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CIÊNCIAS FORENSES

Molecular Forensic Detection of Sexually Transmitted Infections

Ana Catarina Rodrigues Eira

M

2025

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Molecular Forensic Detection of Sexually Transmitted Infections

Ana Catarina Rodrigues Eira

Dissertação de Mestrado em Ciências Forenses
Ano: 2024/2025
Orientador/a: Professora Doutora Laura Cainé
Co-Orientador/a: Doutora Jennifer Fadoni

AGRADECIMENTOS

No final desta etapa tão importante na minha vida gostaria de expressar o meu profundo agradecimento a todos que, direta ou indiretamente, contribuíram para a realização deste trabalho e tornaram esta caminhada mais especial.

À minha orientadora, Professora Doutora Laura Cainé, agradeço toda a orientação prestada, pelo apoio e pelas correções e sugestões feitas ao longo desta dissertação, bem como pela confiança que desde o início depositou em mim. Além disso, agradeço também toda a paciência que teve comigo, ajudando-me de forma inigualável na resolução dos vários problemas que desde cedo apareceram, fazendo-me crescer imenso ao longo deste percurso.

À minha co-orientadora, Doutora Jennifer Fadoni, gostaria de agradecer por toda a ajuda, por todo o conhecimento que me foi transmitido no decurso deste estudo. A sua dedicação e os seus conselhos foram fundamentais para a concretização do mesmo.

A toda a equipa do Laboratório de Análises Clínicas e Médico-Legais do INMLCF, I.P., da Delegação do Centro, principalmente à Magda e à Helena, por me terem recebido da melhor forma possível e por me terem ajudado ao longo da parte prática deste trabalho. Obrigada por me receberem sempre com a maior das alegrias, nunca me vou esquecer de todas as gargalhadas que demos juntas.

Às minhas colegas de Mestrado Rita, Maria e Juliana, agradeço por ter a possibilidade de fazer esta caminhada convosco, espero que agora seja para sempre.

À Andreia, que para mim para além de uma colega de trabalho, se tornou uma grande amiga. Agradeço por todos os conselhos e por toda a ajuda ao longo deste ano, nunca me vou esquecer de todas as conversas, choros e sorrisos que partilhamos.

Às minhas amigas que sempre me apoiaram um obrigada enorme. Beatriz e Malva, seremos sempre o trio maravilha, agradeço por todo o apoio que me têm dado ao longo destes anos, será de Vila Real para sempre. Sara, a minha amiga de longa data que está sempre lá para me levantar, obrigada por tudo, sabes que és como uma irmã para mim. Dantas, obrigada por todas as conversas, passeios e risadas. Letícia, sem dúvida que sem ti a minha passagem por Coimbra teria sido muito mais complicada, levarei sempre comigo tudo o que vivemos e conversamos, será para sempre.

À minha família, principalmente ao meu pai, à minha mãe e à minha irmã, obrigada por me permitirem lutar pelos meus sonhos e me apoiarem de forma incondicional. Só vocês sabem o quanto difícil foi esta jornada, obrigada por estarem sempre a meu lado

e nunca me deixarem desistir. Um agradecimento muito especial também ao meu namorado por acreditar em mim e estar sempre ao meu lado nos momentos mais difíceis.

Por último, gostaria de agradecer ao meu Avô, sei que está sempre comigo seja onde for, será sempre a estrelinha mais brilhante. Espero que estejas orgulhoso de mim.

ORIGINAL PUBLICATION

No decurso da presente tese de mestrado foram desenvolvidos artigos científicos destinados à publicação. Os artigos originais, que integram a presente tese foram publicados em revistas científicas indexadas, cujo fator de impacto se encontram no 1 quartil (Q1) da respetiva categoria.

- ✓ **Eira, A.**, Fadoni, J., Amorim, A., & Cainé, L. (2025). Forensic Microbiology: Challenges in Detecting Sexually Transmitted Infections. *Diagnostics*, *15*(10), 1294. https://doi.org/10.3390/diagnostics15101294
- ✓ Cainé, L., **Eira, A**., Fadoni, J., Franco, M., Correia Dias, H., & Amorim, A. (2025). Sexually Transmitted Infections: Usefulness of Molecular Methods for Microorganism Detection in Stored Sexual Assault Samples. *International Journal of Molecular Sciences*, *26*(17), 8124. https://doi.org/10.3390/ijms26178124

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LIST OF ABBREVIATIONS

WHO	World Health Organization
STI	Sexually transmitted infections
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
NAATs	Nucleic Acid Amplification Tests
TMA	Transcription- Mediated Amplification
POC	Point-of-care
DFA	Direct Fluorescent Antibody
EIA	Enzyme Immunoassay
VDRL	Venereal disease research laboratory
RPR	Rapid plasma reagin
FTA-ABS	Fluorescent treponemal antibody absorption assays
TPPA	*T. pallidum* particle agglutination assay
NaCl	Sodium Chloride
F	Forward
R	Reverse
P	Probe

RESUMO

Os crimes sexuais consistem em atos praticados sem o consentimento da vítima e representam uma preocupação global, com repercussões significativas a nível físico, psicológico e social. Estes atos podem originar consequências com efeitos tanto a curto como a longo prazo, destacando-se, entre estas, as infeções sexualmente transmissíveis (IST), frequentemente associadas a este tipo de agressão. As IST representam um importante problema de saúde pública a nível mundial, podendo provocar complicações graves. São causadas por agentes bacterianos, virais ou parasitários, transmitidos através do contacto sexual.

Entre as IST mais prevalentes na população em geral e entre vítimas de crimes sexuais incluem-se a gonorreia, a clamídia, a tricomoníase e a sífilis. A sua deteção é fundamental não só para o acompanhamento clínico das vítimas, mas também no âmbito da investigação criminal, podendo contribuir para a reconstrução dos factos. No entanto, a elevada taxa de infeções assintomáticas representa um desafio diagnóstico relevante, sobretudo em contexto forense.

O presente estudo teve como objetivo avaliar a aplicabilidade da reação em cadeia da polimerase em tempo real (PCR em tempo real) na deteção de *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae* e *Treponema pallidum*, em amostras biológicas recolhidas a vítimas de agressão sexual e armazenadas, por um período até 18 anos, em condições habitualmente utilizadas em laboratórios forenses.

Foram analisadas 231 zaragatoas de 116 indivíduos, colhidas entre 2004 e 2017, utilizando PCR em tempo real com recurso a *primers* e sondas fluorescentes específicas. Detetaram-se 13 casos positivos de *T. vaginalis* (5,6%) e 11 de *C. trachomatis* (4,8%). Não foram identificados resultados positivos para *N. gonorrhoeae* nem *T. pallidum*.

Estes resultados demonstram a sensibilidade e fiabilidade da PCR em tempo real na deteção de IST em amostras forenses preservadas a longo prazo. A deteção de ácido desoxirribonucleico (ADN) dos microrganismos em estudo, em material arquivado, reforça a utilidade das técnicas de diagnóstico molecular na análise retrospetiva de casos de agressão sexual. Este estudo evidencia o valor das metodologias moleculares como ferramenta complementar na investigação forense e na resposta clínica centrada na vítima.

ABSTRACT

Sexual assault refers to acts committed without consent and constitutes a significant global concern with profound implications for victims. Such offences can lead to both acute and long-term consequences affecting physical, psychological, and sexual health. Among these, sexually transmitted infections (STI) represent a frequent and direct outcome. STI, which remain a major global public health issue, can lead to severe complications and are caused by bacteria, viruses, or parasites transmitted through sexual contact. Common STI among both the general population and victims of sexual violence include *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Treponema pallidum*. Their often asymptomatic nature poses substantial diagnostic challenges, particularly in forensic contexts.

The detection of STI in forensic settings is crucial not only for the clinical management of victims but also for evidentiary purposes in criminal investigations. This study aimed to assess the applicability of real-time polymerase chain reaction (PCR) for the detection of *T. vaginalis, C. trachomatis, N. gonorrhoeae,* and *T. pallidum* in biological samples collected from victims of sexual assault and preserved under standard forensic laboratory conditions for up to 18 years.

A total of 231 swabs from 116 individuals, collected between 2004 and 2017, were analysed using real-time PCR with pathogen-specific *primers* and fluorescent probes. The analysis identified 13 positive cases of *T. vaginalis* (5.6%) and 11 of *C. trachomatis* (4.8%). No positive results were obtained for *N. gonorrhoeae* or *T. pallidum*.

These findings highlight the sensitivity and robustness of real-time PCR in detecting STI in long-term preserved forensic samples. The successful amplification of pathogen deoxyribonucleic acid (DNA) from archived material underscores the value of molecular diagnostics for the retrospective analysis of sexual offences, including cold cases. This study reinforces the relevance of molecular techniques as a complementary tool in forensic investigations and in the clinical care of victims.

CHAPTER I – GENERAL INTRODUCTION

The World Health Organization (WHO) defines sexual violence as *"any sexual act, attempt to consummate a sexual act, or other act directed against a person's sexuality by means of coercion, by another person, regardless of their relationship to the victim and in any context. It includes rape, which is defined as penetration, by physical or other coercion, of the vulva or anus with a penis, other body part, or object"* (WHO, 2023).

Sexual violence is a global public health and human rights issue, associated with substantial physical, psychological, and reproductive consequences. According to WHO data, nearly 30% of women worldwide have experienced physical and/or sexual violence in their lifetime. While all populations may be affected, individuals with disabilities, adolescents, institutionalised persons, and those in conflict settings are particularly vulnerable (Hernández Ragpa et al., 2019; Luce et al., 2010).

Despite the gravity of these crimes, conviction rates for sexual offences—including rape and sexual assault—remain low. Forensic evidence plays a vital role in bridging this gap, supporting victim testimony and contributing to successful prosecution. Among the various forms of biological evidence, the detection of sexually transmitted infections (STI) has emerged as a valuable forensic indicator. When appropriately interpreted, the identification of an STI may support claims of non-consensual sexual contact, particularly if newly diagnosed and temporally linked to the alleged event. (Murphy et al., 2022; Hernández Ragpa et al., 2019).

STI—such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum* and *Trichomonas vaginalis*—are among the most prevalent infectious diseases globally, with an estimated 374 million new cases annually (WHO, 2018). These pathogens are typically transmitted through sexual contact involving mucous membranes or body fluids. Most STI are asymptomatic, which contributes to underdiagnosis and facilitates ongoing transmission. Furthermore, untreated infections can lead to serious health complications, including infertility, pelvic inflammatory disease, ectopic pregnancy, and increased susceptibility to HIV (Fasciana et al., 2022; Pair & Somerall, 2024).

From a forensic perspective, timely and accurate STI detection can provide essential evidence in cases of sexual violence. This is particularly relevant in pediatric cases, where the presence of an STI may strongly suggest abuse (Rogstad, 2023). The

application of molecular diagnostic techniques, such as real-time polymerase chain reaction (PCR), has significantly improved the ability to detect pathogenic deoxyribonucleic acid (DNA) in clinical and forensic samples. Real-time PCR enables continuous monitoring of DNA amplification, allowing for high sensitivity and specificity, even in samples stored over extended periods (Artika et al., 2022).

Biological samples relevant to forensic investigation—such as vaginal, anal, oral, or skin swabs—are routinely collected using sterile cotton swabs and may represent the only physical evidence available in sexual assault cases (Magalhães et al., 2015; Newton, 2013). The preservation of such samples is crucial, requiring controlled temperature and humidity conditions to prevent DNA degradation. Inadequate storage or handling may reduce the quantity and quality of the recovered DNA, thereby compromising downstream analysis (Cătălin, 2011).

Several studies have demonstrated that moist or frozen swabs yield significantly higher DNA concentrations compared to dried specimens. Given that forensic samples are often processed at a later time and sometimes in different facilities, freezing biological material shortly after collection has proven effective in maintaining genetic integrity, as previously demonstrated by Newton, M. (2013) (Newton, 2013). Despite this, the long-term viability of DNA for molecular testing in real forensic samples remains under-investigated, especially within the Portuguese context.

This study aims to address this gap by evaluating the applicability of real-time PCR in detecting *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and *T. pallidum* in biological samples collected from sexual assault victims and stored for up to 18 years under routine forensic casework conditions. By demonstrating the reliability of molecular diagnostics in retrospective analysis, this research seeks to contribute to improved practices in forensic microbiology and support the evidentiary role of STI in legal proceedings.

In addition to its scientific and legal relevance, this study underscores the ethical obligation to ensure justice for survivors. The detection of STI-related DNA in archived samples may offer delayed but meaningful opportunities for accountability, therapeutic intervention and epidemiological insight. Enhancing diagnostic accuracy through sensitive molecular techniques strengthens not only the forensic investigation but also the broader commitment to protecting victims' rights and public health.

Detection of Sexually Transmitted Infections

STI are among the most frequently encountered complications in sexual assault investigations. While often clinically silent, their detection may yield critical forensic information by supporting the victim's testimony, identifying possible transmission pathways, and establishing timelines of exposure.

From a diagnostic standpoint, advances in nucleic acid amplification tests (NAATs)—such as PCR and transcription-mediated amplification (TMA)—have significantly increased the ability to detect pathogens in low concentrations and from samples stored for extended periods. These molecular methods are particularly valuable in forensic contexts, where samples are often collected under suboptimal conditions, transported across institutions, and analysed long after the alleged event.

Gonorrhoea

Gonorrhoea is caused by *N. gonorrhoeae*, a Gram-negative diplococcus that displays marked antigenic variation and growing antimicrobial resistance (Unemo et al., 2019; Caruso et al., 2021; Jefferson et al., 2021). It typically infects the urethra in males and the cervix in females, although it can also colonise extragenital sites such as the rectum, pharynx, and conjunctiva. In women, complications may include pelvic inflammatory disease, infertility, and ectopic pregnancy. Neonates may develop ophthalmia neonatorum following vertical transmission during delivery (Pair & Somerall, 2024; Jefferson et al., 2021).

Diagnostic methods:

• Microscopy: Effective in symptomatic male urethral samples; less sensitive in asymptomatic or female cases (Unemo et al., 2019).

• Culture: Enables antimicrobial susceptibility testing but requires viable organisms and specific media (Ng & Martin, 2005).

• NAATs: Highly sensitive and specific; applicable to urine and a range of genital and extragenital samples (Rodríguez-Granger et al., 2020).

• Point-of-care (POC) tests: Offer rapid results but currently lack sufficient validation for forensic purposes.

Chlamydia

C. trachomatis is an obligate intracellular bacterium and one of the most commonly reported STI (Rodrigues et al., 2022; Clarke, 2011). It is frequently asymptomatic, particularly in women, contributing to its underdiagnosis and increasing the risk of complications such as infertility, chronic pelvic pain, and ectopic pregnancy (Rodrigues et al., 2022).

Diagnostic methods:

• NAATs: The most sensitive and reliable diagnostic option; widely used in forensic and clinical settings (Meyer, 2016).

• Cell culture: Technically demanding, less sensitive, and rarely used in routine diagnostics (Black, 1997).

• Direct Fluorescent Antibody (DFA) and Enzyme Immunoassay (EIA): Historical methods now largely replaced due to inferior accuracy.

• POC tests: May be useful in resource-limited environments but are suboptimal for forensic applications.

In the forensic context, *C. trachomatis* can be detected in a wide variety of sample types, including vaginal, cervical, rectal, and urine specimens, with NAATs offering the highest evidentiary value, particularly when symptoms are absent.

Syphilis

Caused by the spirochaete *T. pallidum*, syphilis evolves through distinct clinical stages: primary (chancre), secondary (systemic manifestations), latent (asymptomatic), and tertiary (neurological or cardiovascular involvement). Direct cultivation of the pathogen is not possible, making serology the primary diagnostic tool (Forrestel et al., 2019; Eickhoff & Decker, 2016; Gullette & Hopkins, 2021; Peeling, R. et al., 2004).

Diagnostic methods:

• Serological tests:

- ✓ Non-treponemal tests [e.g., venereal disease research laboratory (VDRL), rapid plasma reagin (RPR)] are useful for screening but prone to false negative (Forrestel et al., 2019).

- ✓ Treponemal tests [e.g., fluorescent treponemal antibody absorption assays (FTA-ABS), *T. pallidum* particle agglutination assay (TPPA)] are more specific and used for confirmation; they may remain reactive for life (Larsen et al., 1995; Forrestel et al., 2019).

• Darkfield microscopy: Allows visualisation of live spirochaetes in early lesions but is rarely available (Purwoko et al., 2021).

• NAATs: Of limited utility in most cases due to low pathogen load in clinical specimens.

Interpretation of syphilis serology in forensic settings requires particular caution. Seropositivity alone cannot determine the timing of transmission, and persistence of treponemal antibodies may complicate interpretation in retrospective investigations.

Trichomoniasis

Trichomoniasis, caused by the protozoan *T. vaginalis*, is more prevalent in women and often asymptomatic in men. It is associated with vaginitis, urethritis, and an increased risk of acquiring or transmitting other STI (Kissinger, 2015; Petrin et al., 1998; Mielczarek & Blaszkowska, 2015).

