ISOLATION AND CRYPTOPRESERVATION OF TRICHOMONAS VAGINALIS - STUDY FOR A LOT OF PATIENTS WITH SIMPTOMATIC VAGINOSIS

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Abstract

- *Trichomonas vaginalis* is a flagellated protozoon, the major cause of trichomoniasis, a wide-spread, sexually transmitted disease.
- Samples were collected from patients showing vaginal discharge symptoms. Smears and cultures from vaginal discharges from patients with vaginosis were analyzed to assess the type of vaginosis (bacterial or parasitic).
- Bacterial and *Trichomonas vaginalis* strains were isolated, cultured and freeze-dried. The freezing method was comparatively developed (using DMSO and glycerol) and adapted for the common laboratory use.
- Glycerol and DMSO freezing methods were comparatively analyzed after defrosting of *TV* strains;
- Glycerol freezing method revealed a higher viability of parasites than the DMSO one.
Scope of the work

• To assess the specificity and selectivity of culturing media ordinary used for harvesting of *T. Vaginalis*;
• To compare two different freezing methods;
• To assess the efficiency of *TV* freezing in time;
• To compare the slow and fast type of freezing of *TV*;
The samples were collected during a phase II clinical study from clinical sites located in Romania and Estonia. We were focused on reporting the following list of potentially pathogenic bacterial germs and parasites:

- **always pathogenic** in the genital tract:
  - Neisseria gonorrhoeae
  - Trichomonas vaginalis
  - Candida spp;
  - Listeria monocytogenes
  - Haemophilus ducreyi
  - Treponema pallidum

- **potentially pathogenic:**
  - Gardnerella vaginalis,
  - Streptococcus agalactiae (group B)
  - Staphylococcus aureus and
  - Enterobacteriaceae
Structure of the work

- Microbiology Department:
  - smear examination;
  - bacterial culture;
  - fungus culture;

- Clinical Research Department:
  - Laboratory part:
    - TV wet mount;
    - TV culture and direct examination;
    - TV freezing
  - Logistics part:
    - kits preparation;
    - clinical samples transport;
Materials and Methods

- Microscope;
- Bulb Bunsen;
- Bacteriological loop;
- Gas pack System;
- Exsicator;
- In Pouch TV system;
- In Pouch subculture medium (Trichomonas broth);
- Hemacytometer;
- -80°C freezer – for isolate storage;
- Columbia Agar with 5% sheep blood (2 plates: 1 plate for anaerobic incubation and 1 plate for aerobic incubation);
- Gardnerella Selective Agar with 5% Human blood;
- Lactobacillus MRS Agar
- Broth media for enriched growth: Brain Hearth Infusion (BHI) (to support aerobic growth), THIO (to support anaerobic growth), Lactobacillus MRS Broth;
- GASPAK system for anaerobic bacteria;
- Viatek System
- API Coryne system;
- API 20 STREP system;
- API 50 CHL medium; (*Leuconostoc oenos*)
- API 20 A kit system;
- Antimicrobial agent disk: metronidazole 50 µg, trimetroprime 5 µg, sulfonamide 1µg, kanamycin 1000 µg, vancomycin 5 µg, colistin 10 µg, erythromycin, rifampicin and penicillin – if required
- Incubator at 35- 37°C;
- Sterile loops, sterile slides, sterile cotton swabs;
- Microscope Viewing Clip, Disposable gloves, Disposable glass pipettes, PBS, 10% DMSO freezing-medium (Sigma);
- Biosafety Level II hood;
Introduction. *Trichomonas vaginalis*
structure and life cycle

- Flagellated anaerobic protozoa, having an anterior flagellum and recurrent flagellum;
- shapes - ellipsoidal, ovoidal, spherical;
- size of the trichomonad is approximately the same as that of a lymphocyte (10 µm to 20 µm);
- Large nucleus situated in the anterior end;
- Ondulating membrane;
- Cytoskeletons (costa – specific for TV that possesses ondulating membrane, providing mechanical support, pelta-axostyle – bundle of microtubules across cell body as anterior-posterior axis, parabasal filaments, etc.);

