IDENTIFICATION OF 12 STD PATHOGENS IN SEMEN USING POLYMERASE CHAIN REACTION (PCR) AND “FLOW-THROUGH” HYBRIDIZATION TECHNOLOGY

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Sexually transmitted diseases (STD)

• Sexually transmitted diseases (STDs) are caused by several pathogens, including bacteria, viruses and protozoa, and can induce male infertility through multiple pathophysiological mechanisms.

• Sexually transmitted infections (STI)

• Illnesses that have a significant probability of transmission between humans by means of sexual behavior
  – vaginal intercourse
  – anal sex
  – oral sex
19 million new cases of sexually transmitted infections every year in the United State.¹

In 2005, the World Health Organization estimated that 448 million people aged 15–49 were being infected a year with curable STIs (such as syphilis, gonorrhea and chlamydia).²

There were an estimated 34 million people living with human immunodeficiency virus (HIV) in 2010.³

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1. Ochsendorf, F. R. Sexually transmitted infections: impact on male fertility. Andrologia 40, 72–75 (2008).
2. World Health Organization (WHO) Sexually transmitted infections Fact sheet N°110 August 2011 [Internet] Geneva: WHO; c2013
3. UNAIDS. UNAIDS world AIDS day report 2011 [Internet] Geneva: Joint United Nations Programme on HIV/AIDS (UNAIDS); [cited 2013 Jan 30].
High prevalence of sexual transmitted infections around the globe

1http://www.who.int/mediacentre/factsheets/fs110/en/
STDs can be bacterial, viral and parasitic

• Bacterial

- *Neisseria gonorrhoeae (NG)*
- *Ureaplasma parvum (UP)*
- *Chlamydia trachomatis (CT)*
- *Mycoplasma genitalium (MG)*
- *Mycoplasma hominis (MH)*
- *Ureaplasma urealyticum (UU)*

• Viral

- *Herpes Simples Virus 1/2 (HSV 1, HSV 2)*
- *Human Papillomavirus 6/11 (HPV 6, HPV 11)*

• Parasitic

- *Trichomonas vaginalis (TV)*
STDs can lead to serious outcomes

- Mother to child transmission route is possible.¹
- Emerging trend of drug resistant NG. ²
- STDs are associated with HIV infection ³

¹Jaiyeoba, O., Amaya, M. I., Soper, D. E., & Kilby, J. M. (2012). Preventing neonatal transmission of herpes simplex virus. *Clinical Obstetrics and Gynecology*, 55(2), 510-520.
²Unemo, M., & Nicholas, R. A. (2012). Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future microbiology*, 7(12), 1401-1422.
³Nusbaum, M. R., Wallace, R. R., Slatt, L. M., & Kondrad, E. C. (2004). Sexually transmitted infections and increased risk of co-infection with human immunodeficiency virus. *JAOA: Journal of the American Osteopathic Association*, 104(12), 527-535
Reasons to genotype STD pathogens

- Many STDs are asymptomatic
- Different STDs have overlapping symptoms
- Different STDs have different treatments
- Co-infection rate is unexpectedly high
Most STD are asymptomatic

- ~85% of women have asymptomatic CT infection
- HPV is the most common asymptomatic STD among younger patients
- Asymptomatic infection of NG accounts for around 10% in men and 50% in women

1 Eng, T. R., & Butler, W. T. (Eds.). (1997). The Hidden Epidemic:: Confronting Sexually Transmitted Diseases. National Academies Press.
2 Lehtinen, M. (2005). Preparations for implementing human papillomavirus vaccination should begin. Euro Surveill, 10(9), E050915.
3 Shmaefsky, B. R. (2009). Gonorrhea. InfobasePublishing
Different STDs share common symptoms

| Clinical symptoms | Causative pathogens |
|-------------------|---------------------|
| Urethritis        | *N. gonorrhoeae*    |
|                   | *C. trachomatis*    |
|                   | *Mycoplasma genitalium* |
|                   | *Trichomonasvaginalis* |
|                   | HSV 1/2             |
|                   | *Ureaplasmaurealyticum* |
| Cervicitis        | *C. trachomatis*    |
|                   | *N. gonorrhoeae*    |
|                   | *M. genitalium*     |
|                   | HSV 2               |
|                   | *Bacterial vaginosis*(BV) |
STD co-infection: more than you expect