Diagnostic methods:

• Microscopy (wet mount): Offers immediate visualisation but low sensitivity (Schwebke & Burgess, 2004).

• Culture: Moderately sensitive but time-consuming (Soper, 2004; Garber, 2005).

• Antigen detection assays: Provide faster results; however, sensitivity remains inferior to molecular approaches (Garber, 2005; Kissinger et al., 2022).

• NAATs: Represent the most accurate diagnostic method, detecting infections even in asymptomatic individuals or in long-stored specimens.

Across all four infections, NAATs consistently provide the most robust tool for forensic STI detection. Their ability to identify low levels of genetic material in a wide variety of sample types makes them especially suitable for retrospective case analysis, where pathogen viability may be compromised and precise quantification is necessary.

The high frequency of co-infections among sexual assault victims further supports the potential of multiplex PCR platforms, which allow simultaneous detection of multiple pathogens. However, the introduction of such methods in forensic workflows must be

guided by rigorous validation studies, especially in terms of evidentiary admissibility, timing of infection, and specificity.

Finally, the success of STI detection in forensic science depends not only on the choice of diagnostic method but also on the integrity of sample collection, handling, and storage. Establishing and maintaining strict protocols—particularly regarding temperature, humidity, and contamination control—is essential to preserve DNA quality and ensure the reliability of results.

By enhancing the application of molecular diagnostics, forensic practitioners can strengthen legal investigations, improve support for survivors, and contribute to a more comprehensive public health response to sexual violence.

Material and Methods

Sample Collection

The biological samples analysed in this study were collected from sexual assault victims between 2004 and 2017, originally intended for perpetrator identification. Swab samples were obtained from vaginal, oral, perianal, and rectal sites, including the vulva, perivulvar region, anal margin and canal, perineum, and inner thigh.

To detect the presence of microbial pathogens such as *C. trachomatis, T. vaginalis, N. gonorrhoeae and T. pallidum*, a multi-step analytical workflow was implemented. This included the extraction and purification of genetic material, followed by molecular detection, nucleic acid amplification, and subsequent data analysis.

DNA Extraction and Purification

For sample preparation, a section of each swab containing biological material was cut and transferred into a 1.5 mL microcentrifuge tube containing 1.0 mL of 0.9% sodium chloride (NaCl) solution. Each tube was labelled with the corresponding case identifier. To avoid cross-contamination, scissors and work surfaces were disinfected with 70% ethanol between samples. The tubes were vortexed for approximately 10 seconds and incubated in a dry heating block at 56 °C for 30 minutes to inactivate potential pathogens. After inactivation, samples were stored at −20 °C until DNA extraction, which was performed using the BioRobot EZ1 Advanced automated system (QIAGEN,

Hilden, Germany), and EZ1 DSP virus kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol.

Molecular Detection and DNA Amplification

Real-time PCR was used to detect specific sexually transmitted pathogens. Fluorescent signals were measured using FAM (*T. pallidum*) and HEX (*C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*) fluorophores and pathogen-specific *primers* were employed for each target. The sequences of the *primers* and probes used for amplification are listed in table 1.

Table 1 - *Primer* and probe sequences used for STI detection.

Microorganism	***Primer* sequences (5'- 3')**
C. trachomatis	F: AACCAAGGTCGATGTGATAG R: TCAGATAATTGGCGATTCTT P: ROX-CGAACTCATCGGCGATAAGG
N. gonorrhoeae	F: CCGGAACTGGTTTCATCTGATT R: GTTTCAGCGGCAGCATTCA P: CGTGAAAGTAGCAGGCGTATAGGCGGACTT
T. pallidum	F: GGTAGAAGGGAGGGCTAGTA R: CTAAGATCTCTATTTTCTATAGGTATGG P: ACACAGCACTCGTCTTCAACTCC
T. vaginalis	F: AAG ATG GGT GTT TTA AGC TAG ATA AGG T R: CGT CTT CAA GTA TGC CCC AGT AC P: CCG AAG TTC ATG TCC TCT CCA AGC GT

F = forward; R = reverse; P = probe

These probes hybridize with target DNA sequences and emit fluorescence signals whose intensity correlates with the amount of amplified product in each PCR cycle (Mackay,2004).

Each PCR reaction included 12.5 µL of NZYSupreme Multiplex qPCR Master Mix, 5.5 µL of RNase-free water, and 2 µL of the corresponding primer/probe mix. A total volume of 20 µL of this master mix was combined with 5 µL of extracted DNA in each reaction tube.

Amplification was carried out using the CFX96™ Real-Time PCR Detection System (Bio-Rad, CA, USA), under thermal cycling conditions referred to in table 2.

Table 2 - Thermal cycling conditions for real-time PCR.

Pathogen	Temperature	Time	Cycles
T. vaginalis	50 °C	2 min	1
	95 °C	10 min	1
	95 °C / 60 °C	15 s / 1 min	40
C. trachomatis	95 °C	2 min	1
	95 °C / 60 °C	30 s / 30 s	40
N. gonorrhoeae	95 °C	2 min	1
	95 °C / 55 °C	30 s / 30 s	40
T. pallidum	95 °C / 55 °C / 72 °C	30 s / 30 s / 30 s	50

RNAseP Amplification as Internal Control

To confirm the presence of amplifiable human DNA and validate the reliability of the molecular workflow, RNAseP amplification was performed using *primers* and probes targeting the human gene. The fluorescent signal was measured using HEX fluorophores. Each reaction included 12.5 µL of NZYSpeedy One-Step RT-qPCR Probe Master Mix (2×), 6 µL of RNase-free water, and 1.5 µL of RNAseP primer/probe mix. Then, 5 µL of extracted DNA was added, making a final reaction volume of 25 µL.

Amplification was conducted using the CFX96™ real-time PCR Detection System (Bio-Rad, CA, USA), with the thermal cycling conditions referred to in Table 3.

Table 3 - Thermal cycling conditions for RNAseP gene amplification.

Temperature	Time	Cycles
95 °C	2 min	1
95 °C / 60 °C	5 s / 30 s	40

CHAPTER II – STATE OF THE ART

Sexual assault encompasses all forms of non-consensual sexual contact or behavior and constitutes a major global issue, with severe short- and long-term consequences for victims' mental, sexual, and reproductive health (Farahi & Mceachern, 2021).

Globally, the incidence of STI remains poorly characterized, primarily due to the asymptomatic nature of many infections, limited diagnostic infrastructure in high-burden regions, and inadequate or absent surveillance systems (Fasciana et al., 2022). Incomplete epidemiological data further hinder accurate estimation of true infection rates (Jefferson et al., 2021).

The most extensively studied STI include gonorrhea (*N. gonorrhoeae*), chlamydia (*C. trachomatis*), syphilis (*T. pallidum*), and trichomoniasis (*T. vaginalis*). According to WHO data from 2020, approximately one million new infections involving these pathogens occur daily among individuals aged 15 to 49 years, with chlamydia and trichomoniasis accounting for the majority of cases. Despite these figures, data on STI prevalence among sexual assault victims remain limited. A study by Sachs et al. (2023) reported prevalence rates of approximately 12% for trichomoniasis and 2% for chlamydia in this population (Sachs & Thomas, 2023).

Accurate detection of STI is essential and can be achieved through real-time PCR, a technique that enables the amplification and quantification of target DNA sequences in real time. Owing to its speed, sensitivity, and specificity, real-time PCR is highly valuable in both clinical and forensic contexts, especially for detecting pathogen DNA in long-term stored samples where microbial viability may be compromised (Artika et al., 2022). It is routinely used for pathogen identification, species determination, quantification, and various molecular applications.

Forensic evidence—such as clothing, urine, oral, vaginal, and anal swabs, hand and fingernail scrapings and non-intimate skin samples—is fundamental for suspect identification, the exclusion of innocent individuals, and the overall strengthening of legal proceedings. In cases of sexual assault, biological samples are collected using standardized protocols and sterile swabs to ensure procedural consistency and evidentiary reliability (Newton, 2013; Conte et al., 2025).

Maintaining the forensic integrity of biological samples is critical. Proper collection, handling, and storage are essential to preserve DNA quality and quantity. Samples should be kept in dry, temperature-controlled environments to minimize microbial

contamination and DNA degradation (Murphy et al., 2022). Any mishandling at these stages can compromise the viability of the material for downstream molecular analysis (Magalhães et al., 2015). Given that many forensic samples contain only trace amounts of DNA, strict contamination control, precise collection techniques, and appropriate storage conditions are essential to ensure analytical accuracy and legal admissibility (Cătălin, 2011; Newton, 2013; Alketbi, 2024).

Previous studies have shown that dry storage conditions result in significantly lower DNA recovery compared to samples processed immediately or stored in a moist state. Freezing swabs after collection has been demonstrated to preserve DNA yields comparable to those of freshly collected moist samples and markedly higher than those stored dry (Newton, 2013).

Despite such limitations, swabs remain widely used in forensic practice due to their practicality, ease of transport, efficiency in biological evidence collection, and simplicity in laboratory processing (Ferreira‐Silva et al., 2019).

However, a notable gap persists in the literature regarding the long-term stability and diagnostic utility of biological samples stored for extended periods, particularly in the context of sexual assault investigations. Most existing studies focus on short-term diagnostics, and little is known about the persistence of pathogen DNA in long-preserved forensic specimens.

The retrospective molecular analysis of forensic samples for STI detection also raises ethical and legal concerns, as delays in identification may jeopardize access to justice, timely medical intervention, and effective public health surveillance. Thus, preserving sample integrity and employing highly sensitive molecular diagnostics are essential to uphold victims' rights and ensure the scientific robustness and legal reliability of forensic conclusions.

Although real-time PCR is a widely used technique for STI detection, there is still a lack of research assessing its effectiveness in long-term stored forensic samples. This study therefore aims to contribute to filling this knowledge gap by evaluating the applicability of real-time PCR for detecting pathogenic agents in preserved forensic specimens. The findings may help enhance the quality of forensic analyses and support the judicial system in the context of sexual assault cases.

CHAPTER III– ORIGINAL RESEARCH

Article 1- Forensic Microbiology: Challenges in Detecting Sexually Transmitted Infections

diagnostics

MDPI

Review

Forensic Microbiology: Challenges in Detecting Sexually Transmitted Infections

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Abstract: Sexual assault crimes consist of acts committed without consent and represent a major global issue with serious implications for victims. These acts have both short- and long-term consequences on the physical, mental, and sexual health of victims, with sexually transmitted infections (STIs) being one of the direct outcomes of such crimes. Sexually transmitted infections constitute a serious global public health problem and can lead to severe consequences. These infections may be caused by bacteria, viruses, or parasites and are transmitted through sexual contact. Some of the most common STIs among the general population and victims of sexual crimes include gonorrhoea, chlamydia, trichomoniasis, and syphilis. In most carriers, these infections are asymptomatic, making their detection particularly challenging. Considering the importance of further research in this field, the primary objectives of this study are to review the existing literature on the incidence of major STIs in victims of sexual crimes, to identify the various risk factors associated with these infections, and to explore their public health implications. Additionally, this study aims to assess different STI detection techniques, analyzing their advantages and disadvantages. Studies on this topic are crucial for better understanding the role of sexually transmitted infections in the context of sexual crimes. However, throughout this work, it was verified that point-of-care methods are a good option to allow the diagnosis to be faster and more accurate, when compared to other methods of detecting sexually transmitted infections.

Keywords: sexual crimes; sexually transmitted infections; detection methods; gonorrhoea; chlamydia; syphilis; trichomoniasis

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Academic Editor: Francesco Sessa

Received: 31 March 2025
Revised: 7 May 2025
Accepted: 19 May 2025
Published: 21 May 2025

Citation: Eira, A.; Fadoni, J.; Amorim, A.; Cainé, L. Forensic Microbiology: Challenges in Detecting Sexually Transmitted Infections. *Diagnostics* 2025, *15*, 1294. https://doi.org/10.3390/diagnostics15101294

1. Introduction

Sexual violence is a global issue that affects various aspects of health, including medical and psychological domains [1]. It is both a public health and human rights concern and can be perpetrated against both men and women, although women are at a higher risk [2].

In forensic science, the accurate and timely identification of sexually transmitted infections (STIs) plays a crucial role in the reconstruction of criminal events, particularly in cases involving sexual assault. Forensic microbiology has emerged as a key interdisciplinary domain that bridges traditional microbiological diagnostics with legal investigations. The

ability to detect pathogens in biological samples collected from crime scenes or victims can provide essential corroborative evidence, aiding in suspect identification, timeline estimation, and the establishment of contact or transmission pathways. Although the exact prevalence of sexual violence remains unknown, it is a reality for many individuals [1]. According to the World Health Organization (WHO), "Over the course of their lives, one in three women—around 736 million people—are subjected to physical or sexual violence by their partner or sexual violence by a non-partner" [3]. This high incidence necessitates effective strategies for both medical intervention and forensic investigation.

Sexual assault has serious short- and long-term impacts on the victim's physical, sexual, and reproductive health [2,4]. Among the various associated implications, sexually transmitted infections are a direct consequence of sexual crimes. These infections, caused by bacteria, viruses, or parasites, can be transmitted through body fluids or interaction with the skin through vaginal, oral, or anal sex [5]. They affect individuals of all ages, genders, or sexual orientations and can lead to serious health complications if left untreated [6].

The global incidence of STIs varies widely and remains poorly understood for several reasons. Many infections are asymptomatic, diagnostic procedures are scarce in the most affected regions, and surveillance programs are either absent or underdeveloped in many parts of the world [5]. Additionally, inaccuracies in the epidemiological tracking of STIs further hinder the determination of their true prevalence [7].

The prevalence of these infections varies across countries due to economic differences, cultural perspectives, and the lack of education and prevention of STIs [7].

Currently, four sexually transmitted infections have the known highest prevalence and are the most widely studied worldwide: gonorrhoea, caused by the bacterium *Neisseria gonorrhoeae*; chlamydia, caused by the bacterium *Chlamydia trachomatis*; syphilis, caused by the bacterium *Treponema pallidum*; and trichomoniasis, resulting from infection with the protozoan *Trichomonas vaginalis*. These infections have significant lifelong consequences for victims, making the development of effective, low-cost, and rapid detection methods crucial to facilitate timely diagnosis. The methods used for detection can be direct or indirect, and for each type of infection, different techniques are studied to determine the most suitable approach.

The main aims of this article are to review the existing literature on the incidence of major sexually transmitted infections in victims of sexual crimes, to identify the various risk factors associated with these infections, and to explore their implications for public health. Additionally, this work aims to assess different STI detection techniques, analyzing their advantages and disadvantages, to enhance the quality and effectiveness of care provided to victims.

This review focuses on the most prevalent sexually transmitted infections (STIs) and the available diagnostic tools used for their detection, particularly in the context of sexual crimes. While these infections are clinically significant on their own, their identification may also serve as supporting evidence in forensic investigations, helping to confirm sexual contact, assess the time of infection, and support or refute allegations of abuse. The aim is to provide a concise overview of the main STIs, their symptoms, and diagnostic techniques, with emphasis on their forensic applicability.

2. Methods

The analysis of detection methods considered laboratory tests such as polymerase chain reaction (PCR), serological tests, and rapid tests, focusing on their sensitivity, specificity, and effectiveness in the context of sexual crime victims. The present study is a narrative review; therefore, no formal criteria for study selection were employed.

In November 2024, a search was conducted on the PubMed, Scopus, and Google Scholar databases. The search included the keywords "sexual crimes, sexually transmitted infections, detection methods, Gonorrhoea, Chlamydia, Syphilis, Trichomoniasis", and combinations of these terms were used to refine the search.

The selected articles included clinical trials, observational studies (cohort and cross-sectional), and systematic reviews, with no temporal or linguistic restrictions, that addressed the relationship between sexual crimes and STIs, as well as the methods used for their detection, with a particular emphasis on those applied in forensic settings.

Although a single gold standard test was not formally defined for all infections addressed in this review, it is important to note that molecular diagnostic methods—particularly nucleic acid amplification tests (NAATs), including polymerase chain reaction (PCR) and transcription-mediated amplification (TMA)—alongside culture techniques (e.g., cell culture for *Chlamydia trachomatis* or selective media for *Neisseria gonorrhoeae*), are widely recognized in the scientific literature as reference or gold standard methods. The sensitivity and specificity values presented in this review are those reported in original studies that used such reference methods as comparators to assess the performance of alternative or point-of-care (POC) tests. These comparisons are standard practice in diagnostic accuracy studies, especially in the absence of a universally applicable gold standard across all clinical and forensic settings.

Although this is a narrative review and no formal systematic exclusion criteria were applied, studies were excluded if they did not address the association between sexually transmitted infections and sexual crimes, or if they lacked methodological rigor or relevance to the analysis of diagnostic methods. Preference was given to studies that provided data on diagnostic performance and that were applicable to forensic or clinical contexts.

