase in vaginal candidiasis among HIV-infected women with low CD4 counts compared to HIV-infected women with normal CD4 count during pregnancy [6]. Apart from *Candida spp.*, we reported an increase in the prevalence of bacterial vaginosis among HIV pregnant women. Bacterial vaginosis increases susceptibility to HIV infection and other genital tract pathogens, but it is not clear whether HIV increases the risk of developing bacterial vaginosis [7].

Potential biological mechanisms for this inter-relationship of sexually transmitted diseases (STDs), vaginal infection and HIV, include increased shedding of the virus in

genital fluids, recruitment of HIV target cells or HIV-infected cells into the genital tract as part of the inflammatory process, stimulation of immune response to an STD causing increased viral replication, and disruption of protective epithelial barriers [8]. Direct evidence on the association of STDs with HIV was also provided by a large randomized community-based trial in Tanzania which showed that a better targeted treatment of STDs can lower HIV incidence by about 40%.

Depletion of lactobacilli may limit the production of hydrogen peroxide. It has been postulated that low vaginal pH inhibits CD4 lymphocyte activation and reduces HIV target cells in the vagina. Elevated vaginal pH may enhance HIV adherence to vaginal eukaryotic cells.

**3.3. Risk of Vaginal Infection in HIV Patients.** According to Mbu et al. [1], there is evidence that the severity of preinvasive cervical lesion is related to the degree of immunosuppression, suggesting that it contributes (at least in part) to the risk of developing preinvasive lesions [9, 10]. Some studies suggest that the high prevalence of preinvasive cervical lesions may be related to concomitant risk factors related to the mode of HIV transmission [11]. Ahr et al. reported that HIV-positive women with low CD4 count had a higher prevalence of human papilloma virus (HPV) and that preinvasive and invasive cervical lesions of the cervix were more frequent in women with HPV [12], recognized to be a causative agent for such lesions.

**3.4. Perinatal Complications of Vaginal Infections.** As reported in several studies carried out in South Africa, vaginal pathogens were more common in HIV-infected women. Women with positive cultures had slightly higher rates of infectious morbidity than those negative cultures (20.5% versus 15.2%,  $P = 0.052$ ). *Trichomonas vaginalis* and *Group B Streptococcus* were significantly associated with sepsis ( $P = 0.023$  and  $P < 0.001$ , resp.), whereas the presence of *Candida* species seemed to be protective (relative risk (RR) 0.69,  $P = 0.014$ ).

Women with positive cultures had slightly higher rates of infectious morbidity than those without (20.5% versus 15.2%,  $P = 0.052$ ). *Trichomonas vaginalis* and *Group B Streptococcus* were significantly associated with sepsis [4].

In the past bacterial vaginosis had been considered strongly related to perinatal complications [8]. A meta-analysis reported a nonsignificant association of the treatment of bacterial vaginosis in pregnancy with the reduction in perinatal mortality [13]. In their review Kenyon et al. studied the use of antibiotics for preterm premature rupture of the membrane (pPROM) and underlined a statistically significant 43% reduction in risk of chorioamnionitis (RR = 0.57; 95% CI: 0.37–0.86) [14]. However, there was no impact on perinatal mortality or fetal death before discharge (RR = 0.90; 95% CI: 0.74–1.10). Another Cochrane review by Flenady and King on antibiotics for PROM at or near term showed no impact of antibiotics on chorioamnionitis

(RR = 0.60; 95% CI: 0.30–1.18) nor on perinatal mortality (RR = 0.98; 95% CI: 0.14–6.89) [15].

**3.5. HPV Infection in HIV-Positive Pregnant Patients.** Pap smears in an African population show that HIV-positive women are more likely to have preinvasive cervical lesions: low-grade squamous intraepithelial lesion (SIL) (18.2% versus 4.4%;  $P < 0.001$ ) and high-grade squamous intraepithelial lesion (12.1% versus 1.5%;  $P < 0.001$ ) [1]. This is an 8-fold increase in high-grade SIL among HIV-infected pregnant women compared to their noninfected counterparts. A 5-fold increase in cervical intraepithelial neoplasia among HIV-infected women was described in previous studies [16].

