Establishment of a Rapid Detection Method for Microorganisms in the Lower Genital Tract Using MALDI-TOF MS and Its Clinical Application in Infertility

Project source: Hospital-level clinical research at Shenzhen Second People's Hospital

Research Unit: Shenzhen Second People's Hospital

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1. Program Summary

Research Title	Establishment of a Rapid Detection Method for Microorganisms in the Lower Genital Tract Using MALDI-TOF MS and Its Clinical Application in Infertility
Purpose of the study	1) Development and robustness evaluation of a rapid detection method based on Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for the simultaneous detection of multiple pathogens and CST in the lower genital tract 2) Assessment of the correlation between lower genital tract pathogenic infections, CST and infertility using the newly developed assay
Research Hypothesis	Possible correlation between CST, pathogens, and infertility in the lower genital tract
Study Design	Part I: Diagnostic tests; Part II: Case-control

Case group	Part I: MALDI-TOF MS test; Part II: Infertile individuals
Control group	Part I: Microbiological culture; Part II: Clinically proven to be spontaneous pregnancy
Sample size	Part I: 77 cases in each group; Part II: 114 cases in each group
Selection Criteria	The first part of all test samples are acquired standards with no inclusion criteria; the second part: Inclusion Criteria: Case group: 1) Female aged 18-40 2) Regular menstrual cycle 2) Conform to the clinical diagnosis of infertility 3) No sexual life 3 days before the examination control group: 1) Female aged 18-40 2) Regular menstrual cycle 3) Patients who did pre-pregnancy consultation in our hospital, pregnant at the time of 3-month follow-up 4) No sexual life 3 days before the examination - Exclusion Criteria: Case group: 1) Infertility due to malformation of reproductive tract 2) Infertility caused by endocrine factors such as polycystic ovary syndrome, premature ovarian failure and severe hyperthyroidism 3) Infertility due to male factors 4) Patients with malignant tumors 5) Patients with autoimmune diseases 6) Patients with severe heart, kidney and other basic diseases 7) Patients who consumed antibiotics 3 days before the examination

	8) Have the habit of smoking and drinking Control group: 1) Pregnancy by various assisted reproductive means 2) Patients with malignant tumors 3) Patients with autoimmune diseases 4) Patients with severe heart, kidney and other basic diseases 5) Patients who consumed antibiotics 3 days before the examination 6) Have the habit of smoking and drinking
Exclusion Criteria	All samples tested in Part I were acquired standards with no inclusion criteria; Part II: Case group: 1) Infertility due to malformation of reproductive tract 2) Infertility caused by endocrine factors such as polycystic ovary syndrome, premature ovarian failure and severe hyperthyroidism 3) Infertility due to male factors 4) Patients with malignant tumors 5) Patients with autoimmune diseases 6) Patients with severe heart, kidney and other basic diseases 7) Patients who consumed antibiotics 3 days before the examination 8) Have the habit of smoking and drinking Control group: 1) Pregnancy by various assisted reproductive means 2) Patients with malignant tumors 3) Patients with autoimmune diseases 4) Patients with severe heart, kidney and other basic diseases 5) Patients who consumed antibiotics 3 days before the examination 6) Have the habit of smoking and drinking
Ending evaluation	Main indicators: microbiological test results, are recorded as negative, positive

indicators	
Safety evaluation indicators	Pain indicators: pain score; bleeding indicators: bleeding volume assessment (blood staining area method), blood routine; cervical and vaginal infection: white belt routine