3. Sexual Violence and STI Implications

The WHO has defined sexual violence as "any sexual act, attempt to consummate a sexual act, or other act directed against a person's sexuality through coercion (. . .) regardless of their relationship with the victim and in any context. (. . .) defined as the penetration by physical or other coercion of the vulva or anus with a penis, other part of the body or object" [3]. It often involves physical force or psychological manipulation, particularly in contexts of power imbalance, such as among individuals with disabilities, adolescents, incarcerated persons, and those in institutional or conflict settings [1,8].

According to the World Health Organization, nearly 30% of women globally have experienced physical and/or sexual violence by an intimate partner or non-partner during their lifetime. Sexual violence can result in a range of physical, reproductive, psychological, and social consequences, including injuries, unwanted pregnancies, sexually transmitted infections, and long-term mental health disorders. Table A1 summarizes the potential consequences across these domains [3,9].

To better understand the global impact of STIs in the context of sexual violence, Table 1 summarizes WHO-estimated prevalence rates across different regions.

As sexual violence is a major risk factor for the acquisition of STIs, understanding its global distribution is essential. Figure 1 shows the estimated lifetime prevalence of intimate partner violence by region, based on WHO data. However, it is important to note that this figure does not reflect the full spectrum of sexual violence, particularly assaults committed by non-intimate partners. Data on non-intimate partner sexual violence remain scarce and less systematically recorded across global health systems, despite their relevance in forensic and clinical contexts. This gap underscores the need for improved surveillance and reporting mechanisms that capture all forms of sexual violence, regardless of the relationship between victim and perpetrator.

Table 1. Estimated prevalence of major STIs by WHO region. This table presents the estimated prevalence of the four most common sexually transmitted infections—syphilis, chlamydia, gonorrhoea, and trichomoniasis—across different WHO regions, adapted from WHO data (adapted from [3]).

WHO Region	Syphilis (%)	Chlamydia (%)	Gonorrhoea (%)	Trichomoniasis (%)
Africa	3.5	6.0	2.9	11.0
Americas	0.5	4.2	0.7	3.0
Eastern Mediterranean	1.2	3.8	0.9	4.1
Europe	0.3	3.5	0.4	1.2
South-East Asia	0.7	5.0	1.0	5.3
Western Pacific	0.4	4.0	0.6	2.8

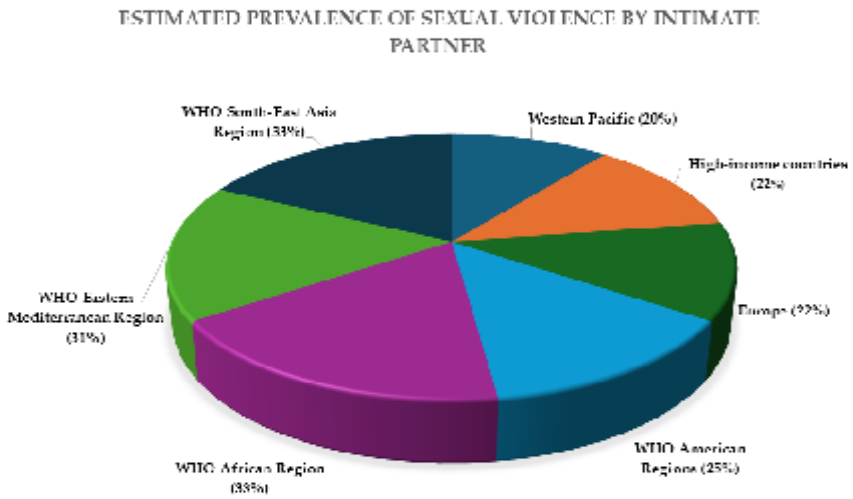

Figure 1. Estimated lifetime prevalence of intimate partner violence in different regions of the world (adapted from the [3]).

4. Sexually Transmitted Infections (STIs)

Often, when STIs are detected in adults, they may be used as evidence of sexual assault or to question the victim's previous sexual behavior. In the case of children, the presence of an STI can be a crucial indicator of sexual violence or may help establish a link between the perpetrator and the victim [10].

Globally, there are approximately 1 million new STI cases every day, with an estimated 374 million new infections annually, including chlamydia, gonorrhoea, syphilis, and trichomoniasis. The prevalence of STIs varies depending on the specific infection and the economic conditions of each country, typically being higher in regions with less-resourced healthcare systems [10].

The WHO [3] reports an increasing number of untreated STI cases worldwide due to treatment failures and the fact that most infections remain asymptomatic in the majority of affected individuals. Although most STIs are non-fatal, they can lead to severe health complications. The greatest risks associated with STIs stem from unprotected sex, an increase in the number of sexual partners, and the emergence of new sexual partners [11].

While global prevalence data for sexually transmitted infections are well established, specific data linking STI incidence directly to cases of sexual assault are limited and often underreported. This is partly due to the sensitive nature of sexual violence, variations in reporting practices, and the asymptomatic nature of many STIs. Studies have shown that sexual assault victims are at elevated risk of acquiring STIs due to forced, unprotected intercourse and potential exposure to multiple pathogens. However, quantifying this risk remains challenging due to inconsistent data collection across regions and the overlap with pre-existing infections. The presence of an STI may support allegations of non-consensual sexual contact, particularly when it is newly diagnosed and the timing of transmission can be reasonably inferred. Nonetheless, additional epidemiological research is needed to more precisely estimate the prevalence and patterns of STI transmission specifically attributable to sexual violence.

The following sections (Sections 4.1–4.4) present the four most prevalent sexually transmitted infections—gonorrhoea, chlamydia, syphilis, and trichomoniasis—commonly observed in the general population and among victims of sexual assault. For each infection, a brief clinical overview is provided to describe key symptoms and possible complications, followed by a summary of the most relevant diagnostic methods, with emphasis on their applicability in forensic contexts.

4.1. Gonorrhoea

Gonorrhoea is a sexually transmitted infection caused by *Neisseria gonorrhoeae*, a Gram-negative diplococcus belonging to the class *Betaproteobacteria* and the family Neisseriaceae [12,13].

This infection is considered a serious global health concern due to the high number of asymptomatic cases, the bacterium's ability to undergo significant antigenic variation on its surface, and the emergence of antibiotic-resistant strains [7].

Neisseria gonorrhoeae is an obligate human pathogen [12] that affects the lower genital tract, pharynx, and rectum, with different complications depending on the sex of the individual. In women, the most commonly affected site is the cervix, whereas in men, it is the anterior urethra. The most prevalent symptoms include purulent cervical or urethral discharge, discomfort, dysuria, urethritis, or cervicitis [7], typically appearing 2 to 10 days after infection [6].

Individuals with rectal infections may experience anal discharge, discomfort, and pain in the affected area. Gonorrhoea is also one of the leading causes of pelvic inflammatory disease (PID), which can result in chronic abdominal pain, dyspareunia, infertility, or ectopic pregnancy [6]. Additionally, this bacterium can be vertically transmitted, meaning it can be passed from the mother to the child during pregnancy, childbirth, or breastfeeding. It has the potential to cause chorioamnionitis, septic abortion, preterm birth, and neonatal conjunctivitis, posing a significant risk to vision [7].

In forensic cases, the identification of *N. gonorrhoeae* in a victim may support allegations of recent sexual contact, particularly when clinical symptoms are present and corroborated by microbiological evidence.

4.1.1. Methods for Detecting *Neisseria gonorrhoeae*

Microscopy

Microscopic analysis is a cost-effective method that does not require highly sophisticated equipment and can be performed during the initial medical examination of the victim [14]. This technique is characterized by its high sensitivity and specificity and serves as a rapid diagnostic test [15].

In symptomatic men, microscopy combined with Gram staining enables the identification of diplococci within polymorphonuclear leukocytes, a key criterion for the presumptive diagnosis of gonorrhoea [15]. Gram staining is a rapid differential technique that distinguishes bacteria based on cell wall structure. In this method, *Neisseria gonorrhoeae*, a Gram-negative diplococcus, appears as pink, kidney-shaped cells often located within polymorphonuclear leukocytes. This allows for a quick presumptive diagnosis, particularly in symptomatic male patients.

Higher sensitivity and specificity have been observed in urethral swab samples from symptomatic men, whereas lower sensitivity has been reported in urethral samples from asymptomatic men and endocervical or urethral samples from women. This discrepancy may be attributed to the lower bacterial load in urethral samples and the presence of other bacterial species in endocervical samples [12].

However, this technique has certain limitations, as its accuracy depends on the technician's expertise, and microscopic examination must be conducted within 10 min of sample collection to ensure reliable results [14].

As an alternative to Gram staining, methylene blue staining has been proposed, demonstrating high sensitivity and specificity in the diagnosis of gonococcal urethritis in men [12].

Culture

The technique of culturing microorganisms on specific media demonstrates good sensitivity and specificity. Although it does not yield results for all sample types, it is effective for urethral and cervical samples, whereas conjunctival, rectal, and oropharyngeal samples require optimal growth conditions for successful culture [15].

For the detection of this bacterium, the primary species must be cultured in the medium of non-selective chocolate agar and selective agar that have antimicrobial agents that inhibit the growth of bacteria and fungi. Culture media that have incorporated antibacterial and antifungal agents are Thayer–Martin, Martin Lewis, and New York City [15].

This technique has evolved over time, enhancing its efficiency and precision, particularly with the integration of MALDI-TOF mass spectrometry [14]. This method allows for the direct identification of bacteria from agar plates, generating a unique spectral profile that correlates with the specific microorganism [15]. Another method is Vitek 2 (BioMérieux, Marcy-l'Ētoile, France), an automated microbiological identification system based on biochemical and enzymatic profiling, which allows for the rapid identification of a variety of bacterial and fungal species, including *Neisseria* species [16].

However, one of the main drawbacks of this technique is its relatively slow process, which can also be costly in certain cases. Additionally, *Neisseria gonorrhoeae* is a fragile bacterium, and its culture may exhibit low sensitivity due to inadequate transportation conditions [14]. Certain transport media have been developed to maintain bacterial viability for up to 48 h at room temperature [17].

Nucleic Acid Amplification Tests (NAATs)

Compared to culture, NAAT (Nucleic Acid Amplification Test)—a group of molecular diagnostic methods that amplify the genetic material of pathogens—offers higher sensitivity and can be applied to a larger number of samples. NAATs include techniques such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), transcription-mediated amplification (TMA), and strand displacement amplification (SDA) while requiring less stringent conditions for transport and storage [17], as they do not necessitate viable organisms for detection [14].

One of the key advantages of this technique is its rapid response time and higher sensitivity compared to other methods. Additionally, this procedure can be automated, performed by individuals with minimal experience, and multiplexed, allowing for the simultaneous detection of multiple microorganisms from a single sample [14].

PCR-based assays

Different types of PCRs, including single-step, nested, real-time, singleplex, and multiplex PCR, are utilized to simplify diagnosis and reduce turnaround time. This technique exhibits very high sensitivity and specificity and is particularly valuable, as it enables the detection of multiple microorganisms within a single sample.

However, its main limitations stem from the requirement for specialized instruments and laboratory facilities, which may not be readily available in all settings [14].

Isothermal and microfluidic amplification tests

Compared to PCR-based tests, this technique is faster and more cost-effective for detecting STIs. In this process, DNA/RNA (deoxyribonucleic acid/ribonucleic acid) amplification occurs at a lower and constant temperature without requiring thermal cycling, thereby reducing the time needed to obtain results. Additionally, results can be observed and evaluated visually by detecting turbidity or color changes in the reaction tube [14].

This technique encompasses several molecular diagnostic methods that involve various chemical reactions. The primary methods used for STI detection include loop-mediated isothermal amplification (LAMP), transcription-mediated amplification (TMA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), and recombinase polymerase amplification (RPA). Furthermore, the enzymes utilized in these methods are less affected by inhibitory substances present in the samples, which reduces both the time and cost associated with sample preparation and extraction [14].

However, these techniques also present certain limitations. False positives may arise during amplification, primer design for detecting bacterial genetic sequences can be challenging, and the final amplification products often vary in length, making them difficult to use in subsequent applications [18].

Sensitive Detection Without Amplification

To minimize sample handling and processing time, alternative molecular methods have been developed that allow direct detection of DNA or RNA from clinical samples without requiring amplification. One example is microwave-accelerated metal-enhanced fluorescence (MAMEF), which extracts nucleic acids via low-power microwaves and uses enhanced fluorescence for detection [14].

It is important to note that, although these methods involve nucleic acid detection, they do not fall under the category of NAATs, as they do not include an amplification step. As such, they represent a distinct class of diagnostic tools that may be suitable for point-of-care use, though further development is needed to adapt these techniques to complex biological matrices such as serum or whole blood.

However, a major limitation of this method is that it is currently validated only for assays involving synthetic or laboratory-prepared samples suspended in buffer solutions. Its application to real biological specimens—such as vaginal swabs, serum, whole blood, or saliva—remains limited and requires further development [19].

This method demonstrates good sensitivity and concordance with PCR, making it a promising approach for use in point-of-care (POC) diagnostic tests [14].

Immunological Tests

This technique allows the detection of antigens or the presence of antibodies produced by the immune system at the time of infection, with lateral flow immunochromatographic

tests being used [14]. When infection caused by the bacterium *Neisseria gonorrhoeae* occurs, there is an increase in IgG and IgA, and these antibodies are detected using immunological methods [20].

These tests exhibit high specificity and can provide results within 25 to 40 min; however, their sensitivity is highly variable, often being low in many cases.

This issue is common across multiple sexually transmitted infections (STIs), and the WHO does not recommend their use until more effective diagnostic methods become available.

The main disadvantages of this method include the visual interpretation of results, which may lead to operator errors, as well as the potential for detecting past infections, rather than active ones [14].

4.2. *Chlamydia*

Chlamydia trachomatis (CT) is a gram-negative, obligate intracellular bacterium [21,22] that belongs to the order *Chlamydiales* within the *Chlamydiaceae* family [23]. Humans are its only natural host [24], and it is the causative agent of chlamydia.

Chlamydia is often an asymptomatic infection, which contributes to a higher risk of transmission [19]. In symptomatic cases, the incubation period typically ranges from 1 to 3 weeks [25].

In women, this infection can lead to long-term complications, affecting both the lower and upper reproductive systems, including the uterus, fallopian tubes, and ovaries. Potential consequences include chronic pelvic pain, pelvic inflammatory disease, an increased risk of ectopic pregnancy, and neonatal complications through vertical transmission, such as conjunctivitis and/or pneumonia. Additionally, it may result in infertility, gynecological tumors [21], endometritis, or peri-hepatitis [24].

In men, *C. trachomatis* infection can cause urethritis [21] and epididymitis, which may lead to fever, scrotal pain, and swelling. Prostatitis can result in pain during and after sexual intercourse, chills, painful urination, and lower back pain [26]. Furthermore, this infection can contribute to male infertility [21], orchiepididymitis, lymphogranuloma venereum [25], ocular infections such as conjunctivitis, and can also affect the throat and rectum [26].

From a forensic perspective, chlamydial infection can be an important marker of sexual exposure, especially in children or individuals unable to provide informed consent, where its detection may strongly suggest abuse.

4.2.1. Methods for Detecting *Chamydia trachomatis*

Culture

This technique requires viable bacteria, and its detection rate ranges from 60% to 80%, even when performed in specialized laboratories by trained professionals [27].

For the detection of this bacterium, the culture method is carried out in cell lines capable of supporting its intracellular growth. The most commonly used are McCoy, HeLa cells, a human epithelial cell line derived from cervical cancer, and BGMK (Buffalo Green Monkey Kidney) cells, a non-human primate epithelial cell line. These cell types provide a suitable environment for the propagation of *Chlamydia trachomatis* [28].

Due to the rigorous care required in the collection, transportation, and processing of samples, this method presents significant disadvantages, as these factors can interfere with its sensitivity, which may vary between 70% and 80%. Other drawbacks include its labor-intensive nature [29] and time-consuming process, as results may take 3 to 7 days to be obtained [28].

Additionally, toxic substances may sometimes be present in clinical samples, and commensal microbes can overgrow, further complicating the analysis [27].

Antigen Detection Methods

In this technique, bacterial viability is not required, and no specific precautions are necessary for sample collection and transport, unlike culture-based methods [29].

A direct fluorescent antibody (DFA) has been developed for direct application to clinical samples. This technique utilizes fluorescein isothiocyanate-labeled monoclonal antibodies that bind to Chlamydia lipopolysaccharide (LPS) or the major outer membrane protein (MOMP). The use of MOMP-specific antibodies has significantly improved specificity to 98–99% and sensitivity to 80–90% compared to culture methods. Additionally, this method is rapid, with a turnaround time of approximately 30 min [29].

Another developed test is the enzyme immunoassay (EIA), which detects specific LPS antigens that are more abundant and soluble than MOMP [29]. Detection can be performed using either monoclonal or polyclonal antibodies; however, this technique requires a specialized technician [30]. The manual procedure typically takes around 3–4 h [28]. A major drawback of this technique is that LPS-specific antibodies can cross-react with LPS from other bacteria, leading to false-positive results [29].

