Development of multiplex Real Time PCR and high-resolution melting assay for detection the sexually transmitted pathogens in a sample of Iraqi patients

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Abstract

Sexually transmitted infections consider the main general health trouble in the world which occasion of severe sickness, long-period complications, medical, mental sequels, infertility, and mortality. The development of new methods for detection bacteria and viruses related with sexually transmitted diseases (STDs) is very important for control and management of these infections. The collection of study samples has taken place at the period collected between September and November 2021 in Baghdad, Iraq, from two of specialized Hospitals and one private hospital, it has included 150 clinical specimens from married couples patients (75 cervical swabs from females and 75 seminal fluids from males), also 50 samples from healthy control (25 couples), during this period, from both gender with age ranging from 18 to 45 years. The current study was conducted to detect sexually transmitted infectious agents (bacteria and viruses) by developing HRM (high resolution melting) assay with multiplex real-time PCR using of set primer of each pathogen that was designated by professional software program (Beacon Designer™ automates the design of real time primers and probes) for the accurate detection of these infections in Iraqi patients. This study conducted for detection seven different sexually transmitted pathogens (3 bacteria, 3 viruses, and one parasite) among 150 suspected patients as married couples. The results of RT-PCR with HRM assay of the positive controls demonstrated different melting curves, where each melting peak represent the amlification of one of the seven pathogens, also the melting peakof the internal control (housekeeping gene). This experiment included three panels, the panel one represent the optimal melting peak for the positive control of *Trichomonas vaginalis, Mycoplasma hominis, Cytomegalovirus* the melting temperature each of them was (80.0℃, 84.90℃ and 88.0℃) respectively. The panel 2 ncluded the melting peak for the positive control of *Human Papilloma Virus, Neisseria gonorrhoeae, and Herpes simplex virus*, the melting temperature for each of them was) 79.6℃, 84.75℃ and 87.75℃) respectively. The panel 3 showed the melting curve for the positive control of *Chlamydia trachomatis* and internal control (Homo sapiens hemoglopin subunit beta (HBB) gene), where the melting temperature was) 79.35℃, and 88.65℃) respectively. The results of STDs detection revealed that the frequency of the sexually transmitted infections (bacteria, viruses and *T. vaginalis*) among 150 of married couple's patients in some of Baghdad hospitals was 108 from 150 (72%). The virus's infections were predominant in comparison with other infectious agents. Out of 108 infections, the outcomes showed the prevalence of virus infections in STDs patients was 63% (68/108), while the bacterial pathogens was 31.5% (34/108) and *T. vaginalis* infections was 5.5% (6/108). The predominant virus in all patients was CMV (31.5%), followed by HSV (24.1%), while HPV was only 7.4%. In bacteria, the high prevalent species was *C. Trachomatis* with 14.8%, followed by *M. hominis* (9.3%) and *N. gonorhoeae* (7.4%). According to the age groups, it was found that the viral infection frequency was higher than other agents in all age groups, where the age group 36-45years was the highest percentage (27.8%). The age group 36-45years was also exhibited the highest frequency for all agents with (41.7%).

Keywords: Multiplex RT-PCR, HRM, Sexually transmitted infections.

1. Introduction

The infections sexually transmitted represent a diversity of clinical symptoms occasion via pathogens gained and transferred by contacting sexual. There were more than 30 pathogens from viral, bacterial, and parasites that were detected until now that transferred sexually) Garcia and Wray, 2022). Infections sexually transmitted consider the main general health trouble in the world which occasion of severe sickness, long-period complications, medical, mental sequels, infertility, and mortality. Further, infections sexually transmitted assist the prevalence of immunodeficiencies virus. It happened around the world 769.85 million conditions of infections sexually transmitted. So, on rate, nearly 1 million populations infected with infections sexually transmitted each day (Fu *et al.*, 2022). The infections sexually transmitted were among almost all critical general

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health troubles, with essential related to illness, death, inability, and reverse pregnancy results (Grant *et al*., 2020).