2. Research Background

1. 1 Research significance

The cervical canal and vagina (collectively referred to as the lower genital tract) are important components of the female reproductive tract, and infections of the lower genital tract are also the most common gynecologic conditions. Infections such as Neisseria gonorrhoeae (NG), Mycoplasma urealyticum (UU) and Chlamydia trachomatis (CT) not only cause discomfort, but may also lead to infertility, ectopic pregnancy, miscarriage and premature birth. These infections not only cause uncomfortable symptoms, but also may lead to infertility, ectopic pregnancy, miscarriage, premature birth and other adverse pregnancy outcomes, which seriously affect women's physical and mental health and family harmony.[1-2] The infection may lead to infertility, ectopic pregnancy, miscarriage, preterm delivery and other adverse pregnancy outcomes, which can seriously affect women's physical and mental health and family harmony. Disruption of the ecosystem of the lower genital tract can lead to overgrowth of pathogens, resulting in vaginal infections and sexually transmitted infections (STIs).[3] The disruption of the lower reproductive tract ecosystem can lead to an overgrowth of pathogens that can cause vaginal infections and sexually transmitted infections (STIs). The community state type (CST) of cervical and vaginal colonizing bacteria is the most important component of the lower genital tract microecosystem and has an important impact on the health of the lower genital tract.[4] It has an important impact on the health of the lower reproductive tract. Recent studies have shown that abnormal CST is strongly associated with infertility and poor pregnancy outcomes.[4-5] . Therefore, rapid, accurate and cost-effective detection of common pathogens and CST in the reproductive tract is a technique that gynecologists are eager to achieve, not only to comprehensively analyze the etiology

of infertility and poor pregnancy outcomes in patients, but also to further investigate the correlation between dynamic changes in CST and various pathogenic infections and diseases in the lower reproductive tract.

Currently, although inflammatory vaginal diseases such as mycosis fungoides, bacterial vaginosis, and trichomoniasis can be detected rapidly (30-60 minutes) by simultaneous routine leukocyte or vaginal microecological evaluation. However, in order to assess the infection status of the lower genital tract, sampling, culture and testing of Neisseria gonorrhoeae, mycoplasma and chlamydia need to be performed separately, which takes a long time (ranging from 2-5 days) and increases the patient's pain and may lead to an increase in the false-negative rate of the later specimens due to repeated sampling, and CST analysis is costly and time-consuming due to the use of 16SrRNA and high-throughput sequencing. Currently, CST analysis is only used in scientific research and lacks practical significance for clinical diagnosis and treatment of patients.

The emerging Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) detection system can detect multiple pathogenic microorganisms and subtypes simultaneously than traditional detection techniques such as fluorescent PCR technology, with high detection stability and accuracy, and lower cost and faster analysis than second-generation sequencing, and has been gradually used in recent years. in clinical testing[6] The project team has previously used MALDI to detect a variety of pathogenic microorganisms. The project team has previously established a cervical fluid-based cell PAX1 methylation detection platform using MALDI-TOF MS (**Patent No. CN202010753067.8., paper submitted**), and has extensive experience in the development of MALDI-TOF MS platform for clinical testing. This study will use the MALDI-TOF MS platform to establish a combined detection system for common pathogens in the lower genital tract and CST to fill this technical gap and address the practical needs of clinicians.

1.2 Current status of domestic and international research.

Sexually transmitted infections (STIs) are a major global health problem, with

127 million and 27 million adults worldwide having Chlamydia trachomatis and gonococcal infections in 2012, according to statistics.[7] . Both infections are mainly asymptomatic and curable, but if untreated or ineffective, they can lead to pelvic inflammatory disease, infertility and ectopic pregnancy, and Chlamydia trachomatis infection is also associated with a variety of adverse pregnancy outcomes, such as chorioamnionitis, preterm delivery and low birth weight[1] . Reproductive mycoplasma infections are as high as 2.0%-7.3% and are thought to be associated with UTIs, cervicitis, pelvic inflammatory disease, and infertility[8-9] .

The vast majority of patients with lower genital tract pathogens are only transiently infected and can be cleared by their own or pharmacological treatment, while only a minority of patients have serious adverse outcomes, suggesting that other factors influence the pathogenic evolution of these pathogens. Previous studies have shown that the type and abundance of long-term parasitic flora in the human cavity are closely related to important aspects of disease development such as immune regulation, inflammatory response, cytogenetic mutation, angiogenesis, and tissue remodeling.[10-11] . Therefore, in recent years, microecology has received a lot of attention from many fields such as immunity and metabolism. Since the discovery of Lactobacillus vaginalis by Albert Doderlein in 1982, a number of studies have been conducted to demonstrate that a decrease in the absence of Lactobacillus vaginalis is a major cause of inflammatory vaginal diseases such as mycobacteria and bacterial vaginitis.[12] However, because early studies on genital tract pathogens were mainly performed by microbiological microscopy, staining, and culture, it was not possible to subtype Lactobacillus, and the correlation between lower genital tract CST and obstetrical and gynecological diseases has only recently been emphasized. Recently, many scholars have studied the relationship between vaginal CST and obstetric and gynecological diseases, and such studies have suggested that abnormal CST in the lower genital tract can lead to pelvic inflammatory disease[13-15] infertility[15-17] and adverse pregnancy outcomes.[15, 18] However, does abnormal CST promote gonorrhea? However, it is unclear whether CST abnormalities promote infection and persistence of infertility-causing pathogens such as Neisseria gonorrhoeae, Mycoplasma