Several EIA-based commercial tests are available, including Chlamydiazyme (Abbott Diagnostics, North Chicago, IL, USA) and Microtrak EIA (Behring, Palo Alto, CA, EUA) [28].

Serological Test

The serological methods developed for detecting Chlamydia infections include the microimmunofluorescence (MIF) test and the enzyme-linked immunosorbent assay (ELISA), which allow the detection of the IgG antibody that is produced during an acute infection caused by the bacteria [28].

The MIF test has been identified as the most sensitive method, capable of providing specific data. However, it has not been widely adopted as a diagnostic tool due to its limitations and high cost [29]. Additionally, this method is highly time-consuming and labor-intensive, and fluorescence signal interpretation is prone to errors [27].

Conversely, the ELISA test, which utilizes lipopolysaccharide (LPS) from the *Chlamydia* genus, can yield false-positive results due to the possibility of cross-reactions with other *Chlamydia* species. Furthermore, as most antibodies produced during infection remain in circulation, a positive result does not necessarily indicate an active infection [29].

Microchip-Based Multiplexed Immunoassay

This type of method enables the simultaneous performance of multiple immunoassays from a small vaginal sample. In this context, the liposomal nanovesicle technique could facilitate the development of a signal amplification system, thereby increasing the sensitivity of this type of assay [31].

This method utilizes small amounts of purified, high-density proteins, which are immobilized on a microscope slide. Specific antibodies are then introduced, and their presence is detected through fluorescent markers or radioisotopes, provided that the antibody binds to the target protein [32].

Multiplex immunoassays are characterized by high clinical sensitivity, as they are capable of detecting antibodies or antigens in patient samples with high accuracy, even when only small sample volumes are available. When an inflammatory disease is triggered by multiple pathogens, it is crucial to detect all of them within a single reaction, a capability provided by multiplex methods [31].

Multiplex PCR techniques enable the simultaneous detection of multiple pathogens from a single clinical sample. In the context of STIs, this approach is particularly valuable for screening infections such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas*

vaginalis in one reaction, improving diagnostic efficiency and reducing the need for multiple tests (Figures 2 and 3).

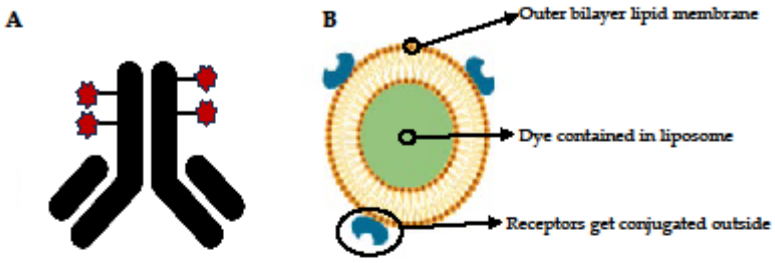

Figure 2. The structure of the liposomal nanovesicles and detection antibody. (**A**) Detection antibody conjugated with biotin. (**B**) Liposomal nanovesicle (adapted from [31]).

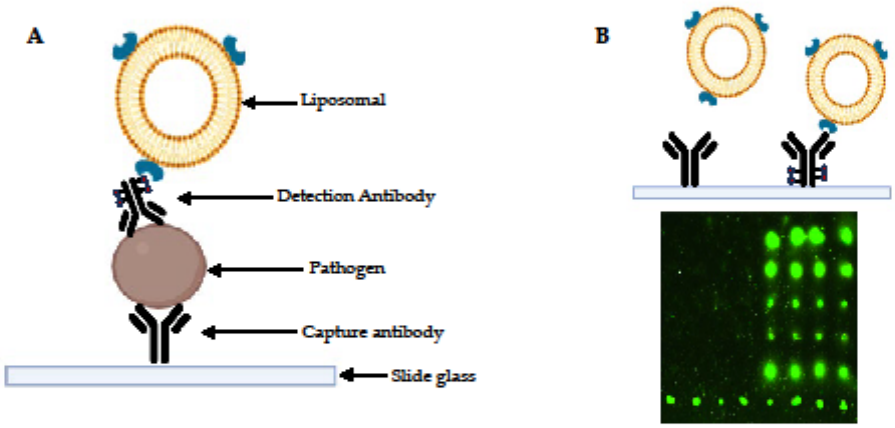

Figure 3. Microorganism detection using a microarray chip and representative examples: (**A**) Overview of the detection process; (**B**) illustrative systems utilizing either native or antibody-conjugated glass surfaces, with positive detection demonstrated in the latter (adapted from [31]).

Molecular Techniques

One of the methods developed for the detection of Chlamydia infections is the nucleic acid hybridization (NAH) technique, with the PACE 2 test (Gen-Probe, San Diego, CA, USA) being the most widely used [29]. The PACE 2 test is a nucleic acid hybridization assay that uses a chemiluminescent DNA probe to detect ribosomal RNA specific to *Chlamydia trachomatis*. The PACE 2C version also allows simultaneous detection of *Neisseria gonorrhoeae*. These tests provide rapid and specific results without the need for nucleic acid amplification [27,29]. The procedure for this test takes approximately 2–3 h, and the technical expertise required is comparable to that needed for enzyme immunoassay (EIA) tests, although automation is possible [28]. These tests provide rapid and specific results without the need for nucleic acid amplification.

Another nucleic acid amplification test (NAAT) test, Hybrid Capture II (Digene Corporation, Gaithersburg, MD, USA), incorporates a signal amplification component to enhance sensitivity [29].

Further advancements in nucleic acid amplification testing (NAAT) have led to the development of polymerase chain reaction (PCR) and ligase chain reaction (LCR) methods

for the diagnosis of Chlamydia infections [29]. These nucleic acid amplification techniques, such as PCR and TMA, exhibit high analytical sensitivity, enabling the detection of minute quantities of bacterial DNA or RNA in clinical samples, often down to a few copies per reaction. In real-world settings, they have also demonstrated high clinical sensitivity, effectively identifying infected individuals, including those with low bacterial loads or asymptomatic cases. When combined with automated nucleic acid extraction, results can be obtained within a few hours [27].

The sensitivity of these methods has been further enhanced through the implementation of pre-analytical steps involving coated magnetic beads, which improve both the quantity and quality of nucleic acid separation [27].

Following these advancements, new molecular technologies have emerged for Chlamydia detection, including TMA-based (transcription-mediated amplification) tests such as APTIMA Combo 2 (Gen-Probe Inc., San Diego, CA, USA) and ProbeTec (BD Diagnostic Systems, Franklin Lakes, NJ, USA) [29]. NAATs (Nucleic Acid Amplification Tests) specifically designed to detect *C. trachomatis* plasmid DNA include the COBAS Amplicor *C. trachomatis* test (Roche Diagnostic Systems, Basel, Switzerland), which employs PCR that has synthetic oligonucleotide primers that bind to specific sequences of this bacterium, and the BDProbeTec™ ET test (Becton Dickinson, NJ, USA), which utilizes SDA (strand displacement amplification) [30].

Another NAAT method, the Cepheid Xpert CT/NG (Sunnyvale, CA, USA), is a rapid and user-friendly real-time PCR assay. It operates within a closed system, requires minimal operator intervention, and delivers quick and efficient results [29].

Despite their advantages, these techniques also present certain limitations. They require trained professionals and specialized laboratory equipment, making them costly and difficult to implement in resource-limited settings [29]. Additionally, these methods may cross-react with other *Chlamydia* species and can be affected by transport conditions [31].

4.3. Syphilis

4.3.1. Overview and Clinical Manifestations

Syphilis is a multisystemic sexually transmitted infection [33] caused by the bacterium *Treponema pallidum*, a spirochete that affects only humans and cannot be cultivated in vitro [34]. It is transmitted primarily through direct contact with infectious mucocutaneous lesions or via vertical transmission from mother to fetus during pregnancy [35]. The disease progresses through four clinical stages—primary, secondary, latent, and tertiary—each with distinct characteristics [36].

- **Primary syphilis** typically presents as a painless ulcer or chancre at the site of inoculation, appearing around 21 days after exposure. It may go unnoticed and spontaneously heals within 3–6 weeks. Regional lymphadenopathy is often presented [34–36].
- **Secondary syphilis** occurs 3 to 12 weeks later and is characterized by a widespread maculopapular rash, mucocutaneous lesions, lymphadenopathy, and systemic symptoms such as fever, malaise, and arthralgia. This stage remains highly infectious and may resolve without treatment [34,35].
- **Latent syphilis** is an asymptomatic stage that follows the resolution of primary and secondary symptoms. It is divided into early latent (<1 year post-infection) and late latent (>1 year). The disease remains serologically detectable but is not transmissible sexually [35].
- **Tertiary syphilis** may develop 10–20 years after the initial infection in untreated individuals. It is associated with severe complications including cardiovascular lesions (e.g., aortitis), gummatous skin or bone lesions, and neurosyphilis [35–38].

- **Neurosyphilis** can occur at any stage and may be asymptomatic or present with neurological and ophthalmic manifestations, such as cranial nerve dysfunction, meningitis, stroke, and progressive dementia. Parenchymatous neurosyphilis may also lead to psychiatric symptoms, motor disturbances, and sphincter dysfunction [34,35,39].
- **Congenital syphilis** results from transplacental transmission and can lead to miscarriage, stillbirth, or severe neonatal complications. It is classified as early (manifesting before age 2) or late (after age 2), with clinical signs ranging from hepatosplenomegaly and skin lesions to sensorineural deafness and dental abnormalities [40].

The presence of syphilitic lesions or serological evidence in alleged victims of sexual assault can support forensic conclusions, particularly when infection is newly acquired and correlated with other findings.

Syphilis progresses through distinct clinical stages—primary, secondary, latent, and tertiary—each with characteristic symptoms and varying degrees of severity. A summary of the signs and complications associated with each stage is provided in Table A2. These stages may include painless chancres, mucocutaneous lesions, neurological and cardiovascular complications, and, in untreated cases, irreversible damage. Congenital and neurosyphilis can also occur and pose significant diagnostic and clinical challenges.

4.3.2. Methods for Detecting *Treponema pallidum*

Animal Inoculation Test

This type of test utilizes various animal models to study the infection capacity of *T. pallidum*, with rabbits being the most commonly used, as the resulting pathological changes can be observed macroscopically. The test demonstrates a sensitivity and specificity of 100% [41].

Samples for this test can be used as long as they have been collected within one hour or have been immediately frozen and stored in liquid nitrogen at temperatures of −78 °C or lower [42]. However, this method has several disadvantages, including the requirement for specialized technicians, ethical concerns due to the use of animals, and a lengthy processing time [41].

Histopathological Staining

In this method, tissue samples are fixed with formalin and stained using silver stain or immunohistochemistry, enabling the visualization of spirochetes or treponemes [34]. However, when silver staining techniques, including Warthin–Starry staining, are applied to tissue from primary or secondary syphilis lesions, the results can be challenging to interpret due to low sensitivity and specificity.

In contrast, immunohistochemical staining, which utilizes fluorescent antibodies against *T. pallidum*, is a more sensitive and highly specific method for detecting the bacterium [34,43].

Microscopy

Dark-field microscopy is particularly useful for early detection of *T. pallidum*, allowing direct observation of its morphology. Detection must be performed within 30 min of sample collection, as bacterial motility is the key characteristic for diagnosis [41].

A positive result provides specific and direct confirmation of syphilis. However, a negative result does not exclude infection, as the bacterial load may be too low for detection, the lesion may be in the healing stage, or an excessive number of surrounding cells may interfere with visualization. The sensitivity and specificity of this technique range from 75% to 100% and 94% to 100% for primary lesions, respectively, while for secondary lesions, sensitivity ranges from 58% to 71%, with a specificity of 100% [44].

Despite its diagnostic value, this method has limitations, including the need for a specialized microscope and trained personnel [34]. Furthermore, it is not recommended for routine diagnosis, as the samples are highly sensitive to light variations and the presence of extraneous particles, which may affect accuracy [41].

Direct Fluorescence Antibody (DFA)

In this method, the antigen–antibody interaction is detected using fluorescence with fluorescein isothiocyanate (FITC), producing a mahogany–green coloration, without requiring live bacteria [41]. This technique can differentiate pathogenic from non-pathogenic treponemes by detecting specific antigen–antibody reactions [42].

Monoclonal antibodies used in this method offer a sensitivity range of 73–100% and a specificity of 100%, while polyclonal antibodies provide a sensitivity of 86–90% and a specificity of 96–97% [41].

However, this technique has several drawbacks, including high costs and the fragility of fluorescent materials, which are easily degraded [41]. Additionally, it requires specialized equipment and trained personnel [34] and has a longer turnaround time, with results typically obtained within 1–2 days [44].

Polymerase Chain Reaction (PCR)

The PCR-based tests developed for syphilis detection include conventional PCR, nested PCR, real-time PCR, and multiplex PCR, all of which offer rapid results [45].

A study conducted by Matt Shields [46] demonstrated that conventional PCR has a sensitivity ranging from 84.6% to 89.1% and a specificity of 93.1% to 100% for primary syphilis cases. However, for secondary syphilis, the sensitivity decreases to 50%, indicating that conventional PCR is most suitable for detecting primary syphilis. In contrast, nested PCR offers higher specificity and sensitivity compared to conventional PCR, as it utilizes a probe that enhances amplification accuracy, achieving a specificity of up to 95%, though its sensitivity remains below 70% [45].

Real-time PCR (qPCR) enables both detection and quantification of pathogen DNA by measuring fluorescence in real time during amplification. Using a standard curve derived from known DNA concentrations, the initial quantity of target DNA in a sample can be estimated, providing an indirect measure of infection load. However, the accuracy of quantification depends on the sample type and DNA extraction process. Despite this limitation, real-time PCR is widely used due to its speed and ease of implementation in laboratory settings [45].

Multiplex real-time PCR enables the simultaneous detection of multiple pathogens and their quantification without cross-interference, making it particularly useful for individuals with suspected co-infections. This technique reduces costs and detection time by allowing multiple amplifications to be monitored in a single reaction [45]. Additionally, a reverse transcriptase PCR test has also been described for syphilis detection [44].

Serological Test

Serological tests for syphilis are divided into two categories: treponemal and non-treponemal tests. Treponemal tests detect antibodies that specifically target *Treponema pallidum* antigens and are generally used to confirm infection, while non-treponemal tests detect antibodies produced in response to cellular damage caused by the infection. The latter are often used for screening and monitoring disease activity or treatment response.

Non-treponemal tests

Non-treponemal tests include the Venereal Disease Research Laboratory (VDRL) test, toluidine red unheated serum test (TRUST), unheated serum reagin (USR), and rapid plasma reagin (RPR) test, with antibodies typically detectable six weeks after infection [41].

These methods rely on detecting tissue damage caused by syphilis through the production of antigens for cardiolipin, cholesterol, and lecithin—common components of human cells [34]. This enables the detection of immunoglobulin M (IgM) and immunoglobulin G (IgG), as well as antibodies produced in response to *T. pallidum* infection [47]. However, false-negative results may occur, particularly in early- or late-stage infections or due to the prozone phenomenon. The latter arises when high antibody concentrations interfere with antigen–antibody binding, which is essential for reactive results [34].

Non-treponemal tests offer several advantages, including widespread availability, low cost, applicability to various sample types, and utility in monitoring treatment effectiveness. However, they also have limitations, such as low sensitivity and the potential for prozone or false-positive results [42].

RPR tests use charcoal particles to facilitate the detection of flocculation, allowing macroscopic analysis of the antigen–antibody complex, while TRUST tests utilize toluidine red dye for visualization. Although most non-treponemal tests are manually performed, some have been automated to improve efficiency and reliability [44].

Treponemal tests

This type of test quantifies the levels of IgM and IgG antibodies that bind to *T. pallidum* proteins. It includes fluorescent treponemal antibody absorption assays (FTA-Abs), agglutination tests such as *T. pallidum* particle agglutination assay (TPPA) and *T. pallidum* hemagglutination assay (TPHA), enzyme immunoassays (EIAs) for various treponemal antigens, rapid diagnostic tests [41], and the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP) [36].

If primary syphilis is suspected and non-treponemal tests yield non-reactive results, treponemal tests should be conducted, as they have a sensitivity close to 100% in primary syphilis and exceed 95% in secondary and tertiary syphilis, respectively [34]. These values correspond to clinical sensitivity, reflecting the performance of these tests in detecting true-positive cases among symptomatic and asymptomatic patients across different disease stages.

These tests use *T. pallidum* as the antigen and can detect antibodies within 2 to 4 weeks after exposure [33,48]. They are employed to verify the reactivity of non-treponemal tests and to confirm syphilis in cases where non-treponemal tests are non-reactive despite clinical symptoms [42].

Individuals with reactive treponemal test results are likely to remain positive for life, as these tests do not differentiate between past and current infections [34]. Additionally, treponemal tests are more complex and expensive to perform and are not suitable for monitoring treatment effectiveness. However, when both treponemal and non-treponemal tests yield reactive results, the specificity of the diagnosis is significantly increased [42].

