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Rapid detection of some STD-causing bacteria using the direct flow chip method

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ABSTRACT

Aims: This study aimed to detect bacterial pathogens that cause sexually transmitted diseases (STD) using multiplex polymerase chain reaction and reverse hybridization.
Methodology and results: Thirty urine samples were collected from male patients aged between 20 and 45 in Dohuk City who were suspected of having an STD. The samples were tested for the presence of five main types of bacteria, namely *Ureaplasma urealyticum*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Mycoplasma genitalium* and *Chlamydia trachomatis* responsible for causing STDs. Nineteen of the thirty urine samples were positive for at least one of the five species of bacteria, yielding a positive rate of 63.3%. *Ureaplasma urealyticum* had the highest diagnostic rate of 68.4% among positive samples, while *C. trachomatis* had the lowest diagnosis rate of 5.2%. Both *N. gonorrhoeae* and *M. hominis* had a 15.7% diagnosis rate, while *M. genitalium* had a 10.5% diagnosis rate.
Conclusion, significance and impact of study: Research findings suggest that *U. urealyticum* was the most common cause of STD, accounting for 68.4% of the positive samples. Conversely, the study identifies *C. trachomatis* as the least prevalent cause, accounting for only 5.2% of the cases. These noteworthy findings shed light on the prevalence of these bacterial pathogens in sexually transmitted diseases, laying the groundwork for more precise and effective diagnostic and treatment options.

Keywords: Bacterial sexual disease, multiplex PCR, reverse hybridization, STD flow chip

INTRODUCTION

In 1997, the Institute of Medicine (IOM) reported that 50% of sexually transmitted diseases (STDs) in adults aged 15 to 24 can have permanent health implications, including infertility, making STDs a "hidden epidemic" with significant health and economic consequences (CDC, 2019). Furthermore, these illnesses are prevalent among the top ten diseases in underdeveloped countries and rank among the most widespread sources of infection globally (Van Dyck *et al*., 1999). In 2008, a study estimated that the lifetime financial burden of supporting specific STD patients, covering expenses for diagnosis, treatment, clinics and other health services, amounted to $15.6 billion (Ho, 2020). The global burden of sexually transmitted diseases is unknown since many of these infections are asymptomatic, diagnostic procedures are unavailable in the most affected nations and there is no surveillance system (Fasciana *et al*., 2022).

Infection of the genitourinary system is produced by endemic microorganisms of the upper genital tract, which contaminate the seminal fluid, affect the parameters of sperms, inflammation in the testicles, epididymis and urethra, and induce genetic arrest of spermatogenesis (Fourie *et al*., 2012; Parekattil *et al.*, 2012). Sexually active young individuals are at a higher risk for sexually transmitted diseases due to biological and behavioral factors. Adolescents between the ages of 15 and 24 account for half of the 20 million cases reported annually (Ho, 2020). In European countries and particularly throughout the past century, there has been an upsurge in sexually transmitted diseases, especially Syphilis, Gonorrhea and *Chlamydia* (Samkange-Zeeb *et al*., 2011). *Ureaplasma urealyticum* is one of the principal bacteria responsible for non-gonococcal urethritis (Liu *et al*., 2022) and has been responsible for 70% of all cases in recent years in the United States (Ho, 2020). Despite significant constraints regarding cost, skills and other forms of support, clinical diagnostic laboratories have played a crucial role in the STD program (Van Dyck *et al*., 1999). Screening for sexually transmitted bacterial infections offers the potential for early treatment of pathological disorders by reducing transmission rates and disease response (Dayan and Ooi, 2003).

Although molecular tests depending on nucleic acid amplification have a high sensitivity of around 86-100% and may diagnose *Chlamydia* and Gonorrhea with 97.1-100% accuracy (Tuddenham *et al*., 2022), the drawbacks

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of conventional microbial culture can be overcome by using nucleic acid detection and diagnosis of sexually transmitted diseases.

The importance of sexually transmitted diseases (STDs) involves identifying the bacterial causal agent precisely and quickly. To meet this critical requirement, current research was focused on evaluating a molecular diagnostic approach. The goal was to identify the bacteria that cause sexual diseases, allowing for earlier and accurate detection for improved treatment and public health outcomes.

MATERIALS AND METHODS

Samples collection

A total of 30 urine samples were collected from January 2022 through July 2022 from male patients suspected of having an STD, according to the doctor`s diagnosis. Urine samples were placed in sterile plane tubes before being brought to the laboratory at a medical center in Dohuk City/Iraq.