urealyticum and Chlamydia trachomatis.

Currently, most clinical tests for Neisseria gonorrhoeae, mycoplasma and chlamydia are performed using microbial cultures, and vaginal CST analysis is performed using 16SrRNA or macrogenomic sequencing.[19-21] . Therefore, performing multiple analyses on a patient at the same time is not only expensive, but also requires multiple sampling, which affects the accuracy of the results and makes it difficult to be widely implemented in the clinic. To the best of our knowledge, there is no accurate and cost-effective method for simultaneous detection of common pathogenic microorganisms in the lower genital tract. Therefore, this study proposes to establish a combined detection system for common pathogens and CST in the lower genital tract based on the MALDI-TOF MS testing platform, which will enable clinicians to accurately detect pathogens and common microorganisms in the lower genital tract with a single sample at a reasonable testing cost, and provide more accurate information and evidence for patient disease status assessment and treatment guidance. In addition, the project team will evaluate and compare the lower genital tract microecology of infertile and healthy control women using the constructed assay, and initially explore the correlation between lower genital tract microecology and infertility, and the correlation between various pathogenic infections and lower genital tract CST.

1.3 References

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3. Purpose of the study

1) Development and robustness of a rapid detection method for microorganisms in the lower genital tract based on time-of-flight mass spectrometry (MALDI-TOF MS) for simultaneous detection of multiple pathogens and CST

2) Assessment of the association between lower genital tract pathogenic infections, CST and infertility using a newly developed assay

4. Study design

The first part of this study is a diagnostic test using using a standard pathogenic strain; the second part is a prospective case-control study

5. Case selection

5.1 Entry Criteria

1. Subjects voluntarily enrolled in the study and signed an informed consent form

2. The inclusion criteria for the case and control groups were as follows.

Case group:

1) Female aged 18-40

2) Regular menstrual cycle

2) Conform to the clinical diagnosis of infertility

3) No sexual life 3 days before the examination

control group:

1) Female aged 18-40

2) Regular menstrual cycle

3) Patients who did pre-pregnancy consultation in our hospital, pregnant at the time of 3-month follow-up

4) No sexual life 3 days before the examination

5.2 Exclusion criteria.

Case group:

1) Infertility due to malformation of reproductive tract

2) Infertility caused by endocrine factors such as polycystic ovary syndrome,

premature ovarian failure and severe hyperthyroidism

3) Infertility due to male factors

4) Patients with malignant tumors

5) Patients with autoimmune diseases

6) Patients with severe heart, kidney and other basic diseases

7) Patients who consumed antibiotics 3 days before the examination

8) Have the habit of smoking and drinking

Control group:

1) Pregnancy by various assisted reproductive means

2) Patients with malignant tumors

3) Patients with autoimmune diseases

4) Patients with severe heart, kidney and other basic diseases

5) Patients who consumed antibiotics 3 days before the examination

6) Have the habit of smoking and drinking

5.3 Exit Criteria

Case group.

1）Unsatisfactory sample nucleic acid extraction

2）Intraoperative detection of developmental malformations or other non-colony related factors that can clearly cause infertility

Control group.