4.4. Trichomoniasis

This infection is caused by *Trichomonas vaginalis* [49], a protozoan parasite belonging to the *Trichomonadidae* family. The incubation period ranges from approximately 5 to 28 days. *T. vaginalis* is a primitive eukaryote with a carbohydrate and energy metabolism similar to that of anaerobic bacteria. It is an obligate human parasite, lacking the ability to synthesize essential macromolecules such as purines, pyrimidines, and lipids, which it acquires from vaginal secretions or through the phagocytosis of host and bacterial cells [50].

The majority of infected individuals remain asymptomatic, with 84% of women and 77% of men exhibiting no symptoms [51]. This sexually transmitted infection (STI) can be classified as acute, chronic, or asymptomatic, depending on the severity of the infection [52].

In cases of acute infection, symptoms include a frothy, yellow or green mucopurulent discharge. Additionally, small punctate hemorrhagic lesions, known as the "strawberry

appearance", may develop on the vaginal and cervical mucosa, occurring in approximately 2% of cases. Chronic infection, on the other hand, presents with milder symptoms, such as itching, dyspareunia, and scant vaginal discharge mixed with mucus. This stage is particularly significant, as individuals with chronic infection are more likely to transmit the parasite [52].

In men, the infection typically resolves within 10 days. Symptomatic cases may present with mild manifestations, including scant, clear to mucopurulent discharge, dysuria, and a mild itching or burning sensation following sexual intercourse [50]. Moreover, *T. vaginalis* infection has been associated with male infertility, as it can contribute to urethritis, prostatitis, epididymitis, and reproductive complications due to inflammatory lesions or impaired sperm production [53].

Approximately 25–50% of women remain asymptomatic during the first six months of infection. If left untreated, *T. vaginalis* can persist indefinitely in the lower urogenital tract [53].

In symptomatic women, vaginitis may develop, and less frequently, adnexitis, which can trigger inflammatory responses in the genital mucosa. This increases the risk of PID through micro-bleeding and heightens susceptibility to cervical neoplasia [53]. Additional symptoms may include vaginal discharge—often diffuse, malodorous, and yellow-green-dysuria, itching, vulvar irritation, and abdominal pain [51]. Furthermore, it may cause vulvar itching and vaginal odor [54], as well as edema, erythema, and colpitis macularis (commonly referred to as "strawberry cervix"), which results from microscopic cervical bleeding [50,55]. The infection can also impact female fertility and contribute to chronic infection of the reproductive tract [53].

During pregnancy, *T. vaginalis* infection can lead to premature rupture of membranes, preterm birth, and low birth weight. Vertical transmission may occur when the newborn comes into direct contact with the maternal genital tract during childbirth or through exposure to infected maternal fluids. In some cases, the infection may also manifest in the infant's respiratory tract [53]. Additionally, *T. vaginalis* has been associated with an increased risk of cervical cancer [55].

Although often asymptomatic, the detection of *T. vaginalis* in post-assault examinations may serve as supportive evidence of sexual contact, particularly when identified in conjunction with other sexually transmitted pathogens.

4.4.1. Methods for Detecting *Trichomonas vaginalis*

Microscopy

This method relies on the presence of a sufficient number of viable, motile microorganisms in the samples prepared for wet mounting [55]. The motility of *T. vaginalis* is temperature-dependent, as these microorganisms can remain viable for approximately six hours at room temperature in phosphate-buffered saline, with their motility gradually decreasing over time. The samples that can be used for this type of technique are vaginal, cervical and secretion samples from the urethra or prostate [56].

The test should be performed within 10 to 20 min of sample collection, as the microorganisms may lose viability. It is an inexpensive and rapid diagnostic method, with a sensitivity ranging from 60% to 70% [57]. However, sensitivity can vary between 38% and 82%, depending on the inoculum size, and when the concentration of *T. vaginalis* is lower than 10^4 organisms/mL, detection is not possible [56].

One major limitation of this method is the requirement for viable samples [56], as well as the need for a microscope and a trained technician, making it less accessible in resource-limited settings [58].

Culture

To perform this technique, the inoculum size must be at least 10^2 organisms/mL, and its growth should be easily identifiable [56]. This method enables the detection of approximately 95% of infections, with high sensitivity (85–95%) and specificity (95%) [59].

The most commonly used culture medium is Diamond's TYI in glass tubes. For the identification of *T. vaginalis*, an incubation period of approximately 2 to 7 days is required [56]. However, this method has several disadvantages, including bacterial contamination, high costs [56], and the extended time required for results. In some cases, culture media are not readily available, and the organisms are sensitive to transport conditions, which may lead to their death [59]. Additionally, since incubation is required for several days before detection, these culture methods are not accessible in all locations [60].

To overcome these challenges, a new method called InPouch (BioMed Diagnostics, White City, OR, USA) was developed. In this technique, samples are placed in a specialized pouch consisting of two chambers [56], the upper chamber is used for sample inoculation under sterile conditions and provides an anaerobic environment suitable for the growth of *T. vaginalis*. After incubation, the lower chamber allows for direct microscopic examination of the culture without the need to transfer the sample, minimizing contamination risk and preserving microorganism motility. This dual-chamber design enhances diagnostic efficiency while maintaining ease of use and safety, allowing the entire culture sample to be analyzed for the presence of *T. vaginalis* [54]. These samples can remain at room temperature for about 30 min before inoculation, and test results are typically obtained within 2 to 5 days [57].

The advantages of this technique include its low sample requirement (only 300 to 500 *T. vaginalis* per milliliter) for successful growth [59]. Additionally, it is a simple method that can be used with urethral samples, allows for direct microscopic analysis through the pouch, and does not require expensive storage conditions [50].

The cell culture method offers even greater sensitivity, enabling the detection of *T. vaginalis* in samples containing as few as 3 organisms/mL. This method requires pretreatment of samples with antibiotics, with Diamond's TYI medium serving as a transfer medium before the samples are introduced into cell cultures [56].

Staining Methods

The Papanicolaou staining method enables the visualization of *Trichomonas* in cervical smears [60]. This technique has a sensitivity of 60% and a specificity of 95% [57].

Staining methods and molecular probes have also been applied directly to clinical samples. These approaches are rapid and exhibit sensitivity comparable to wet mount preparation. However, they present certain disadvantages, including the requirement for specialized equipment, trained personnel, and high costs [55].

Due to the fixative applied during the staining process, these microorganisms may lose their motility, and *T. vaginalis* may not always display its characteristic pear-shaped morphology. Therefore, this technique is most effective when used in conjunction with direct observation methods, such as wet mount preparation, which allows for the assessment of the organism's motility [56].

Nucleic Acid Detection Methods

Nucleic acid amplification methods allow for the detection of *T. vaginalis* DNA or RNA, even in samples with low pathogen concentrations. These tests do not require viable organisms and can be used with a variety of clinical specimens [55].

Sensitivity typically ranges from 85% to 100%, depending on the primers and target sequences employed. These values refer to clinical sensitivity, representing the test's ability

to correctly identify infected individuals based on patient-derived samples. Analytical sensitivity, referring to the minimal detectable DNA quantity, is also high in these methods but varies with the specific platform and protocol used. To enhance the sensitivity of this technique, the development of new primers is necessary. In 1992, Riley et al. identified a set of primers, TVA5 and TVA6, which target conserved regions of the *Trichomonas vaginalis* 18S ribosomal RNA (rRNA) gene. This region is frequently used due to its species-specific sequence and high copy number, which improve both the sensitivity and specificity of detection [57].

NAATs are highly sensitive and specific methods for detecting *T. vaginalis*, but their complexity can vary significantly depending on the platform used. While some assays, such as real-time PCR systems, require specialized equipment and trained personnel, others—like cartridge-based or automated NAATs—are designed for ease of use and are suitable for point-of-care settings. In 2011, the FDA (Food and Drug Administration) approved the Aptima *T. vaginalis* test (Hologic Gen-Probe, San Diego, CA, USA), which detects RNA using transcription-mediated amplification (TMA) and demonstrates approximately 95% sensitivity and 100% specificity. This assay is compatible with automated systems such as Gen-Probe's TIGRIS platform (Hologic Inc., San Diego, CA, USA), facilitating rapid and high-throughput testing [51,59].

It represents an adaptation of the Aptima Combo 2 Chlamydia/Gonorrhoea method (Hologic, San Diego, CA, USA), allowing *T. vaginalis* diagnosis using samples collected for Chlamydia/Gonorrhoea detection [54].

Other FDA-approved NAATs include the BD ProbeTec TV Qx (BD Diagnostics, Sparks, MD, USA), which relies on strand displacement amplification (SDA) and offers a sensitivity of 98.3% and specificity of 99.6% [61]. This test is performed using the BD Viper system (Becton, Dickinson and Company, BD Diagnostics, Franklin Lakes, NJ, USA), an automated platform capable of processing sample preparation through nucleic acid extraction followed by amplification [62]. The BD Max CTGCTV2 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) is a multiplex real-time PCR assay that allows simultaneous detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. It operates on the fully automated BD Max System (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), integrating DNA extraction, amplification, and detection in a single workflow. This method is validated for both vaginal and urine samples and shows sensitivity and specificity values above 90% depending on the matrix. The Roche Cobas TV/MG test (Roche Molecular Systems, Inc., Pleasanton, CA, USA) also demonstrates robust performance across various sample types [61].

Although these methods are highly accurate, their implementation is constrained by high costs, the need for specialized laboratory infrastructure, and trained personnel. As such, they are not universally available, especially in low-resource settings. Nevertheless, their role in forensic diagnostics is increasingly relevant, particularly in cases where rapid and reliable confirmation of infection is necessary [53].

Methods Based on Oligonucleotide Probes

Oligonucleotide probe-based methods are also commercially available and can be performed in clinical settings, with a sensitivity of 80–90% and a specificity of 95% [57].

One such detection method utilizes synthetic DNA oligonucleotide probes, such as the BD Affirm VPIII (Becton Dickinson, Sparks, MD, USA), which has been approved by the U.S. Food and Drug Administration (FDA) for detecting *T. vaginalis*, *Gardnerella vaginalis*, and *Candida* species in a single reaction. This method demonstrates a sensitivity of 91–100% and a specificity of 93–96%, with results available in approximately one hour [61].

T. vaginalis has approximately eight serotypes, and antibody-based techniques, such as immunoblotting, have been studied. Methods including complement fixation, hemagglutination, gel diffusion, fluorescent antibodies, and ELISA have been employed to detect trichomoniasis antibodies. However, these techniques are not specific for detecting recent infections [56].

5. Rapid Point-of-Care Testing

POC tests provide a solution for obtaining rapid diagnoses outside of laboratory settings. These tests can be conducted in hospitals, clinics, or near-patient settings such as emergency rooms or shelters. While theoretically adaptable for use at crime scenes, their application in such contexts is highly limited due to strict legal and procedural requirements, including the need to preserve the chain of custody, ensure sample integrity, and comply with jurisdictional forensic protocols. Therefore, in practice, the use of diagnostic testing for STI detection typically occurs in clinical or forensic laboratory environments following proper evidence handling procedures. They must meet the WHO criteria, including sensitivity, accessibility, specificity, ease of use, speed, robustness, and the ability to function without specialized equipment [14].

For gonorrhoea, POC tests based on molecular polymerase chain reaction (PCR), such as GeneXpert NG/CT (Cepheid, Sunnyvale, CA, USA), have demonstrated superior diagnostic performance compared to laboratory-based NAATs. Other NAAT techniques utilizing isothermal amplification, instead of the thermal cycling required for PCR, help reduce costs and shorten the time needed to obtain results [15]. These DNA/RNA detection-based tests provide faster and more sensitive results than traditional microscopy and culture methods [14].

For the rapid detection of chlamydia, available tests include Clearview (Unipath Ltd., Bedford, UK), TestPack (Abbott, North Chicago, IL, USA), and SureCell (Johnson & Johnson, Rochester, NY, USA) [28]. More recently, the molecular test Xpert (Cepheid, Sunnyvale, CA, USA) has been developed, utilizing nucleic acid amplification techniques with high accuracy. Another advancement is the Io POC Chlamydia test (Atlas Genetics, Bath, UK), which employs PCR in microfluidic systems with electrochemical detection of results. Isothermal amplification tests, such as LAMP and RPA, generate faster results than PCR and enhance the use of NAATs in point-of-care settings [27].

For syphilis detection, the most commonly used rapid tests are immunochromatographic strips coated with recombinant *T. pallidum* antigens, SD Bioline Syphilis 3.0, designed to detect IgM, IgG, or IgA antibodies. These tests exhibit sensitivity and specificity similar to conventional treponemal tests but are unable to distinguish between past and current infections [34]. Their advantages include low cost, rapid turnaround time, ease of use without specialized personnel, and the potential for early syphilis detection [41].

Studies on available rapid treponemal tests have shown a sensitivity of 76–86% and a specificity of 96–99% compared to laboratory assays such as the TPPA [47].

Finally, for the rapid POC detection of trichomoniasis approved by the U.S. Food and Drug Administration (FDA), in addition to wet mount microscopy, lateral flow methods are also available. These include the OSOM® Trichomonas Rapid Test (Sekisui Diagnostics, Bedford, MA, USA) and the AmpliVue helicase-dependent isothermal amplification test (Quidel, San Diego, CA, USA), which provides results within approximately 45–50 min [58], as well as the Solana TV test (Quidel, San Diego, CA, USA) [61].

The OSOM immunochromatographic enzyme assay (Sekisui, Framingham, MA, USA) utilizes a latex-labeled antibody that specifically binds to Trichomonas proteins. It also incorporates a secondary antibody to capture the formed complex within the lateral flow device, enabling detection. Results are obtained within approximately 15 min [54].

The Cepheid GeneXpert TV test (Cepheid, Sunnyvale, CA, USA) is an FDA-approved rapid test that provides results within one hour. Its sensitivity ranges from 99.5% to 100%, and its specificity is between 99.4% and 99.9% when compared to wet mount and culture methods [61]. This method is based on real-time PCR, with fully automated sample preparation [62].

Additionally, an antigen detection test has been developed for point-of-care use, facilitating trichomoniasis detection (Genzyme Corp., Cambridge, MA, USA). Compared to culture, this immunoenzymatic test demonstrates a sensitivity of 78.5% and a specificity of 98.6% [57].

Another detection method is the Kalon TV latex agglutination test (Kalon Biological, Surrey, UK), which has not yet been FDA-approved. This test can be conducted at the POC using latex spheres coated with antibodies specific to *T. vaginalis* proteins. Its advantages include not requiring specialized instruments and delivering results within approximately 10 min. Compared to wet mount and culture methods, this test has a sensitivity of 98.8% and a specificity of 92.1% [63].

To facilitate comparison between the different rapid point-of-care tests discussed in this section, the following Table 2 summarizes their main characteristics, including the type of diagnostic method, time to result, diagnostic performance, and regulatory approval status. This overview highlights the practicality and diagnostic value of these tools in clinical and forensic contexts.

Table 2. Table summarizes their main characteristics, including the type of diagnostic method, time to result, diagnostic performance, and regulatory approval status.

Infection	Test Name	Method	Time to Result	Sensitivity (%)	Specificity (%)	FDA Approved
Gonorrhoea	GeneXpert NG/CT	Real-time PCR	~90 min	95–100	98–100	Yes
Chlamydia	Io POC Chlamydia	PCR (microfluidic)	~30 min	~96	~99	Yes
Syphilis	SD Bioline Syphilis 3.0	Immunochromatographic strip	~20 min	76–86	96–99	Yes
Trichomoniasis	OSOM® Trichomonas Rapid Test	Immunochromatography	~15 min	~95	~97	Yes
	AmpliVue TV	Isothermal amplification	~50 min	~98	~99	Yes
	Cepheid GeneXpert TV test	Real-time PCR	~60 min	99.5–100	99.4–99.9	Yes

5.1. Multiplex Detection of STIs

In addition to single-target diagnostic tests, several multiplex molecular platforms have been developed to enable the simultaneous detection of multiple STIs within a single sample. These include assays such as the Cepheid Xpert CT/NG, BD MAX CT/GC/TV, and Aptima Combo 2, which utilize NAATs to concurrently detect *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*, among others. These systems are particularly beneficial in syndromic management, where overlapping symptoms do not allow for reliable pathogen-specific diagnosis.

Multiplex NAATs exhibit high clinical sensitivity and specificity and are especially valuable in forensic and clinical settings requiring broad STI screening. However, their application is limited in low-resource environments due to their cost, infrastructure demands, and requirement for trained laboratory personnel. Despite these constraints, multiplex NAATs currently represent the most efficient option for multi-pathogen STI detection, and

continued innovation in portable, microfluidic-based platforms may further expand their accessibility and utility in field settings.

5.2. Comparative Reflection on Diagnostic Strategies

Building upon the diagnostic methods detailed in the previous sections, a comparative reflection is warranted to contextualize their applicability, strengths, and limitations. This mid-article synthesis aims to critically assess the suitability of each method across different infections and scenarios.