Sample preparation and DNA molecular detection

The sample was thoroughly mixed using a vortex, followed by 3 min of centrifugation at 12,000 rpm. A Pasteur pipette was used to remove the supernatant. Next, 400 mL of RNase/DNase-free double-distilled water was added to the precipitate (cell pellet) and gently mixed. After the initial washing and centrifugation, the process was repeated.

Molecular detection

The detection system used in this study relies on a process that involves multiplex PCR for simultaneous amplification of bacterial DNA extracted from samples, followed by hybridization on a membrane with specific DNA probes. The molecular analysis was carried out following the manufacturer's instructions as follows:

First: Multiplex DNA amplification

Thirty mL of the cell suspension was used as the DNA template for the PCR reaction. The PCR reaction mixture contained lyophilized PCR buffer, dNTPs(U/T), DNase/RNase-free water, biotinylated primers, hot-start polymerase and uracil DNA glycosylase. The contents were mixed and placed into the thermal cycler using the following PCR conditions: Initial preparation step at 25 °C for 10 min followed by an initial denaturation step at 95 °C for 3 min followed by 40 cycles of amplification including denaturation for 30 sec at 95 °C, annealing for 45 sec at 55 °C and extension for 30 sec at 72 °C (Barrientos-Durán *et al*., 2020).

Second: Denaturation

After multiplex amplification, the PCR product was denatured again at 95 °C for 10 min and tubes were then placed on ice for 2 min.

Third: Reverse hybridization

Before beginning the hybridization procedure, reagent A (hybridization solution) was preheated, and the STD chip was set in the position indicated on the HybriSpot (HS12) platform. Phases of the hybridization process were then initiated. 1.5 mL of reagent A was preheated at 41 °C and then the chip was placed in 300 mL reagent A at 41 °C for 2 min for preliminary washing. The reagent was then extracted using a vacuum pump for 30 sec at 41 °C. Thirty mL of the denatured sample was mixed with 270 mL of reagent A and the mixture was then put on the chip for 8 min. Three consecutive rounds of chip washing with reagent A were performed. After that, the equipment temperature was set to 29 °C and 300 mL of reagent B (blocking solution) was poured onto the chip and left for 5 min. The pump was turned on to remove reagent B and the chip was treated with 300 mL reagent C (streptavidin alkaline phosphate) for 5 min by activating the pump, reagent C was extracted. The equipment was adjusted to 36 °C. The chips were washed four times using reagent D (washing buffer I) while the temperature was set at 36 °C and the pump was turned on between washes. Once the chip reached 36 °C, 300 mL of reagent E (development solution) was added and left to sit for 10 min, then reagent E was extracted using a pump. The chip was then activated, the pump operated, and washed twice with 300 mL of reagent F (washing buffer ll). Finally, HybriSoft software scanned the image and analyzed the results.

RESULTS

Among individuals suspected of having sexually transmitted diseases, examining the sample revealed a high infection rate of more than 50%, with 19 samples out of a total of 30 giving a positive result for one of the bacterial causative agents of sexually transmitted diseases, at a rate of 63.3% and 11 samples being negative, at a rate of 36.7%. The two types of chips, a negative and a positive, are shown in Figure 1.

Most of the positive samples in our study were found to contain only one type of pathogen, as 16/19 (84.3%) samples were positive. In only 3/19 cases (15.7%), the positive samples carried more than one bacterial pathogen. Figure 2 shows the percentage of positive cases and Table 1 shows the total number and percentage of positive and negative cases for all samples tested in the study, as well as the number and percentage of samples infected with one or more bacteria that can cause STD.

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(A) Positive chip

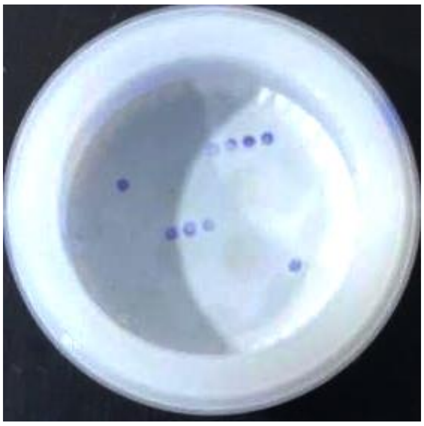
(B) Negative chip

Figure 1: A visual contrast between positive (A) and negative (B) chips, providing an illustrative comparison of the two types.