1) Unsatisfactory sample nucleic acid extraction

2) No spontaneous pregnancy at the time of follow-up

6. estimation of sample size and grouping method

6.1 Estimation of sample size

6.1.1 Part I: Comparison of sensitivity of dose information between the two groups

Single and mixed standard bacterial solutions and blank controls were 11 in total, so the chance of the appearance of four microorganisms in various standard strain solutions was 0.18, and different studies showed that the culture sensitivity of gonococci, lactobacilli, mycoplasma, and chlamydia was 40-60% and specificity 95-98%; time-of-flight mass spectrometry detection was >99% in different studies, and the parameters were brought into

PASS according to α=0.1 Software calculation path: Tests for Two Independent Sensitivities, N=44-72, taking the least integer multiple of 11 for >72, i.e., 77 cases in each group

6.1.2 Part 2: Comparison of independent sample rates between the two groups

Comparison of the independent sample rates of the two groups: the infection rate of at least one pathogen in the whole population is about 0.01%, and the infection rate of patients who visited our hospital for infertility is about 0.46%, and this data was brought into SPSS software: Confidence Intervals for the Difference Between Two Proportions, n=114 cases, so 114 cases were included in the experimental group and the control group each

6.2 Grouping method

Case group were patients with clinical diagnosis of infertility: sexually active, uncontraceptive and infertile for 2 years

The control group was those with spontaneous pregnancy: those with spontaneous pregnancy at the follow-up visit 3 months after consultation

7. Interventions

This study did not involve an intervention

8. Research steps

8.1 Part I. Development of a rapid detection method based on MALDI-TOF MS for simultaneous detection of multiple pathogens and CST in the lower genital tract and evaluation of diagnostic efficacy

8.1.1 Find the relatively conserved regions of virulence factor sequences of each pathogen according to the database, intercept the specific sequences from the conserved regions of each pathogen to design multiplex PCR amplification primers and single base extension (SBE) primers for each strain to construct MALDI-TOF MS assays.

8.1.2 Purchase standard pathogen strains, culture standard concentrations (i.e., high concentrations) of standard pathogens using the culture method to identify standard pathogens and their activities

8.1.3 Formulation of single, mixed and negative control standards with standard pathogens.

8.1.4 Gold standard: The actual pathogen type added to the standard is the gold standard.

8.1.5 Detection of standards at gradient dilution concentrations by incubation method and MALDI-TOF MS, respectively.

8.1.6 Performing diagnostic effectiveness analysis

8.2 Part II Assessment of the association between lower genital tract pathogen infection, CST and infertility using newly developed assays

8.2.1 Select infertile women of childbearing age who meet the criteria for nadir to sign informed consent and be enrolled in the study.

8.2.2 Completion of an information collection form to obtain information on **confounding factors that** may affect this study (age, weight, height, ethnicity, ancestry, place of long residence, number of pregnancies and births, time of last menstruation and menstrual cycle, duration of infertility, relevant diagnoses leading to infertility, etc.), which is collected daily by the information management specialist and kept.

8.2.3 Sample collection: take cervical canal and vaginal secretions during routine gynecological examination and routine leucorrhea examination secretions during routine consultation. ④ Place the swab head in sterile storage solution and tighten the cap ⑤ Label the bottle with patient information ⑥ Store in 4℃ refrigerator.

8.2.4 Isolation and extraction of pathogen DNA.

8.2.5 Detection of microorganisms in the lower genital tract by a constructed MALDI-TOF MS assay.

8.2.5 Uniform disposal of remaining specimens in accordance with medical waste after sample testing is completed.

8.2.6 Statistical analysis to assess the correlation between various pathogenic infections and CST status and infertility separately

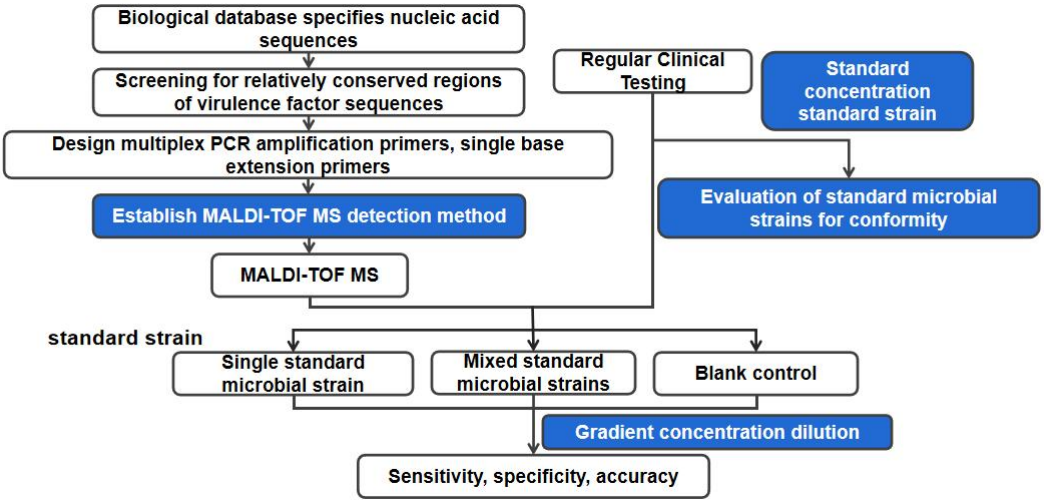
8.2.7 Statistical analysis to assess differences in CST status between infected and uninfected individuals with various pathogens