When comparing diagnostic techniques across the four major STIs reviewed—gonorrhoea, chlamydia, syphilis, and trichomoniasis—it becomes evident that no single method offers universal applicability. Instead, diagnostic suitability is determined by factors such as the pathogen's biology, infection stage, specimen type, and testing context (clinical vs. forensic).

For example, NAATs (including PCR and TMA) show consistently high clinical and analytical sensitivity across all four STIs, making them the most robust option in terms of diagnostic accuracy. However, their reliance on specialized equipment and trained personnel limits their implementation in low-resource or point-of-care settings.

Culture methods, though specific and useful for antimicrobial susceptibility testing (particularly in *Neisseria gonorrhoeae*), are limited by the need for viable organisms, stringent transport conditions, and longer turnaround times. Similarly, microscopy, while rapid and cost-effective, suffers from low sensitivity, especially in asymptomatic cases or for pathogens with low organism load such as *Chlamydia trachomatis*.

For syphilis, the combination of treponemal and non-treponemal tests remains the gold standard, yet interpretation can be complicated due to lifelong seropositivity in treponemal assays and false-positives in non-treponemal methods.

Point-of-care tests, particularly those based on immunochromatography or isothermal amplification, represent a promising compromise between speed and practicality, although variability in sensitivity—especially for antibody-based tests—remains a concern.

In summary, NAATs emerge as the most reliable diagnostic method overall due to their versatility, high sensitivity/specificity, and ability to detect co-infections. Nonetheless, POC methods are essential for rapid, initial screening—especially in forensic and emergency contexts—where laboratory access is limited. Tailoring the diagnostic strategy to the specific clinical and forensic context, while considering the pathogen's characteristics, remains essential for optimizing STI detection in victims of sexual violence.

This comparative overview underscores the need for context-aware diagnostic choices and provides a framework for the concluding discussion that follows.

6. Discussion

Asymptomatic infections and the limitations of symptom-based diagnosis are highly prevalent in sexually transmitted infections (STIs), highlighting the necessity of laboratory confirmation for accurate detection [14]. This step is essential to ensure appropriate treatment for affected individuals and to prevent the further spread of these diseases. To address certain challenges, nucleic acid tests and non-invasive, close-to-patient diagnostic tools have been developed in recent years. These tests can be self-administered without the need for an STI specialist, performed in various settings, and processed at the point of care, delivering results within minutes or hours [10].

Clinical symptoms alone are rarely sufficient to accurately identify a specific STI, making laboratory diagnosis indispensable for confirming the type of infection present [14]. However, careful consideration must be given to the limitations of each test, as supplementary diagnostic methods may sometimes be required to verify results. When a test is conducted for medico-legal purposes, such as in cases of sexual assault or to establish a

link between a victim and a perpetrator, maintaining a proper chain of custody is crucial to ensure the integrity of the evidence [10].

In addition to microbiological testing, DNA profiling plays a central role in the forensic investigation of sexual assault cases. It provides a highly specific form of evidence capable of directly linking a suspect to a victim or crime scene, particularly when biological traces such as semen, blood, or epithelial cells are recovered. While the detection of STIs may support the occurrence of sexual contact—especially in cases where the same pathogen is identified in both victim and suspect—STI testing alone cannot establish the source or timing of transmission. Therefore, DNA analysis offers stronger probative value and is considered the gold standard in forensic identification. Nonetheless, STI detection can serve as a complementary tool, especially in cases where DNA evidence is unavailable, degraded, or insufficient due to delayed reporting. The integration of both approaches enhances the robustness of forensic interpretation, particularly in complex scenarios involving asymptomatic infections or vulnerable populations such as children.

The selection of recommended diagnostic tests depends on various factors, including the victim's circumstances, availability of resources, individual capability, age, type of assault, and the time elapsed since the incident [10].

Choosing the most appropriate diagnostic method is not solely determined by its sensitivity, specificity, and predictive values but also by logistical considerations such as equipment availability, cost-effectiveness, and performance, as well as the intended purpose of the test. In resource-limited settings, the use of highly sensitive and specific tests is often impractical due to their high costs and equipment requirements. Therefore, rapid diagnostic methods are essential to enable immediate treatment for patients [14].

This article has outlined various detection methods developed to diagnose the most prevalent sexually transmitted infections (STIs) worldwide. Traditional microscopy and culture techniques present several disadvantages, including the necessity of examining the sample within 10 min of collection [14], the requirement for viable bacteria [29], reliance on specialized equipment such as microscopes, and the need for trained personnel. These factors make the implementation of such methods challenging in resource-limited areas [58].

To overcome these limitations, alternative diagnostic techniques have been developed, including nucleic acid detection methods, polymerase chain reaction (PCR)-based techniques, serological tests, and rapid POC tests. These POC methods offer a viable solution for the rapid diagnosis of STIs outside of laboratory settings [14]. Additionally, they provide advantages in sample collection, as traditional laboratory-based methods can be invasive. Lower equipment costs also facilitate the establishment of diagnostic facilities closer to patients, increasing accessibility to testing and treatment [29].

Looking ahead, the development of advanced diagnostic technologies remains a priority. Faster diagnostic tools with forensic capabilities are crucial in cases of sexual violence. Innovations in these methods could enhance accuracy and reduce the likelihood of false-negatives. Moreover, the development of non-invasive sample collection techniques, such as saliva or capillary blood sampling, could serve as alternatives or complements to current genital sample collection methods. Such advancements would help minimize the trauma experienced by victims while improving compliance in forensic investigations and medical diagnoses.

These future perspectives emphasize the importance of integrating technological advancements in diagnostics with enhanced collaboration between healthcare services and judicial authorities. By fostering progress in STI detection, healthcare improvements, and victim support systems, it is possible to envision a future where both prevention and care for STI victims are strengthened, contributing to a safer and more just society.

From the authors' perspective, the integration of rapid, reliable, and minimally invasive diagnostic tools into forensic protocols is not merely a technical advancement, but a fundamental step toward more victim-centered forensic practice. In the context of sexual violence, where timely intervention and trauma-sensitive approaches are essential, point-of-care methods offer an opportunity to improve both clinical care and the quality of forensic evidence. However, the implementation of such tools must be accompanied by standardized guidelines, professional training, and strengthened cooperation between healthcare providers and judicial authorities. We believe that diagnostic innovation, when aligned with ethical and legal frameworks, has the potential to significantly enhance the support system for victims of sexual crimes and contribute to a more effective and humane justice process.

6.1. Forensic Relevance and Multiplexing Considerations

Although many of the diagnostic techniques described in this review were developed and validated for clinical settings, their application in forensic contexts presents distinct challenges. In cases of sexual violence, the primary objective of STI detection extends beyond medical treatment; it also includes the potential to support legal proceedings by establishing biological links between the victim and the alleged perpetrator. Therefore, forensic diagnostic tools must adhere to rigorous standards of sample integrity, chain of custody, reproducibility, and evidentiary admissibility. Methods that require complex equipment or involve highly sensitive molecular reactions, such as multiplex NAATs, may be difficult to implement in forensic laboratories with limited infrastructure or where test results must be easily interpretable in court.

While multiplex platforms—capable of detecting multiple pathogens in a single sample—offer practical and diagnostic advantages (particularly for asymptomatic victims or co-infections), they also raise concerns regarding data complexity, legal interpretation, and validation for forensic use. The lack of standardization and variation in performance across test brands may further limit their forensic applicability. Moreover, these tests rarely provide information on time of infection, which is often a critical element in forensic analysis.

Therefore, despite their diagnostic potential, multiplex methods must be carefully evaluated for forensic use. Future research should focus on the development of multiplex-compatible forensic protocols, including robust chain-of-custody documentation, validation studies in forensic scenarios, and interdisciplinary guidelines that integrate medical diagnostics into judicial processes with reliability and ethical rigor.

6.2. Study Limitations

This narrative review provides an overview of current diagnostic approaches for sexually transmitted infections in the context of sexual violence. However, several limitations should be acknowledged. First, the non-systematic nature of the literature review may introduce selection bias, as formal inclusion and exclusion criteria were not applied. Second, the scarcity of forensic-oriented studies focusing on the detection of STIs specifically within the context of sexual crimes limits the depth of analysis concerning medico-legal applicability. Third, the absence of original data collection constrains the ability to compare diagnostic accuracy across different methods in real-world settings. Moreover, variations in healthcare infrastructure, access to diagnostic tools, and laboratory capabilities across different countries may reduce the generalizability of the findings. These limitations highlight the need for further empirical research and standardization of STIs diagnostic protocols, particularly in forensic and resource-limited environments.

7. Conclusions

The literature review revealed that sexual crimes can be associated with the transmission of sexually transmitted infections (STIs), including gonorrhoea, chlamydia, syphilis, and trichomoniasis. Early detection of these STIs plays a crucial role in ensuring not only appropriate medical treatment for victims but also in preventing long-term health consequences and the further spread of these infections.

STIs in victims of sexual crimes represent a complex issue, requiring responses that address both clinical and forensic needs. This review explored the primary STI detection methods within the context of sexual violence, analyzing the challenges, advancements, and limitations of current diagnostic methodologies. New approaches, such as POC methods, have emerged as promising solutions to enhance the speed and accuracy of diagnosis.

Despite significant progress in STI detection techniques, there remain gaps in the effectiveness and accessibility of newer diagnostic methods. Therefore, further research is essential to explore and develop innovative technologies and strategies that optimize the detection and treatment of STIs in cases of sexual violence.

Continued research efforts should focus on improving diagnostic methods to make them less invasive, faster, and more applicable in forensic contexts. Additionally, the implementation of public policies and educational campaigns is vital to raise awareness of STIs, promote timely medical care, and reduce stigma. Enhancing the training of healthcare and forensic professionals, alongside the development of ethical and practical guidelines, will ensure that forensic and clinical care for victims of sexual crimes is conducted with both responsibility and effectiveness.

Ultimately, future studies will contribute to the advancement of early detection methods and the development of new victim care protocols. Strengthening the integration between healthcare and the justice system is fundamental to improving the protection and support available to victims, ensuring a more comprehensive and effective response to sexual violence.

Author Contributions: Conceptualization, A.E.; methodology, A.E.; writing—original draft preparation, A.E.; writing—review and editing, J.F., L.C. and A.A.; supervision, J.F. and L.C.; funding acquisition, L.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: No new data were created or analyzed in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CSF	Cerebrospinal Fluid
CT	*Chlamydia trachomatis*
DFA	Direct Fluorescent Antibody
DNA	Deoxyribonucleic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FTA-Abs	Fluorescent Treponemal Antibody Absorption Assays
HDA	Helicase-dependent Amplification
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

LAMP	Loop-mediated Isothermal Amplification
LCR	Ligase Chain Reaction
LPS	Lipopolysaccharide
MAMEF	Microwave-accelerated Metal-enhanced Fluorescence
MHA-TP	Microhemagglutination Assay for *T. pallidum* Antibodies
MIF	Microimmunofluorescence
MOMP	Major Outer Membrane Protein
NAAT	Nucleic Acid Amplification Test
NAH	Nucleic Acid Hybridization
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
POC	Point-of-care
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
RPR	Rapid Plasma Reagin
SDA	Strand Displacement Amplification
STIs	Sexually Transmitted Infections
TMA	Transcription-mediated Amplification
TRUST	Toluidine Red Unheated Serum Test
TPHA	*T. pallidum* Hemagglutination Assay
TPPA	*T. pallidum* Particle Agglutination Assay
USR	Unheated Serum Reagin
VDRL	Venereal Disease Research Laboratory
WHO	World Health Organization
VDRL	Venereal Disease Research Laboratory

Appendix A

Table A1. Physical, reproductive, psychological, social and relationship consequences that sexual crimes can cause in victims (adapted from [9,64,65]).

	Physical	Reproductive	Psychological	Social and Relationships
Consequences	Generalized and prolonged pain; eating disorders; fibromyalgia; prolonged fatigue, heart, coronary and neurological problems, as well as diabetes; asthma; hyperventilation; feeling of suffocation; sleep problems, restlessness, numbness, weakness; physical injuries (abrasions and bruises to concussions, fractures and gunshot wounds); chronic pelvic pain; chronic pain syndromes; headaches and back pain; irritable bowel syndrome; nausea; vomiting; abdominal pain; diarrhea; bloating; gastrointestinal problems.	Genital lesions; vaginal bleeding or infection; genital irritation; fibroids; chronic pelvic pain; premenstrual syndrome; urinary tract infections; painful menstruation; painful sexual intercourse; lack of sexual pleasure; unwanted pregnancy; sexual dysfunction; dysmenorrhea; menorrhagia; fistulas; abortion; sexually transmitted infections; infertility; complications in childbirth; neonatal deaths; maternal deaths.	Depression, anxiety, phobia, low self-esteem, shame, self-destructive behavior, alcohol and drug abuse; suicidal thoughts; anger, sadness, guilt, disappointment; personality disorder; post-traumatic stress disorder and social phobia; attention deficit disorder (ADD); attention deficit hyperactivity disorder (ADHD).	Difficulties in sex life and relationship with partner; marriage problems; defense against all types of violence; repeated physical, psychological and/or sexual violence in relationships or rape; anxiety and stress in parenthood and motherhood in adolescence; the greatest danger of being violent with your own children; they often seek help from the health system, but don't mention the abuse; receives little or no support from the health system, but receives plenty of medication.

Table A2. Symptoms caused by Syphilis in its different phases (adapted from [34,35,38,40,47,66]).

	Syphilis Stage					
	Primary Syphilis	**Secondary Syphilis**	**Tertiary Syphilis**	**Early Neurosyphilis**	**Late-Onset Neurosyphilis**	**Parenchymatous Neurosyphilis**
Symptoms	Chancre (painless ulcer or papule); regional lymphadenopathy (painless, tender, non-erythematous overlying skin); fever; headache; maculopapular rash on the flank, shoulders, arm, chest, back, palms of the hands and soles of the feet; genital herpes simplex; lymphogranuloma venereum; behcet's disease; inguinal granuloma.	Skin rash; mucocutaneous lesions; lymphadenopathy; glomerulonephritis; headache; arthritis, osteitis periostitis; nephrotic syndrome; fever, weight loss, fatigue, myalgias, arthralgias, sore throat, headaches and/or weight loss; cranial neuropathy, iritis; oral ulceration or mucous patches, hepatitis, nephritis and codilema lata; posterior uveitis or panuveitis (blindness); changes to the nails (fragility, cracks, corrosion, onycholysis, onychomadesis, transverse grooves and Beau's lines).	Mental diseases, dementia, neurological deficits, blindness/deafness, heart disease, aortitis/aortic aneurysm and death; Skin lesions (ulcerative or gummy nodules); aortic valve disease and hostile coronary occlusion; endarteritis; aortic insufficiency, coronary artery stenosis and myocarditis.	Cranial nerve dysfunction; meningitis; cerebrovascular accident; altered mental state, hearing deficits and/or ophthalmic abnormalities.	Tabes dorsalis (gradual demyelination of the spinal cord nerves, causes ataxia, neuropathic pain, blindness and general paresis); mild meningeal symptoms, headache and nausea; paralysis of the cranial nerves, unilateral or bilateral hearing loss; meningovascular syphilis; arthritis (infarcts in the brain or spinal cord, which can cause general paresis, the appearance of dementia, seizures and other psychiatric manifestations).	Irritability, memory loss, personality disorders and insomnia; depression, confusion and disorientation, delirium, paranoia and convulsions (facial tremors, papillary abnormalities, inexpressive facies and hyper-reflexia); bladder and intestinal consequences; visceral changes (intense gastrointestinal pain or laryngeal pain and hoarseness).

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Article 2- Sexually Transmitted Infections: Usefulness of Molecular Methods for Microorganisms Detection in Stored Sexual Assault Samples

International Journal of Molecular Sciences

MDPI

Article

Sexually Transmitted Infections: Usefulness of Molecular Methods for Microorganism Detection in Stored Sexual Assault Samples

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Abstract

Sexual assault is a global public health and human rights concern, with serious physical, psychological and reproductive consequences for survivors. Among these, sexually transmitted infections are particularly relevant due to their frequently asymptomatic nature and potential for long-term complications. The detection of sexually transmitted infections in forensic settings is crucial for clinical management of victims and for evidentiary support in forensic sexual crimes investigations. This study aimed to evaluate the applicability of real-time polymerase chain reaction for detecting *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, and *Treponema pallidum* in biological samples collected from victims of sexual assault and stored under routine forensic conditions, in some cases, for up to 18 years. A total of 231 swabs from 116 individuals collected between 2004 and 2017 were analysed using real-time PCR with pathogen-specific primers and fluorescent probes. The analysis revealed 13 positive samples of *T. vaginalis* (5.6%) and 11 of *C. trachomatis* (4.8%). No positive results were obtained for *N. gonorrhoeae* or *T. pallidum*. These findings demonstrate the usefulness of real-time polymerase chain reaction for detecting sexually transmitted infections in long-term preserved forensic samples. Moreover, the ability to identify pathogen DNA in archived samples highlights the potential role of molecular diagnostics in the retrospective investigation of sexual crimes, including cold cases. It underscores the value of molecular methods as a complementary tool in forensic proceedings and survivor care.