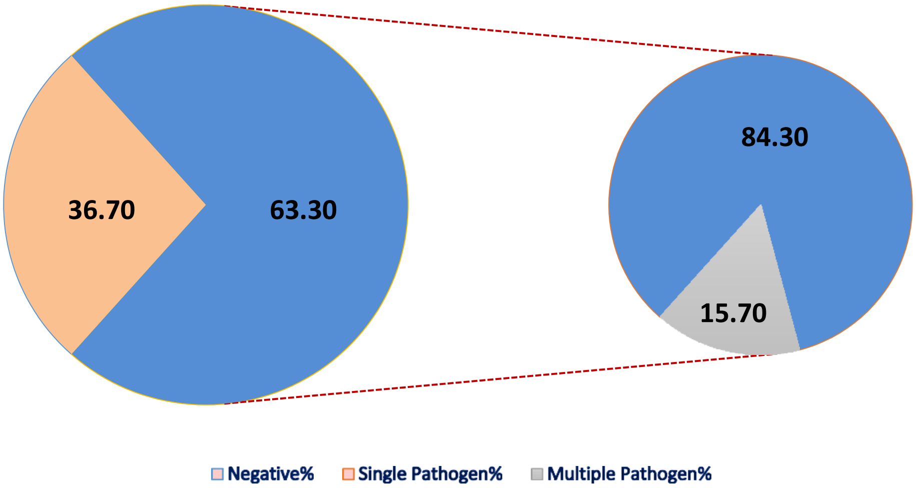

Figure 2: STD infection rates.

Table 1: The number and percentage of positive and negative STD cases among all samples analyzed.

Infection	Total	
	Number	Percentage %
Sample positive	19	63.3
Sample negative	11	36.7
Single pathogen	16	84.3
Multiple pathogens	3	15.7

The detection of STDs is carried out using the extracted DNA according to the manufacturer's instructions. This process involves utilizing a direct flow chip assay on the HybriSpot platform. The negative or positive plate that is infected with one or more sexually transmitted pathogens is detected by photographing the results of each chip membrane with the device's camera and analyzing it automatically using HybriSoft software (Taku *et al*., 2021).

In our investigation, we were also able to identify five different bacterial pathogens that cause sexually transmitted diseases, including *U. urealyticum*, *M. genitalium*, *M. homoinis*, *N. gonorrhoeae* and *C. trachomatis*. Table 2 shows the number and percentage of each of the different types of bacteria that were found during the investigation.

In our analysis, 68.4% of the total number of positive samples were positive for *U. ureaplasma*, an example of software with a positive *U. urealyticum* is shown in Figure 3.

Even though *C. trachomatis* was found in 5.1% of the samples, this was the lowest rate of *C. trachomatis* -positive samples, as shown in Table 2 and Figure 4, which show the positive *C. trachomatis* result.

Other forms of sexually transmitted bacteria besides *U. urealyticum* and *C. trachomatis* were also identified, with a proportion of 41.9% among the positive samples. These types of sexually transmitted bacteria were *N.*