8.3 Sample preservation and handling

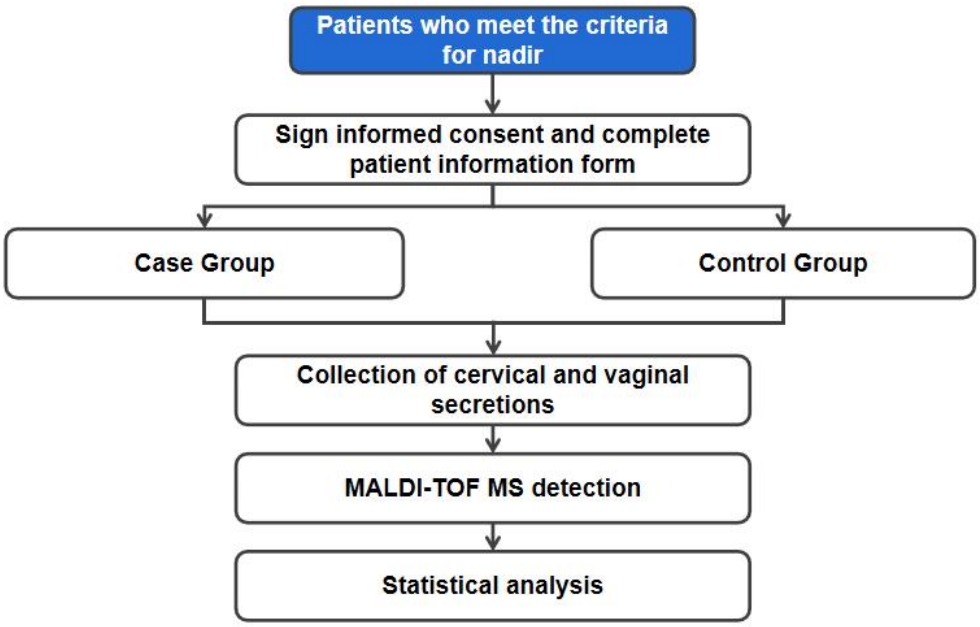
All specimens will be stored in a 4℃ refrigerator on the same day and DNA will be extracted within 24 hours. DNA specimens will be marked with specimen number and sampling time as required and then stored in a -80℃ refrigerator in the gynecology department of the central laboratory, and the remainder will be disposed of in accordance with the medical waste disposal process. After the DNA specimens are tested and the results are analyzed, they will be disposed of according to the medical waste disposal process.

9. Research Flow Chart

Part I.



Part II.



10. Ending evaluation indicators

10.1 Mycoplasma solium, Mycoplasma humanum, Chlamydia antigen, and gonococcus were recorded as positive or negative according to the clinical report.

10.2 MALDI-TOF MS test results were recorded as: microbial type, positive, negative

11. Security evaluation

This study involves only 1 vaginal and cervical secretion collection, which is consistent with HPV sampling during cervical screening in routine clinical testing and does not cause additional collection points or additional consultation costs, and is safe. It may cause pain and a little bleeding to the patient, neither of which will have any impact on the patient's health. Inform the patient before the operation to avoid causing panic. If the patient has intercourse immediately after the test or performs inappropriate transvaginal operations that may lead to infection, the patient is advised to avoid intercourse, sitz baths and other transvaginal operations without professional medical guidance for 3 days after sampling.