WHO's world health -section strategy for infection sexually transmitted (2016–2021) fixed goals to reduce 90% the prevalence of *Treponema pallidum*, and also *Neisseria gonorrhoeae* infection from 2018 to 2030 (WHO,2016). The laboratory methods to detect *Neisseria gonorrhoeae*, *Ureaplasma urealyticum* , *Mycoplasma hominis* , *and Mycoplasma genitalium* that vary from cell culturing to NAAT (nucleic acid amplification tests) (Martín-Saco *et al*., 2022). PCR was utilized to examine different sample types involving men's and women's urine, men's urethra swabs, and women's endocervical or vagina swabs. The spread of classical collecting methods led to some people not performing infection sexually transmitted examinations (Grad *et al*., 2015). As a result of the absence of studies in Iraq on diagnosing sexually transmitted diseases using High-resolution analysis technology for many microorganisms at the same time (bacteria and viruses).Also, it is difficult accurately diagnose STIs. Therefore, this study was designed to facilitate the diagnosis of several STD pathogens at the same time. This study aims to design a professional kit that contributes to the detection of the most important sexually transmitted infectious (STI) through the use of highly professional molecular biology methods by designing primers and the use of high-resolution melting technology Accurate detection of these infections in Iraqi patients in record time and low cost compared to the exorbitant prices of this type of molecular examination.

2. Materials and Methods

DNA extraction and identification of carbapenem genes

Cervical swabs and seminal fluids DNA was extracted from 150 patients specimens using ready kit (Promega, USA). Purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo Scientific, USA). The Primer sequences, used for the detection of pathogens target genes in this study, were showed in Table 1.

Table 1. Designed primers of Multiplex-RT PCR and HRM used in this study.

The microoganism	Target gene	Primer name	Oligonucleotide primer Sequence (5-3)	Anealing temp.	Ambilicon size(bp)
HSV herpes simplex virus 1	***US4***	**HSV F**	**GACTCACCTCAAAGGGAC**	**60**	**830**
		HSV R	**CATCGCACCAATACACAA**		
Human papillomavirus	***L1***	**HPV F**	**CGCCTCTATCCACCTGAAGTCCTAC**	**60**	**235**
		HPV R	**TGCCAGATCCTAATAAGTTTG**		
CMV Cytomegalovirus	**MIE**	**CMV F**	**TGA GGA TAA GCG GGA GAT GT**	**60**	**242**
		CMV R	**ACT GAG GCA AGT TCT GCA GT**		
Mycoplasma hominis	***16SrRNA***	**MH F**	**ACGCTGTGTCGCTCCATCAAG**	**60**	**310**
		MH R	**GCACTTTACAATCCGAAGA**		
Neisseria gonorrhoeae	***rmp***	**NG F**	**AAACCATTTCCCTGTCTGCCAAA**	**60**	**173**
		NG R	**GGACAGATTGGACATTGG**		
Trichomonas vaginalis	***btub2***	**TV F**	**CTCACAACACCAACATACGGCGA**	**60**	**457**
		TV R	**CGAAGCACTTTACGATATTTG**		
Chlamydia trachomatis	***OmpA***	**CT F**	**TCTGGCGGCACTGTGTCCTA**	**60**	**700**
		CT R	**TCTGGCGGCACTGTGTCCTA**		
IC (Homo sapiens)	***HBB***	**IC F**	**TCTGGCGGCACTGTGTCCTA**	**60**	**626**
		IC R	**CTCAGGCCATTAATTGCTA**		

method) from all the samples. These seven chosen genes was divided into 3 penal, the panels 1 and 2 contain three different genes, while Panel 3 contain 2 genes. The conditions of PCR performed with the selected primers outlined in Table(2). Positive results for gene detection and data analysis observed as amplification exponential florescent curves at a specific computer screen attached with the thermal cycle.

Multiplex Real-Time Polymerase chain Reaction panel design

The multiplex Real-time PCR with HRM assay was designed according to Beacon Designer for detection the pathogens target genes. Process conducted by detection of seven different gene and one internal control gene (IC) as housekeeping gene in the purified total genomic DNA (fully automated

Table 2. PCR reaction components for amplified the targeted fragments.