**3.6. Maternal HIV Seroconversion and Vaginal Infection.** A study by Taha on 1196 pregnant women, followed antenatally for a median of 3.4 months, reports that 27 women seroconverted by the time of delivery [17]. Postnatally, 97 seroconversions occurred among 1169 seronegative women who were followed for a median of 2.5 years. Bacterial vaginosis was significantly associated with antenatal HIV seroconversion (adjusted OR = 3.7) and postnatal HIV seroconversion (adjusted OR = 2.3). The approximate attributable risk of bacterial vaginosis alone was 23% for antenatal HIV seroconversions and 14% for postnatal seroconversions. The same study confirms the findings of earlier cross-sectional studies which reported associations between bacterial vaginosis and HIV among sex workers in Thailand, rural women in the Rakai district of Uganda, and urban women in Malawi. The temporal nature of such associations in this study strengthens the inference that the relationship between bacterial vaginosis and HIV acquisition could be causal. *Gonorrhoea*, *Syphilis*, and *Trichomoniasis* were significantly associated with HIV seroconversion either in univariate or multivariate models. *Gonorrhoea* and *Syphilis* showed large relative risks, although their incidence and prevalence rates were not as high as those of bacterial vaginosis or *Trichomoniasis*, the latter being associated with a 2–6-fold increase in risk of HIV transmission [18].

**3.7. Treatment and Perinatal Benefits.** The use of antiseptic or antiviral agents to cleanse the birth canal during labour and delivery has been hypothesised as a possible approach to reducing intrapartum transmission of HIV (WHO/RHT/98.24 UNAIDS/98.44). The efficacy of the use of chlorhexidine lavage to reduce the transmission of *Group B Streptococcus* was demonstrated in Scandinavian studies. A Malawian quasirandomised study compared four-hourly aqueous chlorhexidine 0.25% solution by vaginal swabbing after vaginal examinations and a chlorhexidine wash for the baby, with a control group receiving no wash. No overall reduction was shown in the rate of HIV transmission in the above study group. There was a significant reduction in transmission in mothers who had ruptured membranes for more than four hours. Significant reductions in neonatal and puerperal sepsis were also seen following this intervention. *Benzalkonium Chloride* has been suggested as an alternative antiseptic agent.

## 4. Conclusions

It is not clear whether HIV infection increases the risk of acquisition of vaginal infections, but these conditions are common among sexually active women. Sexually transmitted infections (STIs) and preinvasive cervical lesions were found to be more prevalent among HIV-infected pregnant women compared to their noninfected counterparts [1]. Even comparing African and USA cohorts, HIV pregnant patients present a higher prevalence of vaginal infections compared to HIV seronegative. Women with positive vaginal cultures had slightly higher rates of infectious morbidity than those without (20.5% versus 15.2%,  $P = 0.052$ ). *Trichomonas vaginalis* and *Group B Streptococcus* were significantly associated with sepsis. There is some evidence of possible benefits due to the identification and treatment of vaginal intercurrent infection in HIV-positive patients. Perinatal mortality has not been affected, but some reduction in amnionitis in case of membrane rupture has been demonstrated [13].

According to WHO guidelines, we believe that HIV-positive women should undergo a full physical examination at the first visit.

Particular attention has to be paid to any signs of vaginal thrush or lymphadenopathy. Clinical diagnosis and treatment of vaginal or cervical inflammation, abnormal discharge or STD, should be a priority. A cervical smear has to be performed if this has not been undertaken within the recent past. Colposcopy should be reserved for women who have an abnormal cervical smear result.

Prolonged rupture of membranes should be avoided, as mother-to-child transmission increases where membranes are ruptured for more than four hours [19]. Artificial rupture of membranes should not be performed if progress of labour is adequate. As a general rule, any procedure which breaks the baby's skin or increases the baby's contact with the mother's blood—such as scalp electrodes or scalp blood sampling—should be avoided unless absolutely necessary. Episiotomy should not be performed routinely, but reserved for those cases with an obstetrical indication.

Forceps may be preferable to vacuum extraction, given the risk of microlacerations of the scalp from the vacuum cup. There is an increasing evidence that elective caesarean section may help prevent HIV transmission to the newborn [20]. The operation carries risks of maternal complications and is associated with higher postoperative morbidity in HIV-positive women [21]. Prophylactic antibiotics should be administered for both elective and emergency caesarean sections.

The postpartum care of HIV-positive women should be similar to that for uninfected patients.

HIV-positive women are more prone to postpartum infectious complications, including urinary tract, chest, episiotomy, and caesarean section wound infections. All mothers should be given instructions on perineal care and the safe handling of lochia and blood-stained sanitary pads or materials.

Mothers should be counselled on the need for follow-up care, and advices on contraception systems should be given.

