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SHORT COMMUNICATION

The presence of *Trichomonas vaginalis* in urogenital samples can affect the sensitivity of *Mycoplasma hominis* identification techniques, leading to an underestimation of bacterial infections

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SUMMARY

Trichomonas vaginalis and *Mycoplasma hominis*, two microorganisms causing infections of the urogenital tract, are closely associated in that they establish an endosymbiosis relationship, the only case among human pathogens. As a result, the presence of one microorganism may be considered a sign that the other is present as well. Identification of the two pathogens in clinical samples is based on cultivation techniques on specific media, even though in recent years, new sensitive and rapid molecular techniques have become. Here, we demonstrate that the concomitant presence of *T.vaginalis* in urogenital swabs may lead to a delay in the identification of *M.hominis*, and thus to an underestimation of bacterial infections when cultural techniques are used.

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Mycoplasma hominis is one of the smallest self-replicating organisms, characterized by the absence of a rigid cell wall and an extremely small genome (Taylor-Robinson *et al.*, 2017). Although this bacterium can be isolated in vaginal samples of healthy women as a commensal microorganism, its presence is also correlated with genital infections such as pelvic inflammatory disease, contributing to dysbiosis in bacterial vaginosis (Abou Chacra *et al.*, 2022; Yagur *et al.*, 2021). Moreover, during pregnancy, *M.hominis* can be transmitted to amniotic fluid and fetal membranes, leading to severe post-partum complications (low birth weight, preterm delivery, premature rupture of membranes and subsequent abortion) (Che G, Liu *et al.*, 2023). It is also responsible for infections in newborns (neonatal sepsis and meningitis) (Allen-Daniels *et al.*, 2015). *M. hominis* has been isolated in 40% of amniotic fluid infections (Murtha *et al.*, 2014). Interestingly, *M.hominis* genital infections have also been implicated in male infertility, affecting both quality and quantity of semen (Dehghan *et al.*, 2022; Jamalizadeh Bahaabadi *et al.*, 2014; Nazarzadeh *et al.*, 2022). Finally, chronic infection caused by this microorganism may promote aggressive cervical and prostate cancer (Barykova *et al.*, 2011) and induce DNA damage and cell death in human cells (Amorim *et al.*, 2022).

An intriguing feature of *M.hominis* is its ability to establish an endosymbiotic relationship with *Trichomonas vaginalis*, a protozoan responsible for the most prevalent nonviral sexually transmitted infection in humans, associated with adverse pregnancy outcomes (Fettweis *et al.*, 2019) and with infertility in both sexes (Zhang *et al.*, 2022). This endosymbiotic association represents the first case of symbiosis described involving two obligate human pathogens; both microorganisms can independently induce a genitourinary infection, and their association has been shown to deeply modulate their physio-pathogenicity (Hirt and Sherrard, 2015; Margarita *et al.*, 2016, 2022). The analysis of clinical isolates of *T.vaginalis* demonstrates that the prevalence of protists infected by *M.hominis* ranges from 5 to 89%, regardless of the geographic origin (Margarita *et al.*, 2020).

Since a very high number of *T.vaginalis* strains are stably infected by *M.hominis*, the acquisition of trichomonad infection can imply the simultaneous transmission of *M.hominis*. Similarly, the isolation of *M.hominis* in a urogenital sample could indicate the presence of the protozoan. The identification of both microorganisms from clinical samples can involve a sensitive gene amplification assay (Caza *et al.*, 2023; Leli *et al.*, 2018; Karellis *et al.*, 2022), but the major

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limitation of the molecular techniques is its inability to isolate microorganisms to assess antibiotic sensitivity patterns. Furthermore, molecular diagnostic platforms are still underutilized in clinical microbiology laboratories in developing countries due to high operational costs. Indeed, the gold-standard method for the identification of *T.vaginalis* in clinical samples is based on microscopic observation of wet mount slides and on protozoan cultivation in liquid media. Because of lower sensitivity, especially for wet mount slides, and the particularly long turnaround times of cultural techniques compared to the highly sensitive NAATs, the latter are replacing cultural techniques. The laboratory cultural diagnosis of genital *M.hominis* infection, on the other hand, is based on the use of selective liquid media containing 10 mM arginine (e.g., Hayflick medium) (Pereyre *et al*., 2009). Bacterial growth results in a rise in environmental pH via the arginine dihydrolase (ADH) pathway to generate ATP (Pereyre *et al*., 2009; Kleydman *et al*., 2004), and is detectable because of the color change of the medium. Although less sensitive than molecular techniques, bacterial cultivation is useful to confirm *M.hominis* presence by observing the typical fried-egg appearance colonies on solid media and setting up the antibiotic susceptibility test (Capoccia *et al*., 2013; Taylor-Robinson *et al*., 2017). While the search for *M.hominis* is not recommended in some types of asymptomatic patients, such as the group of males who have sex with males, the identification of this bacterium and the determination of antibiotic susceptibility in samples from pregnant women and in cases of infertility are of particular importance (Horner *et al*., 2018). Given the high frequency of *T.vaginalis/M. hominis* association and the fact that *T.vaginalis* produces acid metabolites during *in vivo* and *in vitro* growth, we hypothesized that the presence of metabolically active protozoa in clinical samples could interfere with the color reaction due to ammonia production by bacteria in Mycoplasma-specific media.