Component	Quantity
PCR Master Mix (Ready to use) SYBR Green	10
Forward primer	0. 5
Reverse primer	0. 5
DNA template	2
Nuclease free water	7
Total volume	20 Ml

components and program conditions for each penal

The mixture of multiplex Real-Time PCR reaction

an initial step of 5 min holding at 95°C (1 cycle) followed by cycles of 5s at 95 °C, 20s at 60°C and 10s at 72 °C. Then followed by (40 repetitions) and holding at 50 s at 55 °C (1 cycle), Melting (60-90) rising by 0.50 degrees, hold for 1 seconds each step, Acquire to [HRM]. Using Multiplex real time thermo cycler according to the protocol recommended by the manufacturer. The PCR conditions as show in the table (3).

1, 2 and 3 are described in Tables 3 (with suitable annealing temperature for each panel as in table 1). Subsequently, multiplex reactions to detect the best temperature for annealing with a total volume of 20 µL.

Reaction mixture for Multiplex real-time PCR was performed in 20 µL of 10 µL master mix (SYBR green), 1 µL panel 1 or panel 2 (forward and Reverse primers), 2 µL DNA template, 7 µL D.w. Program conditions for Multiplex Real-time PCR consisted of

Table 3. PCR Amplification Program.

Steps	Temperature	Time/ sec	Cycles
Hold	95	300	1 cycle
Denaturation	95	5	40 cycles
Annealing	60	20 sec.	
Extension	72	10 sec.	
Hold	55	50 sec.	1 cycle
Melting temperature	60-90 *	Rising by 0.50 degrees, hold for 1 seconds each step, Acquire to (HRM)	
*Dye detection (Filter HRM) for SYBR green.			

3. Results and Discussion

The collection of study samples has taken place at the period collected between September and November 2021 in Baghdad, Iraq, where included two of specialized Hospitals and one private hospital, it has included 150 clinical specimens from married couples patients (75 cervical swabs from females and 75 seminal fluids from males), also 50 samples from healthy control (25 couples), during this period, from both gender with age ranging from 18 to 45 years.

The current study was conducted to detect sexually transmitted infectious agents (bacteria and viruses) by developing HRM (high resolution melting) assay with multiplex real-time PCR using of set primer of each pathogen that was designated by professional software program (Beacon Designer™ automates the design of real time primers and probes) for the accurate detection of these infections in Iraqi patients.

Detection of STD pathogens by using Multiplex real-time PCR with High Resolution Melting (HRM) assay

This study developed and evaluated the Multiplex real-time PCR with HRM reactions that could detect seven different target genes for detection seven different sexually transmitted pathogens (3 bacteria, 3 viruses, and one parasite) among 150 suspected patients as married couples in addition to Homo sapiens Hemoglobin subunit beta (HBB) as internal control, Endogenous positive controls used in this study refer to the use of a native target, reference genes are often selected for this purpose, that is present in the experimental sample(s) of interest. It was used as normalizers and is typically used to correct for quantity and quality differences between samples. The positive controls of this study with the specific TM for each infectious agent, where all targets were amplified with the highest efficiency and specificity under optimum conditions. The multiplex RT-PCR enhanced the separation of the melting peaks for the multiplex reaction via increases in the Tm. HRM assay showed that all of the tested primers were effective, and the Tm value for each target gene was sufficiently separated. The multiplex HRM assays were able to identify STD pathogens by detection the specific target gene of each microorganism.

The results of RT-PCR with HRM assay of the positive controls were showed in figures (1), (2) and (3), where each melting peak represent the amlification of one of the seven pathogens, also the melting peakof the internal contro (housekeeping gene). This experiment included three panels, the panel one as showed in figure (1), which represent the optimal melting peak for the positive control of *Trichomonas vaginalis, Mycoplasma hominis, Cytomegalovirus* the melting temperature for each of them was (80.0℃, 84.90℃ and 88.0℃) respectively.