MG: 30.4% with NG
- MH: 3.4% with UU
- UU/UP: High UU-MH co-infection pattern (13.91%)
- HPV: HSV and CT are associated with HPV infections

1 Mobley, V. L., Hobbs, M. M., Lau, K., Weinbaum, B. S., Getman, D. K., & Sena, A. C. (2012). Mycoplasma genitalium infection in women attending a sexually transmitted infection clinic: diagnostic specimen type, coinfections, and predictors. Sexually transmitted diseases, 39(9), 706-709.
2 Wang, Q.-Y., R.-H. Li, et al. (2014). "Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum and Mycoplasma hominis in female outpatients, 2009–2013." Journal of Microbiology, Immunology and Infection.
3 Zhu, Changtai, Liu, Jinming, Ling, Yang, Dong, Chunlei, Wu, Tingting, Yu, Xiaoyuan, ..., Cheng, Xiaowei. (2012). Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum and Mycoplasma hominis in Chinese women with genital infectious diseases. Indian Journal of Dermatology, Venereology, and Leprology, 78(3), 406.
4 Alberts, C. J., et al. (2013). "Association of Chlamydia trachomatis infection and herpes simplex virus type 2 serostatus with genital human papillomavirus infection in men: the HPV in men study." Sex TransmDis 40(6): 508-515.
STD co-infection: more than you expect

- **TV**: 5-13% with MG\(^1\)
- **HSV 1&2**: 1.4% with CT/NG\(^2\)
- **CT**: Among MG positive samples, 25% with CT co-infection\(^3\)
- **NG**: Among MG positive samples, 73.3% of female with NG co-infection\(^4\)

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1 Getman, D. (2014). Prevalence of M Genitalium, T Vaginalis, C Trachomatis and N Gonorrhoeaein Women Enrolled in a Prospective Multi-Center US Clinical Study. 2014 National STD Prevention Conference, CDC.

2 Vahidnia, A., et al. (2013). "A retrospective study into the prevalence of herpes simplex virus 1&2 in female patients tested for Chlamydia trachomatis and/or Neisseria gonorrhoeaeusing vaginal swabs." Clinical Microbiology and Infection 19(3): E166-E168.

3 Mobley, V. L., Hobbs, M. M., Lau, K., Weinbaum, B. S., Getman, D. K., & Sena, A. C. (2012). Mycoplasma genitaliuminfection in women attending a sexually transmitted infection clinic: diagnostic specimen type, coinfections, and predictors. Sexually transmitted diseases, 39(9), 706-709

4 Gaydos, C., Maldeis, N. E., Hardick, A., Hardick, J., & Quinn, T. C. (2009). Mycoplasma genitaliumas a contributor to the multiple etiologies of cervicitis in women attending sexually transmitted disease clinics. Sexually transmitted diseases, 36(10), 598
HOST FACTORS

• **AGE**: Highest rate are prevalent in 20-24 years old & followed by 25-29 & 15-19 years age groups.

• **GENDER**: Morbidity is higher in men.

• **MARITAL STATUS**: Higher among single, divorced and separated persons than among married couples
DEMOGRAPHIC FACTORS

Certain demographic factors contribute to a higher prevalence rates. They are:

1. Population explosion.
2. Rural to Urban migration.
3. Increasing educational opportunities for women.
4. Delayed marriage due to the afore said.
SOCIAL FACTORS

1. Prostitution (good time girl).
2. Broken homes.
3. Sexual disharmony.
4. Easy money.
5. Emotional immaturity.
6. Urbanization.
7. International travel.
8. Changing behavioural patterns.
10. Alcoholism.
OBJECTIVE
Identify the cause of infertility in Male.

- To access in health care facilities for diagnosis and common pathogens of STDs,
- Those causing infertility and see the cause of Chlamydia trachomatis, Neisseria gonorrhoeae, human papillomavirus (HPV) and Mycoplasma hominis transferred in female.
- Genital wart is a highly contagious sexually transmitted disease caused by some sub-types of human papillomavirus (HPV).
Causes of Infertility
Causes of Male Infertility

- It affects approximately 7% of all men.
- Male infertility is commonly due to deficiencies in the semen, and semen quality is used as a surrogate measure of male fertility.
- One third of fertility problems occur solely in women.
- One third in men.