On the basis of our preliminary observations, 15 out of our 54 pregnant HIV-patients showed clinical signs of vaginitis which was far in excess of the rate in our control population; a third of the HIV patients developed clinical vaginitis versus a 5% expected rate in our general population.

Common candida was by far the most represented pathogen present in almost all cases as a principal or associated agent.

As a result of our investigation, we can state that in our population an abnormal vaginal flora in a pregnant HIV-patient is a very common occurrence. Therefore, a strict screening is mandatory. Our aim is to provide full breakdown of our experience in the near future.

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## Review Article

# Herpes Simplex Virus Infection in Pregnancy

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Infection with herpes simplex is one of the most common sexually transmitted infections. Because the infection is common in women of reproductive age it can be contracted and transmitted to the fetus during pregnancy and the newborn. Herpes simplex virus is an important cause of neonatal infection, which can lead to death or long-term disabilities. Rarely in the uterus, it occurs frequently during the transmission delivery. The greatest risk of transmission to the fetus and the newborn occurs in case of an initial maternal infection contracted in the second half of pregnancy. The risk of transmission of maternal-fetal-neonatal herpes simplex can be decreased by performing a treatment with antiviral drugs or resorting to a caesarean section in some specific cases. The purpose of this paper is to provide recommendations on management of herpes simplex infections in pregnancy and strategies to prevent transmission from mother to fetus.

## 1. Introduction

Herpes simplex virus (HSV) is an ubiquitous, enveloped, and doublestranded DNA virus, belonging to the family of Herpesviridae transmitted across mucosal membranes and nonintact skin, that migrate to nerve tissues, where they persist in a latent state. HSV-1 predominates in orofacial lesions, and it is typically found in the trigeminal ganglia, whereas HSV-2 is most commonly found in the lumbosacral ganglia [1]. Nevertheless these viruses can infect both orofacial areas and the genital tract. In some developed countries type 1 has recently emerged as the prominent causative agent in genital lesions. Changes in sexual behaviours of young adults may partly explain its higher incidence [2, 3].

A first primary infection develops when a susceptible person (lacking of preexisting HSV-1 and HSV-2 antibodies) is exposed to HSV.

Indeed, a first nonprimary episode occurs when a person with preexisting HSV antibodies (against type 1 or 2) experiences a first episode with the opposite HSV type.

Recurrent infection occurs in a person with preexisting antibodies against the same HSV type [1]. Infections during pregnancy may be transmitted to newborns: HSV-1 and

HSV-2 may cause eye or skin lesions, meningoencephalitis, disseminated infections, or foetal malformations.

## 2. Epidemiology

In recent years, genital herpes has become an increasing common sexually transmitted infection. From the late 1970s, HSV-2 seroprevalence has increased by 30%, resulting that one out of five adults is infected [4, 5].

HSV seroprevalence in patients with STD varies from 17% to 40% (6% in the general population and 14% in pregnant women) [6, 7].

Age and sex are important risk factors associated with the acquisition of genital HSV-2 infection. In fact, the prevalence of HSV infection rises with age, reaching the maximum around 40 years [4]. This infection appears related to the number of sexual partners, and regarding sex it is more frequent in women than in men [8, 9].

In addition, ethnicity, poverty, cocaine abuse, earlier onset of sexual activity, sexual behavior, and bacterial vaginosis can facilitate a woman's risk of infection before pregnancy [10, 11].

Regarding pregnant population, there is a high prevalence of genital herpes. Among Italian pregnant women, the seroprevalence varies from 7.6% to 8.4% seroprevalence [9]. Nevertheless it is lower than that reported among pregnant women in other countries. For example, in US, approximately 22% of pregnant women are infected with HSV-2, and 2% of women acquire genital herpes during pregnancy, placing their newborn at risk for herpes infection. In Italy, the number of women who acquire HSV infection during pregnancy is about 3%. The acquisition of genital herpes during pregnancy has been associated with spontaneous abortion, intrauterine growth retardation, preterm labour, and congenital and neonatal herpes infections [12–14].

The risk of neonatal infection varies from 30% to 50% for HSV infections that onset in late pregnancy (last trimester), whereas early pregnancy infection carries a risk of about 1%. When primary HSV infection occurs during late pregnancy, there is not adequate time to develop antibodies needed to suppress viral replication before labour. About 85% of perinatal transmission occurs during the intrapartum period while transmission of HSV from mother to foetus during pregnancy is less common. Moreover, studies in HIV-infected pregnant women show that coinfection with HSV increases significantly the risk of perinatal HIV transmission above all in women who had a clinical diagnosis of genital herpes during pregnancy [15–17].