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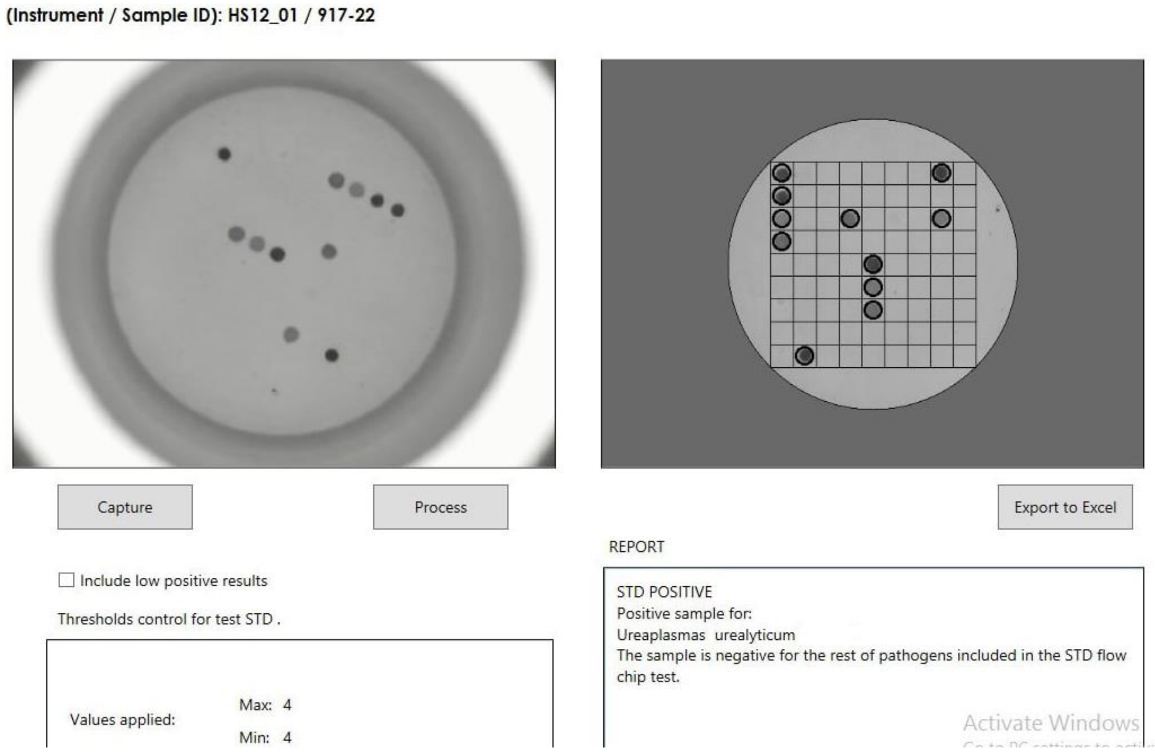

Figure 3: An image showing *U. urealyticum* positive result.

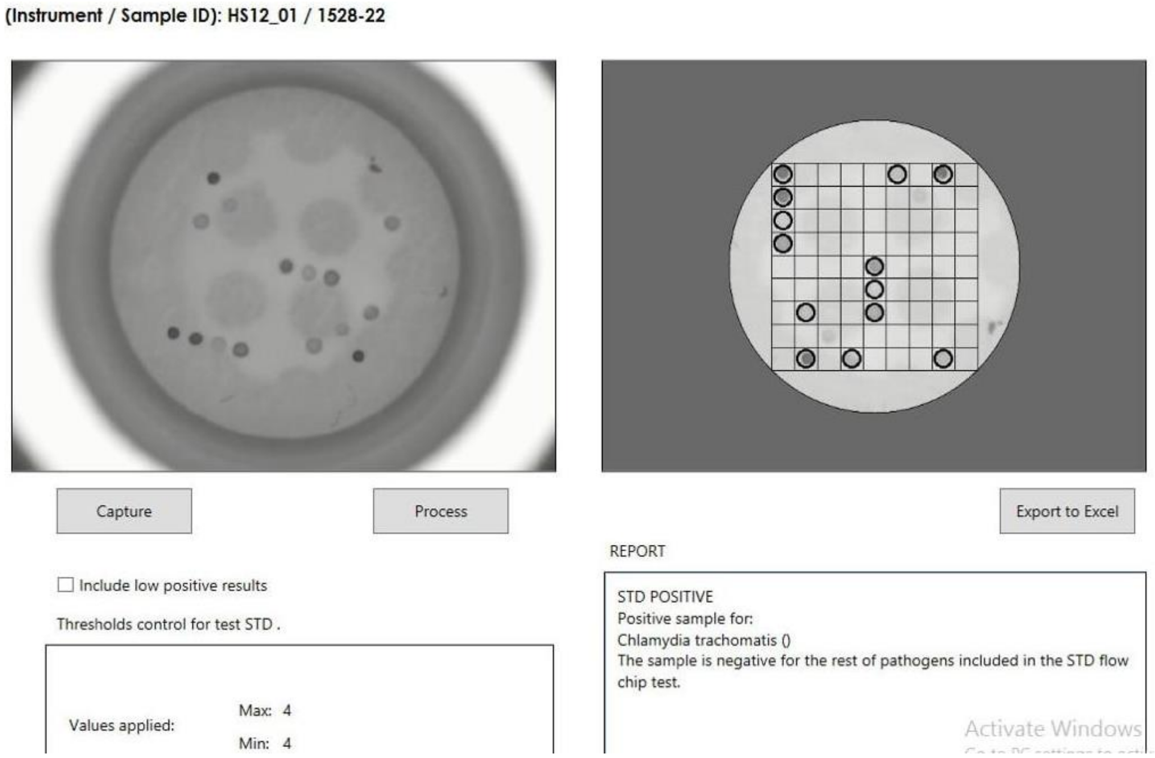

Figure 4: An image showing *C. trachomatis* positive result.