The remaining third are mutual.
Sperm Abnormalities

Major sexually transmitted disease pathogens detected in semen

- Chlamydia trachomatis
- Neisseria gonorrhoeae
- Mycoplasma spp.
- Ureaplasma spp.
- Treponema pallidum

- Human papillomavirus
- HIV-1
- Herpes simplex virus
- Hepatitis B virus
- Hepatitis C virus
- Human cytomegalovirus

Trichomonas vaginalis
Risk factors for male infertility include:

- Aging, which can reduce sperm counts and motility and decrease the genetic quality of sperm
- Sexually transmitted diseases, which can cause scarring in the male reproductive system or impair sperm function
- Lifestyle factors such as smoking and substance abuse
- Long-term or intensive exposure to certain types of chemicals, toxins, or medications
**DNA damage**

- Common inherited variants in genes that encode enzymes employed in DNA mismatch repair are associated with increased risk of sperm DNA damage and male infertility

**Others Causes**

- Environmental toxins such as chemicals
- Radiation
- Pollution
- Drugs/ Street drug use
- Stress
- Cigarette smoking
- Frequent sex/ Lifestyle habits
- Heavy metal exposure/ Poor diet
- Overuse of alcohol
Materials and Methods
STD /11 PATHOGENS

Flow – Through Hybridization

DNA Extraction

STD /11 PATHOGENS

PCR

Screening Strategy I

Normal Count / Infection

Culture / Sensitivity

Screening Strategy II

PCR

Semen Analysis

Basic Analysis

Abnormal Count

Infertility

PCR

Infertility
### PCR-preparation

| Component                        | Volume (μl) |
|---------------------------------|-------------|
| PCR premix                      | 18.6        |
| DNA TaqPolymerase (5U/μl)       | 0.4         |
| 25 x Primer Mix                 | 0.1         |
| DNA template                    | upto 5      |
| Total                           | 25          |

### Thermo cycle

| Temperature (°C) | Duration |
|------------------|----------|
| 95               | 10 min   |
| 95               | 30 sec   |
| 61               | 30 sec   |
| 4                | for ever |

43 cycles
- The uniqueness of "Flow-through" hybridization is by directing the amplicons toward the DNA probes to form duplexes.
- The "Flow-through" hybridization is changing from traditional passive to active channeling process allowing the recombination reactions to complete in seconds.
- DNA hybridization assays are known to have excellent signal-to-noise ratio.
- It enables unambiguous detection of multiple pathogens/disease genotypes in one single reaction.
## Hybridization-at-a-glance

| Solution                  | Volume (µl)    | Incubation | After incubation |
|---------------------------|----------------|------------|------------------|
| **43 °C FT pro**          |                |            |                  |
| Pre-hybridization         | 150            | 2 min      | Drain            |
| Hybridization             | 150 + 25 PCR product | 5 min      | Drain            |
| Hybridization solution wash | 200 x 3       | -          | Drain            |
| **25 °C**                 |                |            |                  |
| Blocking                  | 150            | 5 min      | Drain            |
| Enzyme Conjugate          | 150            | 5 min      | Drain            |
| **36 °C**                 |                |            |                  |
| Wash A solution           | 200 x 4        | -          | Drain            |
| Detection Solution        | 150            | 3 min      | Drain            |
| Wash A solution           | 200 x 3        |            | Drain            |
| Stop                      | 150            | 1 min      | Drain            |
Results
### Screening Strategy I

| Motility     | Rapid progressive | Slow progressive | Non progressive | Immotile |
|--------------|-------------------|------------------|----------------|----------|
| Normal       | Normal            | Watery           | Viscous        |          |
| Viscosity    | ≤ 20%             | ≥ 40%            | ≥ 40           |          |
| Viscosity    | Normal Form       | Head Defective   | Mid Piece & Neck Defective | Tail Defective |
| Sperm Morphology | ≤ 20       | ≥30              | ≥20            | ≥10      | ≥2:1    |
| Debris       | Round cell        | Epithelial cells | Erythrocytes   |          |
| Culture / Sensitivity | Moderate or Heavy | ≥ 10 %           | ≥ 4%           | Present  |
| Normal       | Head to Head      | Head to Tail     | Tail to Tail   |          |
| Agglutination| ≤ 10              | ≥ 20             | ≥ 10           | ≥ 10     |
Detect 11 common STD pathogens:

1. Chlamydia trachomatis (CT)
2. Neisseria gonorrhoeae (NG)
3. Mycoplasma genitalium (MG)
4. Ureaplasma urealyticum (UU)
5. Ureaplasma parvum (UP)
6. Trichomonas vaginalis (TV)
7. Mycoplasma hominis (MH)
8. Human papillomavirus type 6
9. Human papillomavirus type 11
10. Herpes simplex virus type 1
11. Herpes simplex virus type 2
12. Amplification Control (AC)*
13. Positive Control (PC)*
The Target pathogens detected by Gene Flow - Through from Semen sample
The percentage of Target pathogens detected by Gene Flow from Semen sample to see the reason of infertility

| NAME OF THE STD (N=46) | DETECTED | PERCENTAGE |
|------------------------|----------|------------|
| Chlamydia trachomatis   | 02       | 4.3        |
| Neisseria gonorrhoeae   | 05       | 10.8       |
| Mycoplasma hominis      | 11       | 23.9       |
| Treponema pallidum      | 01       | 2.1        |
| Human Papillomavirus    | 01       | 2.1        |
Co-infection rate

| Co-Infection | Percentage |
|--------------|------------|
| CT/NG        | 4.3        |
| CT/HPV       | 6.5        |
| MH/UU        | 2.1        |
| MG/UU        | 2.1        |
Higher infection rate of CT and NG in positive sample

- CT and NG infection: most important STD screening parameters
- NG infection: asymptomatic or often mistaken as virginal or bladder infection\(^1\). May progress to serious complications as skin pustules or petechial, septic arthritis, meningitis, or endocarditis\(^2\).
- \(~85\%\) of women have asymptomatic CT infection\(^3\)

\(^1\)http://www.cdc.gov/std/gonorrhea/stdfact-gonorrhea.htm
\(^2\)Moran, J. S. (2007). Gonorrhoea. BMJ Clinical Evidence, 2007, 1604.
\(^3\)Eng, T. R., & Butler, W. T. (Eds.). (1997). The Hidden Epidemic:: Confronting Sexually Transmitted Diseases. National Academies Press.
CT/HPV co-infection may worsen the clinical outcomes

• Co-infection may prolong both pathogen’s infection, and increasing the risk of cervical cancer or infertility

• CT/HPV co-infection may delay HPV lesion clearance

• Monitoring and control of both pathogens are necessary

• The co-infection effects of other STD pathogens are also concerned

1. Denks, K., Spaeth, E. L., Jõers, K., Randoja, R., Talpsep, T., Ustav, M., & Kurg, R. (2007). Coinfection of Chlamydia trachomatis, Ureaplasma urealyticum and human papillomavirus among patients attending STD clinics in Estonia. *Scandinavian journal of infectious diseases, 39*(8), 714-718.

2. Verteramo, R., Pierangeli, A., Mancini, E., Calzolari, E., Bucci, M., Osborn, J., ... & Degener, A. M. (2009). Human Papillomaviruses and genital co-infections in gynaecological outpatients. *BMC infectious diseases, 9*(1), 16.

3. Simonetti, A. C., de Lima Melo, J. H., de Souza, P. R. E., Bruneska, D., & de Lima Filho, J. L. (2009). Immunological’s host profile for HPV and Chlamydia trachomatis, a cervical cancer cofactor. *Microbes and Infection, 11*(4), 435-442.
Conclusion
STD screening and treatment should be a primary intervention and a standard of care in all health care settings.

Screening for bacterial STI pathogens, *Mycoplasma hominis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are strongly recommended because these pathogens can cause serious reproductive complications such as pelvic inflammatory disease, ectopic pregnancy.

Studies show STD and HIV co-infection increases HIV virus shedding in the patients’ genital secretions.
• *STD infection* rates are common around the globe
• HPV/STD co-infection may *worsen the clinical outcome*
• *Close monitoring* of both HPV and STD infection is necessary
• Cost effect technology should be used
THANK YOU