To demonstrate this, serial dilutions of *T.vaginalis* G3 strain, naturally mycoplasma-free, ranging from 10^4 to 10 cell/ml, were incubated for 48 hours in commercial media commonly used for identification of genital mycoplasmas (Mycoplasma IES, Autobio, Italy, pH 6.3±0.3). The viability of protozoa was observed microscopically, and pH variations were reported. As control, a total of 10^4 *M.hominis* quantified by using qPCR as described (Margarita *et al*., 2022) were inoculated in the same media. Microscopic observation showed that the protozoa remain viable and motile even after 48 hours, although they were unable to multiply, and the pH of the medium dropped to 5.0. As expected, when a total of 10^4 free *M.hominis* were inoculated in the same media, a color change was evident as early as the first 24 hours of incubation (final pH was 8.0).

To further investigate, we inoculated decreasing amounts of *T.vaginalis* reference strain G3, experimentally infected with *M.hominis*, in the same liquid media for identification of genital Mycoplasma. G3 isogenic *M.hominis*-stably infected *T.vaginalis* were obtained as previously described (Margarita *et al*., 2016, 2022); we used isogenic strains in order to avoid strain-to-strain differences in clinical microbial isolates. The Multiplicity of Infection (MOI) of bacteria was 15 *M.hominis*: 1 *T.vaginalis*, as confirmed by using specific qPCR techniques (Margarita *et al*., 2016, 2022). The final concentration of protozoa in the media started from 10^2 *T.vaginalis*/ml up to 10^5 *T.vaginalis*/ml. After 24 hours of incubation, the color change indicating the presence of viable *M.hominis* was detectable only in samples with the lowest number of *T.vaginalis* (starting from a concentration of 10^2 cells/ml). Under these conditions, the concentration of the protozoa is too low to be able to change the pH, while the bacteria can multiply and lower the pH of the medium (*Table 1 A*).

To confirm the results obtained, two separate vaginal samples were taken from three healthy women. The swabs (E-swab, Copan, Italy) were divided in two groups: the first group was experimentally infected with a total of 10^4 G3 *T.vaginalis* parasitized by *M.hominis* (MOI, 15 bacteria/protist); the second group was infected with the corresponding amount of free *M.hominis* (i.e., $1.5x10^5$). The vaginal swabs were separately inoculated in Mycoplasma selective media, as previously described. After a 24 hours of incubation, only the samples positivized by free *M.hominis* (without *T.vaginalis*) showed the color change, while the presence of the same amount of *M.hominis* symbiotically associated with *T.vaginalis* needed 24 additional hours of incubation to demonstrate bacterial growth (*Table 1 B*).

These results suggest that the presence of *T.vaginalis* in genital specimens can mask the simultaneous exis-

Table 1A - *Color change of the medium after 24 and 48 hours of incubation with M. hominis and T. vaginalis alone, and by different concentrations of M. hominis associated with T. vaginalis.*

Incubation	*M.hominis* 10^4/ml	*T.vaginalis* 10^4/ml	*T.vaginalis* 10^2/ml *M.hominis* $5x10^3$/ml	*T.vaginalis* 10^3/ml *M.hominis* $5x10^4$/ml	*T.vaginalis* 10^4/ml *M.hominis* $5x10^5$/ml	*T.vaginalis* 10^5/ml *M.hominis* $5x10^6$/ml
24 h	+	–	+	–	–	–
48 h	+	–	+	+	+	+

Table 1B - *Color change of the medium after 24 and 48 hours of incubation of vaginal swabs positive for M. hominis alone or positive for M.hominis in association with T. vaginalis.*

Incubation	*Vaginal sample positive for M.hominis*	*Vaginal sample positive for T.vaginalis and M.hominis*
24 h	+	–
48 h	+	+

tence of *M.hominis* when using selective liquid media to identify the bacterium. The presence of *M.hominis* in clinical specimens may only be detected with a delay of at least 24 hours. Thus, the endosymbiotic relationship between *T.vaginalis* and *M.hominis* may be responsible for diagnostic failure or delay in the identification of *M.hominis* infections, and highlights that a negative *M.hominis* culture test with positive *T.vaginalis* might not rule out the bacterial infection if the microbiologic analysis is not performed for an adequate time.

Although the influence of endosymbiosis on the pathophysiology of *T.vaginalis* and *M.hominis,* such as modulation of virulence, immune response to infections, and regulation of expression of molecules related to metabolism and pathogenicity, have been well-described in previous studies (Dessì et al., 2019; Fettweis *et al*., 2019; Hirt and Sherrard, 2015; Pereyre *et al*., 2009), this work is the first to emphasize the diagnostic implications of the close endosymbiotic relationship between the two pathogens on the routine diagnostics of *M.hominis* infections.

The results confirm that clinical samples should always be incubated for an adequate period (at least 48 hours) before ruling out an infection by *M. hominis*. In fact, if the observation of color change is limited to only the first 24 hours of incubation, the consequence is an underestimation of the presence of *M.hominis*. This issue is especially important when complete eradication of *M.hominis* infection is necessary. If the detection of *M.hominis* in a clinical specimen is limited to only 24 hours of incubation time, the presence of the bacterium may not be reported to the clinician, leading to diagnostic errors that may be particularly significant in cases of infertility diagnosis or in high-risk pregnancies, preterm delivery and neonatal morbidity and mortality (Thu *et al*., 2018).

Overall, these findings underscore the need for caution when interpreting results obtained from genital clinical specimens and the importance of using appropriate diagnostic techniques to accurately detect the presence of *M.hominis.* Only the simultaneous use of more sensitive and rapid NAATs, coupled with cultural techniques aimed at potential tests to assess antimicrobial resistance, ensures the maximum diagnostic success.

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Conflicts of Interest statement

The authors declare no conflicts of interest.

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