<table>
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<tr>
<th>Kingdom</th>
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<tr>
<td>Phylum</td>
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<td>Species</td>
<td>T. vaginalis</td>
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</table>
Introduction (cont’d)

TV dimensions on gram smear

TV movie

TV life cycle
**TV diagnostic - past and present**

- Wet mount microscopy – the most cost effective, still not so precise, as described in the literature;
- Different stains and smears including Giemsa, Gram, Papanicolaou and acridine orange;
- Enzyme immunoassay (EIA);
- Monoclonal antibody staining of direct specimens;
- Latex slide agglutination test;
- Broth culture technique (Diamond’s TYI medium in glass tubes) – contamination with bacteria is a major problem; method expensive;
- Cultivation on cell cultures – very expensive method, require pretreatment of the TV specimens with antibiotics using Diamond TYI medium transfer medium, followed by subsequent passage onto cell cultures;
- PCR method;
- The XenoStrip test: immunochromatographic, capillary flow dipstick technology to detect the presence of stable *T. vaginalis* antigens from vaginal samples, utilizing immunoglobulin G1 monoclonal antibodies XDTv1 and XDTv2 to target intracellular and surface secretory proteins;
- Direct examination using InPouch TV system;
TV diagnostic – past and present (cont’d)

- In Romania, Bucharest Cantacuzino Institute harvested *Trichomonas vaginalis* since 1970-1980 using Loeffler medium (bovine blood serum) enriched with rice starch and streptomycin or beef spleen extract diluted in Ringer’s solution (no rice starch) enriched with streptomycin;
- Vaginal swabs were discarded in warm physiological serum and cultured on the above mentioned media;
- *TV* was identified after 24/48/72 hours by microscopically examination;
InPouch TV system structure

- System which provide an accurate identification and culture confirmation of TV from female and male urogenital sites;
- It maintains the viability of TV for up to 72 hours in ambient conditions; this made possible the transportation of the TV samples from overseas clinical sites;
- Contains trypticase, proteose peptone, yeast extract, maltose and other sugars, amino acids, salts, antifungal and antimicrobial agents in normal PBS;
InPouch TV system advantages

• Directly microscopic examination;
• It’s the only kit approved by FDA for TV transportation;
• Maintains TV viability for up to 72 hours in ambient conditions;
• Competitive performance – can detect a single trichomonad;
• Showed 23 to 57% more high sensitivity than Diamonds or Tricosel media;
• Prevention of sample contamination;
• No slide preparation or centrifugation is necessary;
• Convenient transporting system;
• Can be stored at room temperature whereas other media require refrigeration;
• Low cost;
Patients samples

- Were obtained on a period of 10 months from 7 different sites from Romania and Estonia, from patients over 18 years old, not pregnant, showing symptomatic vaginitis (discharge, itching, burning, irritation, edema, erythema and/or excoriation of the vagina/vulva, painful sexual intercourse (dyspareunia);
- Were transported in ambient conditions to our laboratory;
- Were collected using the following kit:
  - 3 sterile cotton swabs
  - 1 In Pouch TV’ system
  - 1 ACT 1 tube
  - 1 port-slide with 2 glass blades
  - 1 thermo bag
  - 1 requisition form and barcode labels
  - 1 cardboard box;
Inoculation of InPouch TV system

1. Squeeze the fluid from the top of the InPouch downward toward the bottom of the upper chamber;
2. Tear off the plastic above the closure;
3. Open the InPouch and insert the cotton swab containing the vaginal discharge;
4. Squeeze the top of the upper chamber and move the fluid downwards;
5. Roll the upper chamber down to the blue label;
6. Fold the tabs over to prevent leakage;
7. Apply the label/if applicable;
Laboratory tests

The following laboratory tests were performed:

- Gram stain smear;
- Bacterial and fungi culture;
- Gardnerella vaginalis inoculation and culture;
- Lactobacillus spp. inoculation and culture;
- Anaerobic germs inoculation and culture;
- *Trichomonas vaginalis* wet mount;
- *Trichomonas vaginalis* culture;
Gram stain smear

- Is prepared directly from the pathological sample;
- Is microscopically examined for the presence of the following items (according to Nugget scheme, but no score was determined as it was not required by the study sponsor);

- Clue cells/lpf;
- Yeasts, hyphae and/or buds/hpf;
- Large, gram-positive bacilli (Lactobacillus spp. morphotypes)/hpf;
- Small, gram-variable bacilli (Gardnerella spp. morphotypes)/hpf;
- Curved, gram-negative or gram-variable bacilli (Mobilluncus spp. morphotypes)/hpf;
- Trichomonas vaginalis;

Gram-stained smear positive for TV
Direct examination of inPouch TV system Trichomonas vaginalis

• InPouch TV system could be directly examined under optical microscope using the viewing clip;
• Roll the EMPTY upper chamber down to the top of the label, fold the tabs over to prevent the InPouch from reopening;
Direct examination of inPouch TV system Trichomonas vaginalis

- InPouch TV system must be squeezed with the thumb and forefinger;
- Hold the bottom of the pouch with the other hand and move the medium from the top chamber to the lower chamber by pulling it upward across the edge of the counter/table in a “shoe shine” motion;
Direct examination of inPouch TV system Trichomonas vaginalis

- Place the viewing clip horizontally over the lower chamber;
- Close the clip. (Using the clip is optional). Place the pouch on the stage and scan under 10 x and 20x. Confirm under 40x;
InPouch TV systems processing

- InPouch TV direct microscopic examination
- InPouch TV wet mount

If POSITIVE (motile trichomonads observed)

- Incubation at 37°C

If NEGATIVE (no motile trichomonads observed)

Further subculturing and passages

If POSITIVE (motile trichomonads observed)

Discard the sample

If NEGATIVE (no motile trichomonads observed)

InPouch TV System examination after 24/48/72 hours
Trichomonas vaginalis freezing methods

There are two different freezing methods described in the literature, one is performed using glycerol, and one using DMSO. Both have been used for Trichomonas vaginalis freezing.

- Glycerol TV freezing method, suggested by BioMed Diagnostics;
- DMSO freezing method, suggested by the clinical study sponsor;
DMSO freezing method

- TV isolated were harvested using subculture pouch TV/InPouch TV system/Vagicult /Trichomonas broth (Sanimed);
- After 48/72 hours, subculture pouch TV, InPouch TV, Vagicult and Trichomonas broth were checked for exponential growing;
- After 48/72 hours, the cultures were transferred into 10 ml sterile glass tubes and washed with 37 °C PBS. They were centrifuged at 1000xg for 10 minutes for separating the trichomonads (as specify the sponsor request);
- Trichomonads were hemacytometrically counted;
- $1 \times 10^5$ trichomonads were aliquoted in 1mL DMSO 10%;
- The aliquots were placed in the -80 °C freezer;
DMSO freezing method (cont'd)

48/72 hours exponential grown TV culture

37°C PBS washing

PBS washing step was dropped due to the dilution of the sample

37°C PBS washing

centrifugation at 100xg, 15 minutes

hemacytometrically counting of TV in sample

no aliquoting was possible

Total number of TV/mL < 1x10^5 trichomonads/mL

Hemacytometrically counting of TVs in the sample

Aliquoting of 1x10^5 trichomonads/mL in 1mL 10% DMSO

freezing at -80°C
Glycerol freezing method

- TV isolated were harvested using subculture pouch TV/InPouch TV system/Vagicult /Trichomonas broth (Sanimed);
- After 48/72 hours, subculture pouch TV, InPouch TV, Vagicult and Trichomonas broth were checked for exponential growing;
- After 48/72 hours, the cultures were hemacytometrically counted for the number of Trichomonads;
- $1 \times 10^5$ trichomonads were aliquoted in a new InPouch TV system;
- 50 µL of glycerol were added to the aliquot;
- The aliquots were placed in the -80 °C freezer;
Glycerol freezing method