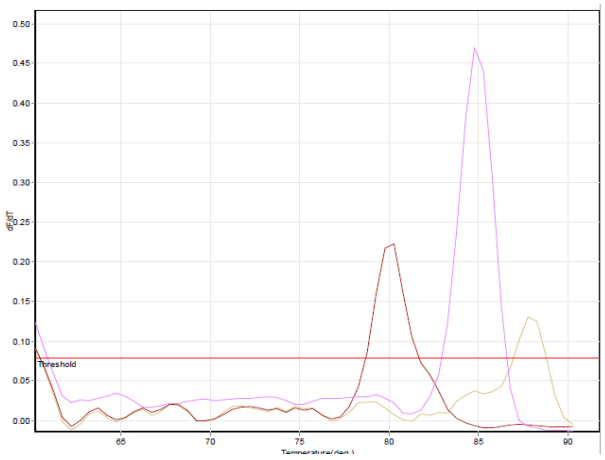

Figure (1). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay for the detection of T. vaginalis, M. hominis, and Cytomegalvirus of positive control (PC); with the specific TM values (80.0℃, 84.90℃ and 88.0℃), respectively. (Panel 1).

The panel 2 as demonstrated in figure (2) showed the melting peak for the positive control of *Human Papilloma Virus ,Neisseria gonorrhoeae, and Herpes simplex virus*, the melting temperature for each of them was (79.6℃, 84.75℃ and 87.75℃) respectively.

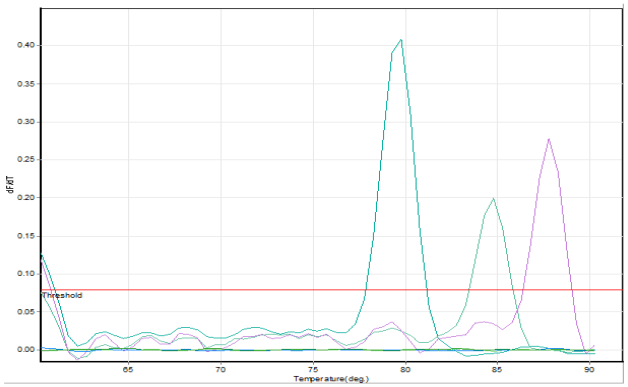

Figure (2). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay for the detection of Human papiloma virus, N. gonorhoeae, and Herpes simplex virus of positive control (PC); with the specific TM values (79.6℃, 84.75℃ and 87.75℃), respectively.(Panel 2).

The panel 3 as demonstrated in figure (3) showed the melting curve for the positive control of *Chlamydia trachomatis* and internal control (Homo sapiens hemoglopin subunit beta (HBB) gene), where the melting temperature was (79.35℃, and 88.65℃) respectively.

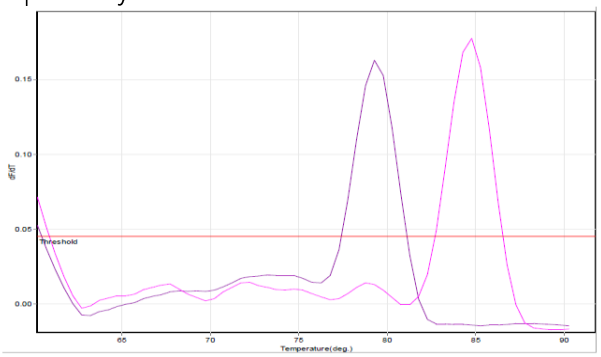

Figure (3). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay for the detection of C. trachomatis, and internal control (Homo sapiens hemoglopin subunit beta (HBB) gene) of positive control (PC); with the specific TM values (79.35℃, and 88.65℃), respectively. (Panel 3).

The results of STDs detection among married couples patients by Multiplex real-time PCR with HRM reactions for detection seven different sexually transmitted pathogens (3 bacteria, 3 viruses, and one parasite) among 150 suspected patients as married couples in addition to Homo sapiens Hemoglobin subunit beta (HBB) as internal control, were demonstrated in the figures from (4) to (9).