Keywords: sexual crimes; sexually transmitted infections; forensic microbiology

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Academic Editor: Vittorio Fineschi

Received: 28 July 2025
Revised: 19 August 2025
Accepted: 20 August 2025
Published: 22 August 2025

Citation: Cainé, L.; Eira, A.; Fadoni, J.; Franco, M.; Correia Dias, H.; Amorim, A. Sexually Transmitted Infections: Usefulness of Molecular Methods for Microorganism Detection in Stored Sexual Assault Samples. *Int. J. Mol. Sci.* 2025, 26, 8124. https://doi.org/10.3390/ijms26178124

1. Introduction

The World Health Organization (WHO) defines sexual violence as "any sexual act, attempt to consummate a sexual act, or other act directed against a person's sexuality by

means of coercion, by another person, regardless of their relationship to the victim and in any context. It includes rape, which is defined as penetration, by physical or other coercion, of the vulva or anus with a penis, other body part, or object" [1].

Sexual violence has profound and multifactorial implications for survivors' health, affecting physical, reproductive, and psychological domains. The severity and nature of these effects vary across age groups and may present as both acute and chronic conditions [2,3]. Among the most significant long-term health outcomes are sexually transmitted infections (STIs), which rank among the most prevalent infectious diseases worldwide [4].

STIs are primarily transmitted through sexual contact and constitute a major global public health challenge [5]. These infections may result from bacterial, viral, or parasitic pathogens and can affect individuals regardless of age, gender, or sexual orientation. If left untreated, STIs may lead to serious complications such as infertility, chronic pelvic or genital pain, and increased vulnerability to coinfections [6].

Chlamydia trachomatis (*C. trachomatis*) and *Trichomonas vaginalis* (*T. vaginalis*) are among the most prevalent sexually transmitted pathogens worldwide. Both infections may be symptomatic or asymptomatic and are associated with significant reproductive health complications, including urethritis, pelvic inflammatory disease, infertility, and increased susceptibility to coinfections [7–10]. The frequently silent nature of these infections complicates timely diagnosis and highlights the importance of sensitive detection methods, particularly in forensic contexts where early intervention may not be possible.

According to the WHO, the global burden of untreated STIS remains substantial, largely attributable to treatment failures and the frequently asymptomatic presentation of these infections. It is estimated that approximately one million new cases of STIS occur daily worldwide, culminating in nearly 374 million new infections annually [1]. The prevalence and incidence of STIS vary depending on the causative pathogen and are significantly influenced by socioeconomic determinants, with elevated rates commonly observed in regions characterized by under-resourced healthcare infrastructures [11].

Biological samples of forensic significance are frequently encountered in cases of physical assault and hold particular importance in the investigation of sexual offences. Such biological evidence is critically relevant to forensic investigations, as it may occasionally constitute the sole proof of the occurrence of sexual assault and enable the identification of the perpetrator [12]. It is imperative that these samples are collected within specialized facilities dedicated to victims of sexual violence or in clinical environments where individuals can receive comprehensive medical care, psychological support, and screening for sexually transmitted infections [13].

Forensic evidence—including clothing, urine, oral cavity swabs, hand and nail scrapings, as well as non-intimate skin samples—can facilitate the identification of perpetrators, the exclusion of suspects, and contribute to the successful prosecution of criminal cases [14]. In both victims and suspects of sexual assault, the majority of biological samples are conventionally collected using sterile cotton swabs [14].

To preserve the evidentiary value, sample collection must be performed with meticulous care to ensure the integrity and forensic reliability of the biological materials. Such materials should be stored under dry, temperature-controlled conditions to prevent microbial proliferation and degradation of deoxyribonucleic acid (DNA) [13]. Improper collection or handling procedures may compromise the suitability of samples for forensic analysis [12]. Given that often only trace amounts of DNA are available, stringent precautions are essential to avoid contamination, guarantee proper collection, and maintain optimal storage conditions [15], thereby preserving both the quality and quantity of the genetic material [14].

The detection of STIS is of critical importance and can be performed using various methodologies, including polymerase chain reaction (PCR) in real-time. This technique is a modification of conventional PCR that enables continuous monitoring of the amplification process in real time, while simultaneously facilitating the synthesis of multiple copies of a targeted DNA region. Real-time PCR has a wide range of applications, including pathogen detection and quantification, species identification, among others [16]. This study aims to evaluate the utility of real-time PCR for the detection of *C. trachomatis* and *T. vaginalis* in biological specimens obtained from sexual assault victims and stored for up to 18 years, for some samples. By demonstrating the reliability of this approach, we seek to emphasize the importance of molecular diagnostics in the forensic investigation of sexual crimes. The present work is therefore particularly relevant, as it involves the analysis of real forensic samples collected from sexual assault victims and stored under routine casework conditions. This research will contribute to the development of the national scientific literature and to the improvement of diagnostic practices in forensic and clinical contexts.

2. Results

Among the studied samples, 13 tested positive for *Trichomonas vaginalis* (5.6%) and 11 for *Chlamydia trachomatis* (4.8%). No positive results were observed for *Neisseria gonorrhoeae* or *Treponema pallidum*. These outcomes are illustrated in Figure 1. In the case of *C. trachomatis*, two positive samples were collected 18 years prior to analysis, one 10 years ago, five 9 years ago, and three 8 years ago (Figure 1). For *T. vaginalis*, two positive cases corresponded to samples collected 11 years ago, six to samples from 9 years ago, and five to those collected 8 years ago (Figure 1).

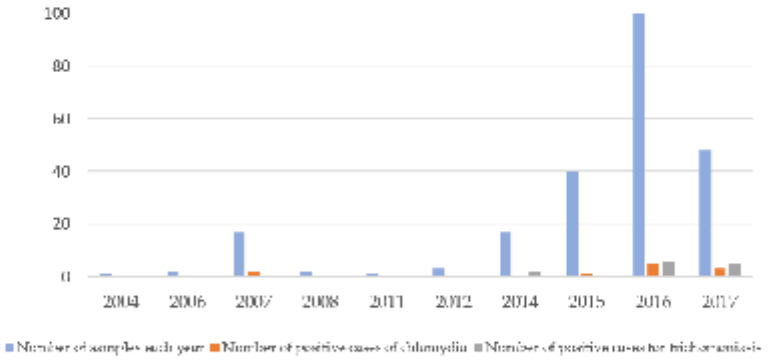

Figure 1. Representation of the number of positive cases for chlamydia (orange bars), the number of positive cases of trichomoniasis (gray bars), and the number of total samples studied per year, between 2004 and 2017.

Amplification of the human ribonuclease P (RNAseP) gene was successful in all specimens analysed, confirming the presence of amplifiable human DNA in the extracted material. The detection of human DNA after long-term storage confirms both the integrity of the stored sample and the robustness of the extraction protocol in recovering amplifiable DNA under these conditions.

All the studied samples were obtained from female individuals aged between 15 and 37 years. A total of 14 positive results were identified from vaginal samples, six from vulvar samples, and one from a combined perianal and oral sample. With regard to *T. vaginalis*, the pathogen was detected in three vulvar samples, eight vaginal samples, one perianal sample, and one oral sample. In the case of *C. trachomatis*, it was detected in four vulvar

samples and seven vaginal samples. One vulvar sample (IST200) tested positive for both *T. vaginalis* and *C. trachomatis*.

In this study, the obtained positive results correspond to 12 individuals with samples collected from different anatomical regions in some cases. The cases were analyzed between 2004 and 2017 as part of the forensic casework of the National Institute of Legal Medicine and Forensic Sciences. Genetic profiles were obtained from each sample, whenever possible, through short tandem repeat (STR) analysis. Regarding autosomal STR, each sample was processed using the GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA), and for the Y-STR analysis, it was carried out using the Yfiler™ Plus PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA). It was possible to detect a mixture of female and male genetic profiles in certain individuals, as well as the presence of the Y-chromosome haplotype. In the case of individual 12, as indicated in Table 1, a single male genetic profile was identified.

Table 1. Information regarding each alleged sexual assault victim who tested positive for *C. trachomatis* and/or *T. vaginalis*, including year of collection, victim's age, sample type, STI detected, and genetic profile identification.

Alleged Victim	Year of Collection	Victim's Age	Sample ID	Swab Type	STI Detected	Genetic Profiles Obtained
Individual 1	2007	22	IST015	Vulvar	*Chlamydia trachomatis*	No information
			IST016	Vaginal	*Chlamydia trachomatis*	
Individual 2	2014	36	IST032	Vulvar	*Trichomonas vaginalis*	No male profile identified
			IST033	Vaginal	*Trichomonas vaginalis*	
Individual 3	2015	37	IST073	Vaginal	*Chlamydia trachomatis*	Female and male admixture
Individual 4	2016	14	IST092	Vulvar	*Trichomonas vaginalis*	No male profile identified
			IST104	Vaginal	*Trichomonas vaginalis*	
Individual 5	2016	Unknown	IST095	Vaginal	*Chlamydia trachomatis*	Female and male admixture
Individual 6	2017	23	IST142	Vaginal	*Chlamydia trachomatis*	Male profile (STR) + 2 different Y haplotype profiles
			IST147	Vulvar	*Chlamydia trachomatis*	
			IST148	Vaginal	*Chlamydia trachomatis*	
Individual 7	2017	26	IST154	Perianal	*Trichomonas vaginalis*	Female and male admixture profile + Y haplotype
			IST158	Vaginal	*Trichomonas vaginalis*	
			IST197	Vaginal	*Trichomonas vaginalis*	
Individual 8	2016	15	IST169	Vulvar	*Chlamydia trachomatis*	Female and male admixture profile + Y haplotype
			IST171	Vaginal	*Chlamydia trachomatis*	
			IST196	Vaginal	*Chlamydia trachomatis*	
			IST200	Vulvar	*Trichomonas vaginalis* and *Chlamydia trachomatis*	
Individual 9	2017	17	IST190	Vaginal	*Trichomonas vaginalis*	No male profile identified
Individual 10	2016	25	IST198	Oral	*Trichomonas vaginalis*	Admixture profile of two Y haplotypes in a sample

Table 1. *Cont.*

Alleged Victim	Year of Collection	Victim's Age	Sample ID	Swab Type	STI Detected	Genetic Profiles Obtained
Individual 11	2017	31	IST199	Vaginal	*Trichomonas vaginalis*	Male profile (STR) + Y haplotype
Individual 12	2016	37	IST204 IST232	Vaginal	*Trichomonas vaginalis* *Trichomonas vaginalis*	Male profile identified

This study was designed as a descriptive analysis; therefore, no inferential statistical tests were conducted.

3. Discussion

Sexual crimes are associated with a wide range of long-term consequences for survivors, among which STIS are particularly significant due to their frequently asymptomatic nature and their potential to cause chronic health complications [4]. Despite their clinical and forensic importance, investigations specifically addressing the detection of STIS in biological evidence from sexual assault cases remain limited. This scarcity of research may contribute to the underestimation of infection rates, diagnostic delays, and missed opportunities for timely medical or forensic intervention.

The retrospective analysis of stored forensic samples for STI microorganism detection also raises important ethical and legal considerations. Reliable identification of pathogens years after the assault may contribute to delayed justice, therapeutic intervention, and epidemiological tracking. Thus, preserving the integrity of biological evidence and applying sensitive molecular techniques are essential for ensuring the rights of survivors and the robustness of forensic conclusions. The biological samples analyzed in the study were collected during forensic medical examinations conducted as part of criminal investigations into sexual assault cases in Portugal. These samples were obtained under judicial authority and used exclusively for forensic purposes, including the identification of perpetrators. According to Portuguese law, biological samples from cases that occurred more than two years ago may be used for research purposes, provided that full anonymity is maintained. All samples included in the study were anonymized prior to analysis, and the corresponding criminal cases had been fully processed and legally closed. The study was approved by the Ethics Committee of the National Institute of Legal Medicine and Forensic Sciences. From a legal standpoint, the ability to detect pathogen DNA with high accuracy even after prolonged storage may introduce new probative elements into previously inconclusive cases, enabling the reconsideration of cold cases and the reopening of judicial proceedings when new scientific evidence corroborates survivor testimony or strengthens forensic links to alleged perpetrators.

The implementation of real-time PCR to detect STI microorganisms in forensic settings represents a significant methodological advancement. This molecular technique was selected for this study due to its multiple advantages, such as high sensitivity and specificity, rapid processing time, reduced risk of cross-contamination, and efficient target amplification [17], and offers the ability to detect pathogen DNA in samples that are often unsuitable for conventional culture-based diagnostics [16]. The real-time PCR approach used in this study enabled the detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* in forensic samples stored for up to 18 years, with prevalence rates of 4.8% and 5.6%, respectively.

These results are consistent with those reported by Sachs et al. (2022), who observed comparable prevalence rates of *C. trachomatis* and *T. vaginalis* among victims of sexual violence [18]. Similar findings have been reported by other studies using PCR-based diagnostics, including those by Kebbi-Beghdadi et al. (2022), Schirm et al. (2007), Caliendo et al.

(2005), and Šoba et al. (2015) [19–22]. In contrast, a notably higher prevalence of *T. vaginalis* was reported by Elsherif et al. (2013), which may be attributable to the preselection of culture-positive samples prior to PCR analysis [23]. These discrepancies in prevalence rates likely reflect variations in sample selection criteria, population characteristics, methodological approaches, and storage or handling conditions [24].

Comparison of our findings with previously published molecular studies highlights considerable heterogeneity in the reported prevalence of *C. trachomatis* and *T. vaginalis*. This heterogeneity underscores the relevance of population-specific and methodological factors in STI detection and reinforces the diagnostic value of molecular tools in forensic casework.

The observed detection rates—particularly in samples stored for up to two decades—emphasize the importance of integrating STI screening into routine forensic protocols. The frequent asymptomatic nature of these infections, combined with extended storage durations between sample collection and analysis, poses a challenge for conventional diagnostic methods. In our study, pathogen DNA was successfully amplified from long-term preserved samples, including IST015 and IST016 from individual 1, collected in 2007, and confirmed positive for *C. trachomatis*. These findings confirm that, when appropriate preservation protocols are followed, real-time PCR remains a reliable tool for STI detection in archived forensic materials.

The results obtained indicate a higher prevalence of *T. vaginalis* and *C. trachomatis* in women aged between 15 and 37 years, which, according to the WHO, is the age group most vulnerable to these infections [1]. A greater number of positive cases was observed in vaginal and vulvar samples, suggesting that these are the primary targets for detecting such infections. Furthermore, the detection of STI microorganisms in perianal and oral samples highlights important considerations regarding the inclusion of these anatomical regions in sampling protocols, particularly in cases of sexual assault.

Simultaneous detection of *T. vaginalis* and *C. trachomatis* was observed in a single vulvar sample (IST200). This finding is of particular significance, as coinfections have the potential to augment the risk of transmission and may complicate therapeutic management for the affected individual. Consequently, these results highlight the importance of implementing multiplex diagnostic approaches, given that the identification of one sexually transmitted infection may be indicative of the concurrent presence of additional pathogens.

According to the study by Silva et al. (2020) [25], the prevalence rates of *N. gonorrhoeae* and *T. pallidum* were 1.3% and 1.0%, respectively. In this context, the absence of positive results for these pathogenic agents in our study may indicate a lower prevalence of these infections in the population investigated. Nonetheless, the potential for DNA degradation over time cannot be excluded, as it may compromise the integrity of the genetic material and, consequently, diminish the sensitivity of molecular diagnostic methods. This consideration emphasizes the critical importance of appropriate preservation and handling of biological specimens, particularly with regard to the stability of nucleic acids, in order to ensure the reliability of diagnostic outcomes—especially in cases where the pathogens are present in low abundance.

It should be noted that, considering the obtained positive results for *T. vaginalis* and *C. trachomatis*, the simultaneous presence of both female and male genetic profiles was observed in some of these samples, including the identification of Y-chromosome haplotypes. This finding confirms the presence of male genetic material, which may be indicative of sexual contact and holds particular relevance in forensic and criminal investigative contexts.

In the specific case of individual 12, an exclusively male genetic profile was identified, which may contribute to the forensic identification of the perpetrator. Similarly, in those individuals in whom a Y-chromosome haplotype was detected, the same evidentiary

potential exists, provided that an appropriate reference sample is available for comparative analysis and the identification of a potential suspect. This detection supports the possibility of genetic material transfer between individuals. The presence of multiple genetic profiles—both female and male—also introduces important considerations for the interpretation of results, as the coexistence of DNA from multiple contributors can complicate the individual identification and the forensic attribution of genetic material.