11.1 Vital signs observation and laboratory tests

Pain: WHO Pain Classification

Bleeding: ①Bleeding volume assessment: (according to the bleeding area assessment method in the Guidelines for the Diagnosis and Treatment of Abnormal Uterine Bleeding); ②Blood count

Infections: white belt routine, blood count

11.2 Adverse Events

(List of possible adverse events, all possible adverse reactions in the study should be fully considered, and should include possible adverse reactions in all aspects of laboratory tests (e.g. blood collection, ECG, imaging, etc.), interventions (e.g. drug treatment, surgical treatment, etc.) and other non-interventions (e.g. postural tests, scale tests, questionnaires, etc.), and evaluate the severity of the adverse reactions . (The same should also be reflected in the informed consent form, fully informed to the subject.)

Pain: pain can be graded (0-4), ≥1 noted as an adverse event

Grading: Grade 0: No pain.

Grade 1 (mild pain): painful but not severe, tolerable, sleep is not affected

Grade 2 (moderate pain): pain is obvious and unbearable, sleep is disturbed,

analgesics are requested

Grade 3 (severe pain): severe pain, unbearable sleep severely disturbed, analgesics needed

Bleeding: small amount 0.1-1ml, medium amount 1-5ml, large amount >5ml, medium and large amount of bleeding recorded as adverse events

Infections.

Mild infection - increased cervical or vaginal discharge, white belt routine indicating cleanliness grade III or leukocytes +++

Moderate - Large amount of discharge with abnormal characteristics, white belt routine indicating cleanliness IV or white blood cells ++++ or BV positive

Severe - with pain or pressure and the presence of infection are recorded as adverse events

11.3 Pre-assessment of relevant risks and disposal plans

Pain: ≥ grade 1 may be given pain medication

Bleeding: Vaginal gauze is used to stop heavy bleeding

Infection: mild infection - vaginal douching; moderate - vaginal medication based on routine leucorrhoea test results; severe - vaginal medication combined with oral anti-infection therapy based on routine leucorrhoea test results

*The study sponsor will bear all the costs related to the above adverse events and exempt the subjects from the costs related to the adverse events, including all the costs related to the adverse events during and after the end of the trial.

12. Data management and statistical analysis

(The following points should be clarified.

a. Whether data collection, processing and analysis are managed by a dedicated person and the manager has a GCP certificate.

b. the use of questionnaires, completed by patients with the assistance of the investigator, to complete the questionnaires and collect data.

c. **Using Empower statistical software,** sensitivity (Se), specificity (Sp), positive predictive value (PV+), and negative predictive value (PV-) were used for the first part **of the diagnostic efficacy assessment**, and chi-square test, rank sum test, stratified analysis,

and logistic regression correlation analysis were used for the second part.

d. Establish standard operating procedures and quality control procedures for each part of the operational process, and the experimental test report form must be complete with all items and all reports written using uniform standards to ensure **accurate and valid data**.

e. All data are collected and managed in a confidential account, and the paper files are stored in the department's clinical research file safe, so that any non-data administrator conducting data analysis cannot obtain patients' personal privacy information such as name, ID number, telephone number, and address.

13. Confidentiality measures

The results of the research through this project may be published in medical journals, but the investigators will keep the patients' information confidential as required by law and the patients' personal information will not be disclosed. When necessary, the government administration and the hospital ethics committee and its related personnel may have access to the patient's information as required.

14. Ethical standards

The study protocol and informed consent for this study should be approved in writing by the Clinical Research Ethics Committee of Shenzhen Second People's Hospital before proceeding.

All original informed consent forms signed and dated by the subject or their legal representative and the person conducting the informed consent process should be kept in a copy by the investigator.

The investigator or a person authorized by the investigator will be responsible for explaining the benefits and risks of participation in the study to each patient, the patient's legal representative or notary witness, if any, and shall obtain written informed consent prior to the patient's entry into the study.

This study is a non-interventional study. Only one additional tube of cervical and vaginal secretions was collected during routine gynecological examinations, and the sampling method was the common clinical method for HPV DNA testing. All subjects participating in the study were exempted from registration fees and gynecological examination fees (which are necessarily incurred in the course of routine treatment) as financial subsidies for study-related visits and follow-ups.