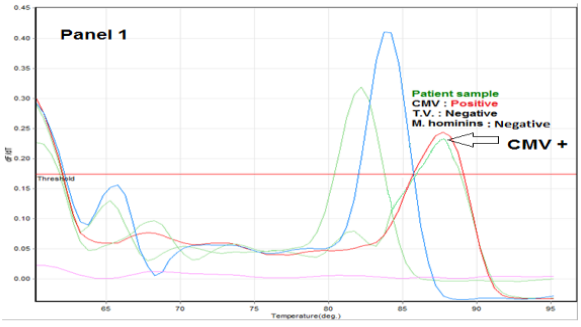

Figure (4). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 1, the specific melting peak identified CMV with TM: 88℃.

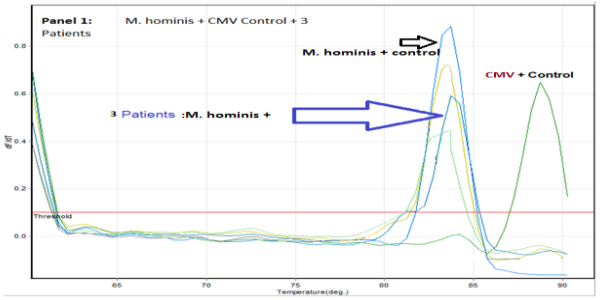

Figure (5). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 1(3 patients), the specific melting peaks identified CMV with TM: 88℃, and M. hominis (84.90℃).

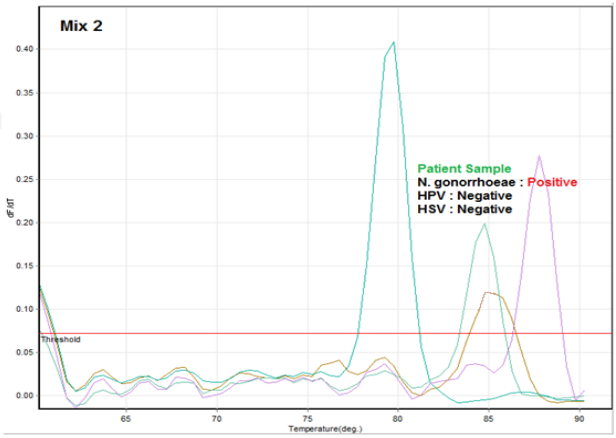

Figure (6). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 2, the specific melting peak identified N. gonorhoeae with TM: 84.75℃.

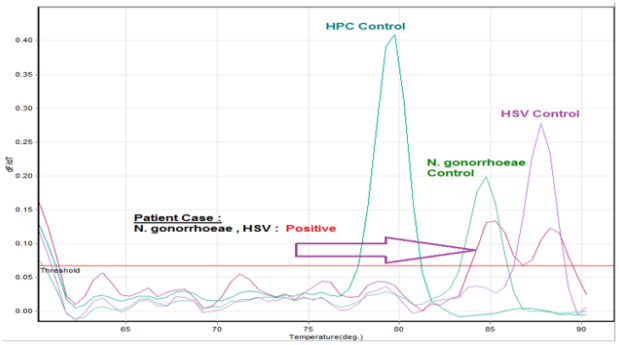

Figure (7). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 2, the specific melting peak identified N. gonorhoeae with TM: 84.75℃, HSV: 87.75℃.

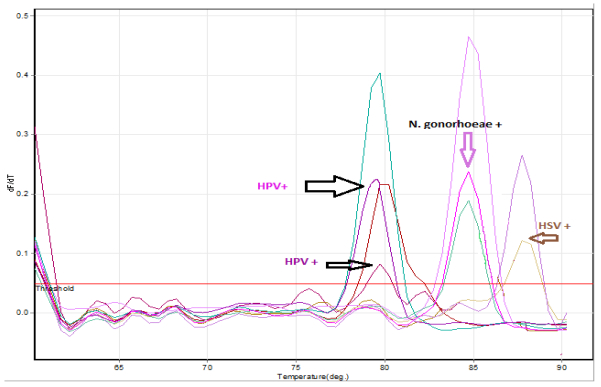

Figure (8). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 2, the specific melting peak identified N. gonorhoeae with TM: 84.75℃, HSV: 87.75℃, and HPV: 79.60℃,