The newborn could be also infected by HSV-1, that may represent almost one-third of all new genital HSV diagnoses.

### **3. Diagnosis**

Primary symptomatic genital herpes, that occurs after an incubation of a period of 2–20 days, lasts up to 21 days [4, 18]. Within women it causes blistering and ulceration of the external genitalia and cervix leading to vulval pain, dysuria, vaginal discharge, and local lymphadenopathy [18]. Vesicular and ulcerative lesions of the internal thigh, buttocks, perineum or in perianal skin are also observed. Both in man and in woman primary infection may be complicated by systemic symptoms such as fever, headache, myalgia (38% in men, 68% in women), and occasional meningitis and by autonomic neuropathy resulting in urinary retention, mainly in women [9, 11].

All suspected herpes virus infections should be confirmed through viral or serological testing. A diagnosis of genital herpes based on the clinical presentation alone has a sensitivity of 40% and specificity of 99% and a false-positive rate of 20% [19].

The tests used to confirm the presence of HSV infection can be divided into two basic groups: (1) viral detection techniques and (2) antibody detection techniques. Primary viral DNA testing techniques are viral culture and HSV antigen detection by polymerase chain reaction (PCR). The antibody detection techniques include the use of both laboratory-based and point-of-care serologic tests to detect the presence of antibodies to either HSV-1 or HSV-2. With viral detection techniques, negative results do not rule out the presence of infection [20]. The diagnosis of HSV should be confirmed

either serologically or with viral culture. Isolation of HSV in cell culture is the preferred virologic test for patients who seek medical treatment for genital ulcers or other mucocutaneous lesions and allows differentiation of the type of virus (HSV-1 versus HSV-2) [21]. The sensitivity of this test is limited because of several issues related to sampling and transportation of the specimen. Additionally, as the lesions heal, they are less likely to be culture positive [21]. Thus, a positive genital culture provides conclusive evidence of genital HSV infection; however, a negative result does not exclude the presence of infection. Polymerase chain reaction techniques involve the amplification of particular sequences of DNA or RNA before detection and can thus detect evidence of viral DNA at low concentrations. In one very large study, PCR results were three to five times more likely to be positive than were cultures. Cultures were more likely to be positive at increasing concentrations of virus. Polymerase chain reaction techniques are commercially available and can differentiate between HSV-1 and HSV-2. Polymerase chain reaction provides increased sensitivity over culture and may ultimately replace culture as the standard of care for diagnosis [22].

At the first prenatal visit also the partner history should be investigated. In case of positive history in the male partner, he should be strongly advised to have no oral and sexual intercourse at the time of recurrence in order to avoid infection (in particular during the third trimester of gestation). Moreover, use of condoms throughout pregnancy should be recommended to minimize the risk of viral acquisition, although the male partner has no active lesions [23].

### **4. Congenital and Neonatal Infection**

It is necessary to distinguish between congenital infection and neonatal infection with HSV. In fact, HSV infection of the newborn can be acquired during pregnancy, intrapartum and postnatally. The mother is the most common source of infection for the first two routes of viral transmission. Congenital infection is very rare due to the acquisition of the virus in utero; it comes to the neonatal HSV infection when the appearances of the lesions are more than 48 hours after birth [24, 25].

Intrauterine HSV infection accounts for 5% of HSV infections in neonates. The highest risk of intrauterine infection has been observed in pregnant (about 50%) who develop disseminated HSV infections and 90% of those are related to HSV-2. Both primary and recurrent maternal infection can result in congenital disease, even if the risk after recurrent infection is small.

Intrauterine viral transmission is highest during the first 20 weeks of gestation leading to abortion, stillbirth, and congenital anomalies. The perinatal mortality is 50% [24].

In 85–90% of neonatal HSV infections, HSV is acquired at the time of delivery and 5–10% are caused by early postnatal viral acquisition. A percentage of 70–85% of neonatal HSV infections are caused by HSV-2, whereas the remaining cases are due to HSV-1. The HSV-2 infection carries a graver prognosis than that caused by HSV-1 [26].

The disease transmission to the newborn is dependent on the type of maternal genital infection at the time of delivery.

In fact, neonatal herpes is much more frequent (50%) in babies from mothers with a primary HSV infection with respect to babies from mothers with recurrent HSV infection (<3%). However, most neonatal HSV infections (about 70%) result from exposure to asymptomatic genital HSV infection in the mother near delivery [27].