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Table 2: Each diagnosed bacterial species percentage in the study.

Organism	Total positive	
	Number	Percentage %
Ureaplasma urealyticum	13	68.4
Neisseria gonorrhoeae	3	15.7
Mycoplasma hominis	3	15.7
Mycoplasma genitalium	2	10.5
Chlamydia trachomatis	1	5.2

gonorrhoeae, *M. homoinis* and *M. genitalium*, with percentages of 15.7, 15.7 and 10.5, respectively.

DISCUSSION

The DNA FLOW technique by STD direct flow chip kit identifies many kinds of bacteria responsible for sexually transmitted diseases. This technique is distinguished by its quick diagnosis and interpretation of results in less than 4 h and its ability to analyze a range of clinical samples without the need for a DNA extraction step. The study findings were obtained by first performing a multiplex amplification process using the PCR mix tube and then entering it into the PCR thermocycling program, followed by reverse hybridization (Fernández *et al*., 2015; Barrientos-Durán *et al*., 2020). The flow chip technique represents a potential alternative to standard diagnostic processes since it enables the immediate examination of samples and the identification of a wide variety of infections (Barrientos-Durán *et al*., 2020).

Along with heterosexual behavior, not using condoms is one of the main reasons why the number of sexually transmitted diseases is going up (Kularatne *et al*., 2018). This outcome was consistent with the findings of Barrientos-Durán *et al*. (2020), who examined the etiology of sexually transmitted illnesses using 94 semen samples and a positive sample rate of 57.4%. While the findings of the study done by Wendt *et al*. (2019), using the multiplex real-time PCR technique on 67 distinct samples used to analyze the etiology of sexual illnesses, the proportion of positive samples was 38.8%, which is less than 50% of the total samples evaluated. In recent years, the frequency of sexually transmitted bacterial diseases has gradually increased worldwide. This necessitates a greater emphasis on sexually transmitted diseases in educational agendas (Tenaw, 2022).

The percentage of single pathogen samples in our study was 84.6% and the percentage of samples with more than one pathogen was 15.3%. This is similar to a study by Wendt *et al*. (2019) that was mentioned above. The results also matched the study by Fife *et al*. (2017). In only two of 16 cases of *U. urealyticum* positive infections, we noticed the presence of another STD causing pathogen such as *C. trachomatis* and *N. gonorrhoeae*, for a rate of 12.5%. Our findings differed from those of Wetmore *et al*. (2011), who investigated the association between *Ureaplasma* and non-gonococcal urethritis in males and discovered that 56.2% of the samples were positive (co-infection). According to a study published by Lee *et al*. (2022), the rate of co-infection varied depending on the pathogen. Patients with *N. gonorrhoeae* and *C. trachomatis* infections had a low risk of co-infection, but those with *M. genitalium* and *Trichomonas vaginalis* infections had a high rate of infection with other causes of sexually transmitted diseases.

U. urealyticum. is one of the causes of non-gonococcal urethritis in men and cervicitis in women, and it is one of the most often detected cases among men's illnesses (Fife *et al*., 2017). This pathogen was isolated in various proportions among urethral and urine swab samples, particularly among sexually active college students, and its presence in the urethra leads to contamination of semen following ejaculation. The initial exposure to *U. urealyticum* causes a greater inflammatory response than subsequent exposures because past exposure to *U. urealyticum* impairs the immune response to subsequent infection cases (Wetmore *et al*., 2011).

Esen *et al*. (2017) investigated the occurrence of *U. urealyticum* in sexually transmitted diseases and identified bacterial species that were identical to the bacterial species detected in our study, *M. genitalium*, *M. homoinis*, *N. gonorrhoeae* and *C. trachomatis*. In addition, their study demonstrated that the proportion of *U. urealyticum*, which was 52.9%, represented the largest percentage of positive cases. In a study carried out in Turkey (Sarsar and Aydin, 2010), the prevalence of *U. urealyticum* and *M. genitalium* infection cases were investigated using PCR technique. The proportion of positive samples for *U. urealyticum* was only 50%, which is close to our results. While in Kriesel *et al*. (2016) study on urine samples from both sexes suggested that the infection rate of *U. urealyticum* was 6.1%, which was a lower percentage and a different proportion compared to our study.