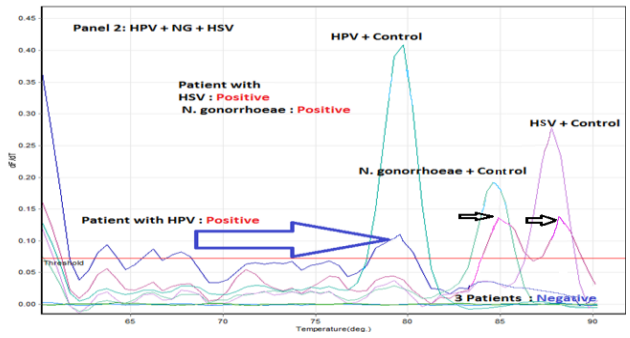

Figure (9). Derivative melt curve results of multiplex RT-PCR with high-resolution melting assay of panel 2, the specific melting peak identified N. gonorhoeae with TM: 84.75℃, HSV: 87.75℃, and HPV: 79.60℃,

Our results converged with other studies when discriminating the microorganisms through melting curves using HRM real-time PCR. In one study is identified of *C. trachomatis* in urogenital, where it was diagnosed 11 genotypes by nest real-time PCR VS2 (variable segment) and HRM. The HRM (high-resolution melting) outcomes harmonized with the sequencing results of VS1–VS2. Comparing with other techniques, HRM showed cost efficient, simple, and rapid. The green saturating dye utilized in HRM can transferred in usual temperature and store at 4 °C, also, frequent freeze and thawing does not lead to degrade and dissolution of fluorescence. The genotyping by HRMA was used because it is depending on computer in data analysis instead of electrophoresis and risk ethidium bromide. Besides, the contamination was declined. Further, the product PCR by HRMA can analysed through gel electrophoresis and sequencing because of the stain that used in HRM is non devastating to ds DNA (double-stranded) (Li *et al.*, 2010).

Ajitkumar *et al.*(2012) applied in their study HRMA to fast detection of the nine bovine pathogens that cause mastitis including (*Arcanobacterium pyogenes, Escherichia coli, Streptococcus dysgalactiae, Lactococcus lactis, Staphylococcus aureus, Streptococcus uberis, Klebsiella pneumoniae, Mycoplasma bovis, Corynebacterium bovis*) these bacteria identified through differences the melting temperatures of them by HRMA, the region of the melting curve was relied on in identifying the pathogen through clustering of melting curves conforming to the line before melting(pre-melt region) of dsDNA , through the melting and post melting, the pre melt region was 80.8 - 81.2° C and the post melt region 89.5-90.0° C Moreover, other study used multiplex real-time PCR by analysis melting curve, for identification of nine pathogenic of STD, involving *Chlamydia trachomatis, Mycoplasma genitalium, Trichomonas vaginalis, HSVI, HSVII, Ureaplasma parvum, Neisseria gonorrhea, Ureaplasma urealyticum,,* and *Mycoplasma hominis.* The multiplex real-time PCR depending on fluorescence probe of melting curve test which depended on melting temperature via temperature denaturation of the probe hybrid .Where the outcomes appeared that the rate of positivity was 100% to all pathogens tested (Hu *et al.*,2019). Another study employed HRM for identifying six species of Listeria, these species were showed difference melting temperature where *Listeria innocua* was 83.51 °C, *Listeria ivanovii* was 83.88 °C, *Listeria seeligeri* was 84.29 °C, *Listeria grayi* was 85.05 °C, *Listeria monocytogenes* was 85.42 °C, Listeria *welshimeri* was 86.12 °C (Jin *et al.*, 2012).