Nonetheless, several limitations must be acknowledged. Although the total number of swabs analysed ($n = 231$) is considerable within the context of forensic casework, the absence of stratified subgroups based on variables such as the age of the victim or the duration of sample storage has limited the scope for comparative analyses. This, in turn, constrains the capacity to identify patterns or associations that may hold relevance for clinical or forensic interpretation.

Additionally, the study did not include inferential statistical analyses, as the retrospective and descriptive nature of the dataset was primarily intended to assess the feasibility of molecular detection of STI microorganisms in long-term stored forensic samples. Future studies should incorporate larger, stratified samples and statistical approaches to assess associations over time or by sample type.

Another important limitation is the absence of direct comparison with conventional diagnostic techniques, such as culture or microscopy. Without this, it is not possible to fully assess the diagnostic accuracy and specificity of real-time PCR in forensic applications. This is particularly relevant for its integration into standard forensic workflows, where complementary diagnostic validation is often necessary.

Nonetheless, previous studies have shown that real-time PCR generally demonstrates higher sensitivity and faster turnaround times compared to conventional culture methods, particularly when applied to samples with low pathogen load or compromised viability [20,26]. These characteristics make PCR particularly advantageous in forensic contexts, where sample integrity may be affected by delayed collection and long-term storage.

Despite these constraints, the present findings support the long-term applicability and forensic value of real-time PCR. This technique has demonstrated robust performance even in archived samples, making it a promising candidate for routine integration into forensic and clinical workflows involving sexual assault cases. Future research should aim to harmonize sample processing protocols, investigate the influence of different storage conditions on diagnostic yield, and validate these results in multicenter and prospective settings.

4. Materials and Methods

4.1. Sample Collection

A total of 231 biological swabs collected from sexual assault victims between 2004 and 2017 were included in our study. Samples were initially collected only with a focus on aggressor genetic profile detection. So, they were initially studied with that focus and stored at −20 °C in a specific cold storage room for up to 18 years.

Samples were obtained from diverse anatomical locations, including 112 vaginal, 57 vulvar, three peri-vulvar, one perineum, one inner thigh, 32 anal canal, three rectal, 12 perianal, and 10 oral.

These samples were selected taking into account the number of old sexual assault cases available for subsequent analysis in 2024.

4.2. DNA Extraction and Purification

The extraction and purification of DNA are critical steps in microbiological investigations, as they directly impact the sensitivity and reliability of downstream molecular analyses [27]. Various protocols—both manual and automated—are available, with

selection depending on the type of biological sample, time constraints, and specificity requirements [28].

In this study, DNA was extracted and purified using an automated platform, the QIAGEN EZ1 (QIAGEN, Hilden, Germany), with DSP Virus Kit, according to the manufacturer's protocol. This kit offers several advantages, including high reproducibility, reduced contamination risk, and efficient nucleic acid recovery [27].

For sample preparation, a portion of each swab containing biological material was cut and transferred into a 1.5 mL microcentrifuge tube containing 1.0 mL of 0.9% sodium chloride (NaCl) solution. Each tube was labeled with the corresponding case identifier. To minimize the risk of cross-contamination, scissors and work surfaces were disinfected with 70% ethanol between the processing of each sample. The tubes were vortexed for approximately 10 s and incubated in a dry heating block at 56 °C for 30 min to inactivate potential pathogens. After inactivation, samples were stored at −20 °C until DNA extraction, which was performed using the BioRobot EZ1 Advanced automated system (QIAGEN, Hilden, Germany), following the standard protocol. In each extraction, a negative control is used to monitor contamination.

4.3. Molecular Detection and DNA Amplification

Real-time PCR enables the detection of bacterial DNA through the use of sequence-specific primers, oligonucleotide probes, and fluorescent reporter molecules. These probes hybridize with the target DNA sequence and emit a fluorescence signal, which produces an amplification curve, indicating the presence of the microorganisms' DNA [29].

In this study, fluorescence signals were detected using FAM and HEX fluorophores, and pathogen-specific primers were employed for the identification of each targeted sexually transmitted infection. The target genes/sequences, primers, and probes used for the detection of microorganisms are presented in Table 2. In this work, the primers and probes were used at a concentration of 400 nM and 100 nM, respectively.

Table 2. Target genes/sequences, primers, and probes for the detection and amplification of microorganisms and human RNAseP [20,30–33].

Target Gene	Primer Sequences (5′3′)
Cryptic plasmid from *C. trachomatis*	F: AACCAAGGTCGATGTGATAG R: TCAGATAATTGGCGATTCTT P: ROX-CGAACTCATCGGCGATAAGG
Por A pseudogene from *N. gonorrhoeae*	F: CCGGAACTGGTTTCATCTGATT R: GTTTCAGCGGCAGCATTCA P: CGTGAAAGTAGCAGGCGTATAGGCGGACTT
polA gene from *T. pallidum*	F: GGTAGAAGGGAGGGCTAGTA R: CTAAGATCTCTATTTTCTATAGGTATGG P: ACACAGCACTCGTCTTCAACTCC
2 kb repeat sequence from *Trichomonas vaginalis*	F: AAG ATG GGT GTT TTA AGC TAG ATA AGG T R: CGT CTT CAA GTA TGC CCC AGT AC P: CCG AAG TTC ATG TCC TCT CCA AGC GT
Human RNase P gene	F: AGATTTGGACCTGCGAGCG R: GAGCGGCTGTCTCCACAAGT P: TTCTGACCTGAAGGCTCTGCGCG

For each reaction, the PCR master mix was prepared as follows: 12.5 µL of NZY-Supreme Multiplex qPCR Master Mix, 5.5 µL of RNase-free water, and 2 µL of the corresponding primer/probe mix.

A total of 20 µL of this master mix was combined with 5 µL of the extracted DNA sample in each reaction tube. Amplification was conducted using the CFX96™ Real-Time

PCR Detection System (Bio-Rad, Hercules, CA, USA). For *Trichomonas vaginalis* DNA, the real-time PCR amplification program consisted of an initial hold at 50 °C (2 min), denaturation at 95 °C (10 min), followed by 40 cycles at 95 °C (15 s) and at 60 °C (1 min) [20]. The thermal cycling conditions for *Chlamydia trachomatis* DNA and *Neisseria gonorrhoeae* DNA consisted of an initial hold at 95 °C (2 min), followed by 40 cycles at 95 °C (30 s) and 60 °C (30 s) for *C. trachomatis*, and 55 °C (30 s) for *N. gonorrhoeae* [30]. For *Treponema pallidum* DNA, amplification conditions involved 50 cycles, including an initial denaturation at 95 °C (30 s), followed by 55 °C (30 s) and 72 °C (30 s) [31,32].

In each extraction run, a negative control was used to monitor contamination. The positive controls for each microorganism used in these experiments were certified reference-specific DNA solutions for each microorganism, from a commercial source, to guarantee that the amplification process was successful. To confirm the integrity of the samples, the human RNAseP gene was amplified in all analyzed specimens. RNAseP is widely used as an internal control in molecular diagnostics, serving as a marker of sample adequacy and amplification reliability.

Amplification of RNAseP was carried out using primers and probes specific to the human gene. For each reaction, a master mix was prepared containing 12.5 µL of NZYSpeedy One-step RT-qPCR Probe Master Mix (2×), 6 µL of RNase-free water, and 1.5 µL of RNAseP primers/probes. To this mixture, 5 µL of the extracted sample DNA was added, resulting in a final reaction volume of 25 µL.

The amplification was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad, CA, USA) under thermal cycling conditions as follows: initial denaturation at 95 °C (2 min), followed by 40 cycles at 95 °C (5 s) and 60 °C (30 s) [33]. The successful amplification of the human RNAseP gene confirmed the presence of human DNA and validated the overall reliability of the molecular workflow. The primer and probe sequences are listed in Table 1.

All PCR assays included positive and negative controls, and a cycle threshold (Ct) value below 35 was considered indicative of a positive result.

Both the extraction and amplification processes were carried out in duplicate for each sample. In cases where discrepancies were observed between duplicates, the samples were repeated to ensure confidence in the results.

5. Conclusions

Although we do not have information regarding the detection of STIS in these samples at the time of collection, this study proves to be extremely important due to the fact that we obtained positive STI results after 18 years of storage, in some samples. The successful amplification of pathogen DNA from long-term preserved forensic samples highlights the robustness of real-time PCR and underscores its applicability in contexts where sample integrity is frequently compromised. These findings emphasize the value of molecular diagnostics for retrospective forensic investigations, support clinical decision-making in the care and follow-up of survivors, and contribute to epidemiological surveillance by addressing diagnostic gaps in underreported or unresolved cases.

Real-time PCR proved to be a promising tool in both medical and legal responses to sexual violence. Future research should aim to validate these findings across diverse settings, expand the range of detectable pathogens—including viral agents—and standardize storage and handling protocols. Multicenter, prospective studies stratified by variables such as anatomical sampling site and storage duration will be essential to fully establish the role of molecular diagnostics as a reference method in forensic casework involving sexual assault.

Author Contributions: Conceptualization, L.C.; methodology, A.E., M.F. and H.C.D.; writing-original draft preparation, L.C. and A.E.; writing-review and editing, J.F. and A.A.; supervision, J.F., A.A. and L.C.; funding acquisition, L.C. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Instituto Nacional de Medicina Legal e Ciências Forenses, Portugal.

Institutional Review Board Statement: The study was approved by the Ethics Committee of the National Institute of Legal Medicine and Forensic Sciences (CE-22/2024) on 25 July 2024.

Informed Consent Statement: Informed consent was waived due to the retrospective nature of the study and use of anonymized data.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

WHO	World Health Organization
STI	Sexually transmitted infections
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
Ct	Cycle threshold
RNaseP	Ribonuclease P
INMLCF	National Institute of Legal Medicine and Forensic Sciences
STR	Short tandem repeat
NaCl	Sodium chloride
F	Forward primer
R	Reverse primer
P	Probe

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CHAPTER IV – DISCUSSION

Asymptomatic infections and the limitations of symptom-based diagnosis are common in STI, underscoring the need for laboratory confirmation to ensure accurate detection (Malleswari, K. et al 2023). When testing is conducted for medico-legal purposes, such as in cases of sexual assault or to establish links between victims and perpetrators, maintaining a proper chain of custody is essential to preserve the integrity of the evidence (Van Der Pol, 2014).

Despite their clinical and forensic significance, investigations specifically targeting the detection of STI in biological evidence from sexual assault cases remain scarce. This gap may contribute to the underestimation of infection rates, delays in diagnosis, and missed opportunities for timely medical and forensic interventions.

The present study aimed to assess the feasibility of real-time PCR for detecting *T. vaginalis*, *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* in biological samples collected from victims of sexual assault and preserved under standard forensic laboratory conditions for up to 18 years.

The implementation of real-time PCR in forensic settings represents a methodological advancement. In this study, real-time PCR enabled the detection of *C. trachomatis* and *T. vaginalis* in archived forensic samples, with prevalence rates of 4.8% and 5.6%, respectively. These findings are consistent with those reported by Sachs et al. (2023), who observed similar prevalence among victims of sexual violence (Sachs & Thomas, 2023).

Comparable results have been reported in other PCR-based studies, including those by Kebbi-Beghdadi et al. (2022), Schirm et al. (2007), Caliendo et al. (2005), and Šoba et al. (2015) (Kebbi-Beghdadi et al., 2022; Schirm et al., 2007; Caliendo et al., 2005; Šoba et al., 2015). In contrast, a significantly higher prevalence of *T. vaginalis* was found by Elsherif et al. (2012), likely due to preselection of culture-positive samples prior to PCR analysis (Hamed Elsherif & Fatah Youssef, 2012). Variations in prevalence across studies may be attributed to differences in sample selection, population characteristics, methodologies, and storage conditions (Dhawan et al., 2014).

This heterogeneity reinforces the importance of population and methodological context in interpreting STI data and underscores the diagnostic value of molecular tools in forensic applications.

Detection rates observed in samples stored for up to two decades highlight the value of integrating STI screening into routine forensic protocols. The asymptomatic nature of many STI, combined with prolonged storage intervals, presents challenges for conventional diagnostics. In this study, pathogen DNA was successfully amplified from long-term preserved samples, including two (IST015 and IST016) collected in 2007 and confirmed positive for *C. trachomatis*. These results confirm that, under appropriate preservation conditions, real-time PCR remains a reliable method for detecting STI in archived forensic material.

A higher prevalence of *T. vaginalis* and *C. trachomatis* was observed in women aged 15 to 37, aligning with WHO data identifying this group as particularly vulnerable (WHO, 2023). Vaginal and vulvar samples showed the highest number of positive results, confirming their importance as primary anatomical targets in STI detection. The identification of infections in perianal and oral samples also highlights the relevance of including these sites in forensic sampling protocols, especially in sexual assault cases.

Co-infection with *T. vaginalis* and *C. trachomatis* was identified in a single vulvar sample (IST200). Co-infections may increase transmission risk and complicate treatment, emphasizing the utility of multiplex diagnostic approaches. Identifying one STI may suggest the presence of others, justifying broader pathogen screening.

The absence of positive results for *N. gonorrhoeae* and *T. pallidum* could reflect a lower prevalence within the study population. However, DNA degradation over time cannot be ruled out as a factor, potentially reducing the sensitivity of molecular detection. This highlights the critical importance of specimen preservation to maintain nucleic acid stability and ensure accurate diagnostics—particularly for pathogens present in low abundance.

Among the twelve individuals who tested positive, samples were collected from multiple anatomical regions. In several, mixed genetic profiles were detected, including the presence of Y-chromosome haplotypes, indicating male DNA. These findings are particularly relevant in forensic investigations, as they support evidence of sexual contact and may assist in identifying perpetrators.

In one case (individual twelve, see table 1, Chapter III- Article 2), an exclusively male genetic profile was observed, reinforcing the potential value of these findings in forensic identification. In cases with Y-chromosome detection, the same evidentiary potential applies if appropriate reference samples are available for comparative

analysis. The detection of multiple contributors introduces complexity in interpretation and requires careful evaluation in forensic contexts.

Overall, these findings support the long-term applicability and evidentiary value of real-time PCR in forensic science. The method demonstrated robust performance in aged samples, reinforcing its suitability for routine use in both forensic and clinical investigations involving sexual assault.

Nonetheless, some limitations must be acknowledged. Although 231 swabs were analysed—a notable sample size for forensic studies—stratification by variables such as victim age or storage duration was not performed, limiting comparative analyses. The study also did not include inferential statistical methods, given its retrospective and descriptive design aimed at evaluating the feasibility of molecular detection.

Additionally, no comparison was made with conventional diagnostics such as culture or microscopy, limiting the ability to assess real-time PCR's specificity and sensitivity in this context. Future studies should aim to include stratified samples, statistical analyses, and parallel testing using conventional methods to fully validate the forensic application of molecular diagnostics.

CHAPTER V – CONCLUSIONS

STI are a frequent consequence of sexual violence and represent a significant public health concern. This study demonstrated that real-time PCR is a precise and reliable method for detecting the microorganisms *C. trachomatis* and *T. vaginalis* in biological samples collected from victims of sexual assault, even after storage periods of up to 18 years.

The ability to amplify the genetic material of these pathogens in long-term preserved samples highlights the robustness of real-time PCR, particularly in contexts where sample integrity may be compromised and conventional diagnostic methods prove insufficient.

These findings emphasize the relevance of real-time PCR not only in retrospective forensic investigations but also in supporting clinical diagnosis and long-term follow-up of victims. Additionally, they contribute to improved epidemiological surveillance by enabling the detection of underreported or previously undiagnosed cases.

The accurate identification of infectious agents in prolong storage samples may yield novel probative elements in previously inconclusive cases, allowing for the reopening of investigations, reinforcing forensic evidence, and ultimately advancing justice and victim protection.

These findings also highlight the importance of integrating molecular diagnostic advancements into routine forensic practice, promoting both scientific progress and a more equitable judicial response.

CHAPTER VI – FUTURE PERSPECTIVES

The results of this study support the promising potential of real-time PCR for the detection of STI in stored samples, and thereby enable future research and applications in both clinical and forensic contexts.

The development of faster and more accurate diagnostic tools with demonstrated forensic applicability remains a priority—particularly in cases involving sexual violence. Innovations that reduce false-negative results and support less invasive sample collection (e.g., saliva or capillary blood, if applicable) may help minimize retraumatization of victims and enhance compliance across both healthcare and judicial settings.

Equally important is the harmonization of sample processing protocols and a deeper understanding of how storage conditions affect diagnostic performance. Standardizing procedures for collection, preservation, and handling will be essential to ensure reproducibility and inter-laboratory consistency.

To establish real-time PCR as a reference method in forensic casework, future research should prioritize multicenter, prospective studies that include a broader spectrum of pathogens—particularly viral agents—and stratify results based on key variables such as anatomical sampling site, victim age, and storage duration.

These perspectives highlight the importance of integrating technological innovation in molecular diagnostics with strengthened collaboration between healthcare and judicial systems. Advancing STI detection methods not only improves the quality of forensic investigations and victim care but also contributes to a more effective and equitable response to sexual violence.

CHAPTER VII – REFERENCES

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