According to the World Health Organization (2008), *C. trachomatis* is the most common cause of treatable sexually transmitted diseases in Europe and the United States. It is also the most studied type of infertility, including low sperm concentration and an increase in semen morphological abnormalities. Since *C. trachomatis* infection has been associated with urethritis in some men, it is now advised that all men receiving treatment for urethritis should also receive treatment for *C. trachomatis* infection. This bacterial infection, which can affect the urethra, rectum and even the throat, is more prevalent among men, particularly sexually active young men. *C. trachomatis* infection has also been connected to the risk of subsequent infertility and miscarriage (Ahmad *et al*., 2017; Priyadarshi *et al*., 2019; Tenaw, 2022).

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In South Africa, Kularatne *et al*. (2018) discovered that 6% of men had *C. trachomatis*, which is comparable to the results of our study. In another study, Kriesel *et al*. (2016) evaluated nine different kinds of sexually transmitted infections using multiplex PCR technology for urine samples from unrestricted sexual activity people; the *C. trachomatis* rate was 39.6%, which was the highest proportion among STD-positive samples and differed from the percentage found in our study.

Neisseria gonorrhoeae is a disease-causing bacterium that spreads via sexual contact. Albert Neisser first isolated this pathogen in the discharge from an inflamed urethra and cervix in 1879. Humans are the only natural reservoir for *N. gonorrhoeae,* and it takes between 1 to 14 days to become active. *Neisseria gonorrhoeae* infection is asymptomatic and can affect the urethra, endocervix and rectum. Additionally, it is believed to be the second-most common bacterial disease that can be transmitted sexually. The number of new cases of gonorrhea is usually high in developing countries, where men with symptoms are more likely to seek treatment and preventive care to avoid complications. It is recommended that people who are sexually active and most at risk be checked regularly (Buder *et al.*, 2019; Tenaw, 2022).

In terms of *Mycoplasma*, *M. homoinis* and *M. genitalium*, two forms of genital mycoplasmas were discovered from both the male and female reproductive systems. Even though they have been isolated from men for over a decade, scientists still do not know how important this factor is in the development of male infertility. Neither *M. genitalium* nor *M. homoinis* were associated with aberrant sperm characteristics. Studies on how *M. genitalium* and sperm interact have shown that this relationship is critical for both spreading the infection to the partner and clumping sperm, which prevents men from having children (Ahmad *et al*., 2017).

The rates of isolation for *N. gonorrhoeae*, *M. genitalium* and *M. homoinis* in our study are similar to those obtained in a study by Esen *et al*. (2017) on male urine samples (17.6%, 17.6% and 11.7%, respectively). When we compared our results to those of Kriesel *et al*. (2016), we noticed that the percentages of *N. gonorrhoeae* and *M. genitalium* in urine samples were about the same, 15.5% and 8.7%, respectively. Even though the percentages of *N. gonorrhoeae* and *M. genitalium* were different in our investigation, the proportion of *M. homoinis* was 16.6% which was identical to what was observed in Barrientos-Durán *et al*. (2020). In their investigation, no *N*. *gonorrhoeae* was recovered and only 7.4% of *M. genitalium* was isolated, which was lower than what we detected. According to our results, only 6% of patients had *M. homoinis*, whereas 12.1% of the patients in Yokoi *et al*. (2007) investigation of patients with Gonococcal urethritis had *M. genitalium*. Also, Wendt *et al*. (2019) showed that the rate of *N*. *gonorrhoeae* isolation was 38%, which was higher than what we found in our investigation. Finally, DNA of both *M. genitalium* and *M. homoinis* were detected in the semen of infertile Tunisian males. The percentages of DNA found in their semen were 4.8% and 9.6%, respectively (Ahmad *et al*., 2017), which are lower percentages than what was revealed in our study and are different from the percentages found in other studies.

CONCLUSION

STDs are a significant public health problem. The diagnostic procedures are unavailable in resource poor countries and the availability of rapid methods is urgent due to the high infection rate, especially in polygamous and hypersexual people. The direct flow chip method used in this research was shown to be both accurate and rapid in the isolation of many STDs-causing bacteria. This method provided results within 4 h only.

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