The prolonged rupture of membranes is a risk factor for acquisition of neonatal infection [28]. Congenital intrauterine infection is characterized by skin vesicles or scarring, eye lesions (chorioretinitis, microphthalmia, and cataract), neurologic damage (intracranial calcifications, microcephaly, seizures, and encephalomalacia), growth retardation, and psychomotor development. Infants infected intrapartum or postnatally by HSV can be divided into three major categories:

- (1) HSV disease localized to the skin, eye, and/or mouth (SEM); this syndrome is associated with a low mortality but it has a significant morbidity, and it may progress to encephalitis or disseminated disease if left untreated;
- (2) HSV encephalitis with or without skin, eye, and/or mouth involvement which causes neurologic morbidity among the majority of survivors;
- (3) disseminated HSV which manifests as severe multi-organ dysfunction (including central nervous system, liver, lung, brain, adrenals, skin, eye, and/or mouth) and has a mortality risk that exceeds 80% in absence of therapy [27, 28].

At diagnosis, symptoms are found with the following frequency: skin vesicles 68%, fever 39%, lethargy 38%, seizures 27%, conjunctivitis 19%, pneumonia 13%, and disseminated intravascular coagulation 11%. Symptoms may occasionally be present at birth, but occur in 60% later than 5 days after birth and sometimes are present after 4–6 weeks of life [21].

Localized infections have been found in 50% of the affected neonates, involvement of the central nervous system (CNS) in 33%, and disseminated infections in 17% of the cases [19, 23]. Several studies have demonstrated that disseminated HSV infections are characterized mainly by liver and adrenals failure associated with shock symptoms and disseminated intravascular coagulopathy [29–31]. Other symptoms of HSV disseminated infection include irritability, seizures, respiratory distress, jaundice, and frequently the characteristic vesicular exanthem that is often considered pathognomonic for infection. However, over 20% of infants with disseminated infection do not develop skin vesicles during the course of their illness. Encephalitis appears to be a common component of this infection form, occurring in about 60–75% of infants with disseminated HSV infection.

Mortality in the absence of therapy exceeds 80% [29]. The prognosis of infants with disseminated HSV disease or neurological manifestations is poor. The mortality in cases with neurological involvement by about 5% with 50% of children with neurological sequelae, while in cases with

multiorgan involvement mortality, is 30% and the percentage of sequelae of 20% [28, 32].

## **5. Management of First Infection with HSV in Pregnancy**

In 2008, the Society of Obstetricians and Gynaecologists of Canada published guidelines on the management of HSV in pregnancy [33].

The risk of infection to the infant appears to be higher when the first infection occurs during the third trimester of pregnancy. In this case there may not be sufficient time for the development of maternal IgG and their passage to the fetus, and the risk of neonatal infection is 30 to 50% [34].

If infection occurs in the first trimester of pregnancy, this seems to be linked to an increase in spontaneous abortions and cases of intrauterine fetal growth restriction. Only in rare cases there is the transmission of the virus transplacentally, resulting in a very severe congenital infection that can occur with microcephaly, hepatosplenomegaly, intrauterine fetal death, and IUGR. The use of antivirals is also permitted in the first trimester of pregnancy if the mother's injuries are particularly serious. At the moment there are enough data to define acyclovir safe to use during pregnancy [35].

When primary infection is acquired during the first two trimesters of pregnancy, it is advisable to carry out sequential viral cultures on genital secretions from 32th week of gestation [36]. Both viral culture and the nucleic acid amplification tests (NAATs) are considered as a test of choice for symptomatic patients. As in Western Europe and the United States, there are no comprehensively validated and approved commercial NAATs available for detection of HSV in many eastern European countries. However, some NAATs for HSV detection have been developed and are available in Eastern Europe, but have not been validated against their internationally acknowledged analogues.

However, if two consecutive cultures result negative and there are no active herpetic genital lesions at the time of delivery, it is possible to perform a vaginal delivery. If seroconversion is completed at the time of delivery, caesarean section is not required since the risk of HSV transmission to the foetus is low, and the neonate should be protected by maternal antibodies.

If primary genital infection is acquired during the third trimester of pregnancy, the optimal way of proceeding is not well defined. Most guidelines propose caesarean section for women developing a primary clinical infection within the last 4–6 weeks of gestation, because they cannot complete their seroconversion prior to the time of delivery, and therefore they could infect the neonates. When vaginal delivery is irreversible, since the risk of vertical transmission is high (41%), a maternal and neonatal intravenous acyclovir therapy is recommended [37–39].