1. 48/72 hours exponential grown TV culture
2. Hemacytometrically counting of TVs in the sample
3. Aliquoting of $1 \times 10^5$ trichomonads/mL in a new InPouch TV System
4. Add 50μL of glycerol

Sub-processes:
- Transfer in dry ice & ethanol (fast freezing)
- Transfer in -80°C freezer (slow freezing)
- Transfer direct in -80°C freezer (slow freezing)
## Results and discussion

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<thead>
<tr>
<th></th>
<th>Total</th>
<th>Site 1</th>
<th>Site 2</th>
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<td>52</td>
<td>163</td>
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![Graph](image)
Results and discussion (cont’d)

<table>
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<tr>
<th>Pathogen name</th>
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<td>Total positive samples</td>
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<td>Klebsiella pneumoniae</td>
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<td>Streptococcus agalactiae</td>
<td>33</td>
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<tr>
<td>Trichomonas vaginalis</td>
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</table>
Results and discussion

Trichomonas vaginalis freezing scheme
Results and discussion

- Subculture pouch TV showed a very less growing after 48/72 hours, therefore it was dropped as passaging system;
- Vagicult system showed a high exponential growth after 48 hours, then a decrease;
- Trichomonas broth showed a high exponential growing after 72 hours and a high specificity;
- The best exponential growing was realized using the InPouch TV system for culturing;
- The washing step with 37 °C PBS forwarded by centrifugation at 1000xg for 10 minutes for separating the trichomonads was dropped as it was noticed that, due to the high motility of trichomonads, no separation occurred, only a dilution of the samples and the aliquoting was not possible;
- Trichomonads were also hemacytometrically counted direct from the sample (without washing). As a sufficient number was found, aliquoting was possible.
Results and discussion (cont’d)

• Defrosting was performed from both fast frozen and slow frozen isolates;
• Defrosting was performed from both DMSO frozen isolates as well as glycerol frozen isolates;
• Defrosting of the frozen TV isolates was performed at three time intervals: at 72 hours after freezing, at 2 weeks after freezing and at 1 year after freezing;
Reconstruction of *Trichomonas vaginalis* after defrosting

1. After removing from -80°C freezer, InPouch TV kits/DMSO bottles containing the frozen TVs were incubated immediately (in vertical position) at 37°C for 24h;
2. After 24h, InPouch TV kits were microscopically examined under low power (10X); the best location is above the bottom edge; before reading, the InPouch TV kits were mixed by gently pulling the InPouch up and down across the edge of a table 3-4 times; from DMSO bottles, a smear was prepared and microscopically examined;
3. Further incubate the InPouch for another 24h;
4. Repeat step 2
5. Repeat step 3
6. Repeat step 2
Results and discussion (cont’d)

[Bar chart showing recovery percentage over time for Glycerol fast freezing and DMSO freezing.]
Results and discussion (cont’d)

• It was noticed that, if reconstituted after 72 hours, TV frozen isolated become viable and shown a total recovery; the same for 2 weeks;
• If reconstituted after 1 year, the TV reconstitution was totally unsuccessful;
• All viable reconstituted TV strains were previously fast frozen (with dry ice and ethanol);
Conclusions

• InPouch TV system has been shown to be the appropriate system for TV transport and culture;
• Pouch TV subculture medium didn’t show an exponential growing for neither of the TV samples;
• Trichomonas broth showed a high specificity for TV culturing, but it will not be further used due to the high cost; also, it is not suitable for TV sample shipment;
• Reconstituting of the TV cultures after defrosting from 10% DMSO was totally unsuccessful;
• Reconstituting of the TV cultures after defrosting from glycerol was successful after 72 hours of freezing and after 2 weeks of freezing;
• Reconstitution of the TV frozen strains at regular time intervals will be the aim of a next research project; moreover, the TV isolates freezing in liquid nitrogen will be analyzed;
THANK YOU!