It was demonstrated the using internal control has many different targets including as the indicator for purification of nucleic acid, control of reverse transcription, normalization (Ruhanya et al.,2017). Also monitoring of inhibition PCR (Gomez et al.,2018). Where the internal control is amplified with the goal sequence in same of PCR reaction, or PCR run. Further the internal control not to contest with the goal region and can be distinguishable through distinct fluorophore marker from the goal (Wagner,2013). Tamburro and Ripabelli (2017) demonstrated in their study the important HRM technique, it was widely applied in molecular detecting for Gram-positive and negative pathogenic bacteria, they considering this technique as tool to detect phylogenetic diversity and mutation detection and estimate antibiotics resistance. Besides, Other study applied HRM for detection *Shigella* sp., the study used HRM that targeted rrsA gene to recognize for 4 species of *Shigella* were 49 isolates for food and clinical samples. It was confirmed a good sensitivity (0.01–0.1 ng) of DNA templates with specificity (100%) to distinguish between Shigella sp. PCR-HRM test had ability to distinguish of species for all 49 isolates from *Shigella* correctly (Pakbin *et al.*,2022).

The High-Resolution Melting (HRM) assay has emerged as a low-cost and fast method for bacterial typing, particularly promising for epidemiological applications (Perini *et al.*, 2020). Cost-effective molecular diagnostic tools are required for rapid AMR detection, especially in low–middle-income countries. Using a diagnostic tool such as HRM, which is affordable and where multiple targets can be run in a single analysis, is an option for use in antimicrobial stewardship, Cost-effective molecular diagnostic tools are required for (Mitchev *et al.*, 2022). HRM is a single-step procedure for the discrimination of sequence variants on the basis of their melting temperature. This method allows performing bacterial typing in less than five hours (Perini *et al.*, 2020). Bentaleb *et al.* (2017) and Otaguiri *et al.* (2018) demonstrated that HRMA based methods have an improved level of sensitivity when compared to culture and, thus, can detect lower levels of bacteria, thus, using a sensitive and precise method such as HRMA and specific primers could identify strains such as MBL and NDM producing strains.

4.3. The Prevalence of infectious agents in STDs patients

The frequency of sexually transmitted infections

among Iraqi patients was summarized in table (11). The present study revealed that the frequency of the sexually transmitted infections (bacteria, viruses and *T. vaginalis*) among 150 of married couple's patients in some of Baghdad hospitals was 108 from 150 (72%).

Table (11). Distribution the main pathogens among Iraqi patients (married couples) with STDs.

Sexually Transmitted Infectious agent	Male (N=108)	Total of Infectious agent
Chlamydia trachomatis	16(14.8%)	34 (31.5%)
Mycoplasma hominis	10 (9.3%)	
Neisseria gonorrhorea	8 (7.4%)	
CMV	34 (31.5%)	68 (63%)
HPV	8 (7.4%)	
HSV	26 (24.1%)	
Trichomonas vaginalis	6 (5.5%)	6 (5.5%)

The virus's infections were predominant in comparison with other infectious agents. Out of 108 infections, the outcomes showed the prevalence of virus infections in STDs patients was 63% (68/108), while the bacterial pathogens was 31.5% (34/108) and *T. vaginalis* infections was 5.5% (6/108).

The predominant virus in all patients was *CMV* (31.5%), followed by *HSV* (24.1%), while *HPV* was only 7.4%. In bacteria, the high prevalent species was *C. Trachomatis* with 14.8%, followed by *M. hominis* (9.3%) and *N. gonorhoeae* (7.4%).

According to the age groups, as showed in table 12, it was found that the viral infection frequency was higher than other agents in all age groups, where the age group 36-45years was the highest percentage (27.8%). The age group 36-45years was also exhibited the highest frequency for all agents with (41.7%).

Table (12). Distribution the main pathogens according to the age groups among Iraqi patients (married couples) with STDs.