## **6. Management of Infection Recurrent HSV in Pregnancy**

A pregnant woman with HSV lesion that has already presented a first infection in the past has circulating IgG,

TABLE 1: Recommended doses of antiviral medications for herpes in pregnancy [20].

Pregnancy	Antiviral drug	First episode			Recurrent episodes		
		Recommended daily dosage	Length of therapy	Antiviral drug	Recommended daily dosage	Length of therapy	
Episodic treatment	Acyclovir	Orally: 5 × 200 mg	10 days	Acyclovir	Orally: 5 × 200 mg	5 days	
	Valacyclovir	Orally: 2 × 500 mg	10 days	Valacyclovir	Orally: 2 × 500 mg	5 days	
Suppressive treatment	Acyclovir	Orally: 3 × 400 mg		Acyclovir	Orally: 3 × 400 mg		
	Valacyclovir	Orally: 2 × 250 mg	From week 36 until delivery	Valacyclovir	Orally: 2 × 250 mg	From week 36 until delivery	

which are then able to pass the placenta and reach the fetus. It is so unusual that the fetus develops the infection with HSV. If the lesion is present in genital skin during delivery, the risk of infection for the baby will be 2–5% [28].

Instead, a woman with periodic reactivations of the virus and asymptomatic at birth has a low risk (1%) to eliminate the virus with vaginal secretions, so the risk of fetal infection is even lower (0.02–0.05%) [28].

Randomized trials showed that the use of antiviral drugs from the 36th week of pregnancy reduces the risk of spreading of the virus in the absence of clinically visible lesions and the risk of viral reactivation with decreased percentage of caesarean sections [21].

The use of antiviral drugs is allowed before the 36th week in case of very serious events in the mother, or if there is an increased risk of preterm delivery.

The therapy includes the administration of acyclovir 400 mg tablets 3 times daily or acyclovir 200 mg tablets 4 times a day from week 36 until delivery, and viral cultures on cervical-vaginal secretions from 36th week of gestation are required. Recent studies also suggest the use of valacyclovir at a dose of 200 mg 2 times a day.

In absence of clinical herpes lesions but with positive viral cultures at delivery, caesarean section is recommended. On the contrary, if all viral cultures are negative, in the absence of clinical lesions, a spontaneous delivery is indicated.

Finally, in presence of clinical genital HSV lesions at the onset of delivery, if it may be assumed that the foetal lungs are mature, a caesarean section should be performed as quickly as possible within 4–6 hours after membranes rupture [20, 21, 34].

## 7. Therapy

Pregnant women with a first clinical episode or a recurrence may be treated with acyclovir or valacyclovir at the recommended dosages (Table 1). Since acyclovir and valacyclovir are not officially approved for treatment of pregnant women, patients should be informed to give consent before the administration [20]. However, no increase of foetal abnormalities was ascribed to these treatments, although long-term outcomes were not evaluated [20].

Treatment with acyclovir and valacyclovir by 36 weeks of pregnancy to term reduces the frequency of clinical manifestations, vertical transmission, elimination of the virus during birth by reducing the percentage of caesarean (Table 1) [28].

## 8. Conclusions

Genital herpes is a preventable chronic disease. Although most HSV infections are subclinical, clinical disease can be associated with substantial physical and psychosocial morbidity. The clinical manifestations are diverse; hence a suspected diagnosis of HSV should be confirmed by laboratory tests. The management of genital herpes should be tailored to the individual and should include counselling about the variable natural history appearance of lesions, education about prevention of transmission, the link between HSV and HIV, and discussion to assess the psychosexual effects of the disease. Antiviral therapy is safe and effective, both for episodic treatment and chronic suppression of HSV.

A large amount of information on the transmission of herpes from male to pregnant partner, on the mode of transmission from mother to newborn, mainly by maternal first-time infection in the third trimester of pregnancy, has been published in literature.

Since the increasing prevalence of genital HSV infection and apparent increase in the incidence of neonatal herpes, we have focused our attention on prevention of maternal foetal transmission as well as on the management of infected pregnant women and neonate. Further studies are needed to monitor the changing HSV-1 and HSV-2 trends and to develop effective strategies to prevent HSV infection. Finally, the major vaccine strategies under development should take into account the three important features of herpesviruses: the viral latency, the herpes immune escape, and the high seroprevalence.

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