Age (years)	No. (%) of couples samples	Pathogenic bacteria			Pathogenic viruses			Parasite
		CT	*MH*	*NG*	*CMV*	*HPV*	*HSV*	TV
18-25	28 (25.9%)	4	5	1	11	3	4	2
26-35	35 (32.4%)	8	2	3	17	1	4	0
36-45	45 (41.7%)	4	3	4	6	4	20	4
Total	108	16	10	8	34	8	26	6
		14.8% (16/108)	9.3% (10/108)	7.4% (8/108)	31.5% (34/108)	7.4% (8/108)	24.1% (26/108)	5.5% (6/108)
Healthy control	50	0	2% (1/50)	0	0	0	4% (2/50)	2% (1/50)

Healthy control (18-45 years), *CT: Chlamydia trachomatis, MH: Mycoplasma hominis, NG: Neisseria gonorrhoeae, TV: Trichomonas vaginalis, CMV: Cytomegalovirus, HSV: Herpes simplex virus, and HPV: Human Papiloma virus.*

In one study conducted in Lebanon, which included 41.5% (248 women) and 58.5% (349 men) were infected with STIs, where 53.5% of samples were positive (one or more pathogens). *Ureaplasma urealyticum* was 49.3%, *Gardenerella vaginalis* was 33.5%, *Chlamydia trachomatis* was 5.36%, *Mycoplasma genitalium* was 5.16%, Herpes simplex virus was 2.5%, *Neisseria gonorrhoeae* was 2.5%, and *Trichomonas vaginalis* was 1.39%. They were diagnosed by using multiplex real-time PCR where the dominance infection of *Neisseria gonorrhea*, and *Chlamydia trachomatis* in male was 92.5 and 85% respectively (Beayni *et al.*, 2021). The spread of STD may be associated with raised resistance of antibiotic to many bacterial species as in *Neisseria gonorrhea* (Hamze *et al.*, 2016).

In other case-control studies in Iraq there were correlated to the prevalence *Chlamydia* infections with infertility in women the percentage was 8-29% (Ahmed, 2012). In addition, other study demonstrated men with infertility have infection with *Chlamydia* at percentage 4-17% (Ali and Al-Kazaz , 2018).

Besides, Kriesel *et al.* (2016) appeared in their study the prevalence of sexually transmitted pathogens in patients, demonstrated that the percentage prevalence of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, herpes simplex virus 1 , herpes , simplex virus 2 were 13%,7%, 3%, 2%, and 2% respectively. In other epidemiological study of the prevalence of syphilis, chlamydia, gonorrhea, genital herpes, trichomoniasis in world from 1990-2019, the cases of these STDs pathogen raised by 58.15% (Fu *et al.*,2022).

Another study illustrated HPV infection prevalence was 15.5%, where the spread elevated in women to 32.2% which had age 25 year or less, *C. trachomatis* prevalence was 4.6% in young women had age 25 years or less increased infection to 16.1% (Dos Santos *et al.*, 2022). Infections of the genital tract by *C. trachomatis* infection are related with Pelvic inflammation disease, abortion, and avertable infertility whereas HPV associated with the malignant diseases (Peder *et al.*, 2018; Hoenderboom *et al.*, 2019). On the other hand, Ali *et al.* (2018) illustrated in their study the percentage of HSV infection in women, those in the age of 25-34 and 35-44 years were the percentage of 50%. Another Iraqi study recorded the rate infection with HSV for women in Waset city with 6.6% (Kamal *et al.*, 2013). Whilst, in the other countries the rates infection for HSV in women more than the current study where in Syria, Qatar, Iran, Turky was 52%, 26.3%, 43.75%, 63.1 % respectively (Barah,2012; Shahraki *et al.*, 2010 ; Ghazi *et al.*,2002).

4. Conclusion

The viral pathogens (CMV and HSV) were the most prevalent among married couple's patients with STDs in Baghdad hospitals. *Chlamydia trachomatis* was recorded as the highest among the bacterial agents, while the parasite *T. vaginalis* was in lower frequency. High accurate with high specificity at a short time for molecular detection of sexually transmitted pathogens by Multiplex real-time PCR with High Resolution Melting Analysis according to Beacon designer software, especially with conventional PC results.

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