Materials and Methods
MATERIALS AND METHODS

I. ISOLATION

1. Study Population

A. Patients

The study group consisted of in and out patients from the Institute of Venereology of the Government General Hospital, Madras, Rajah Sir Ramaswamy Mudaliar Lying in Hospital, Madras and Kasturba Gandhi Hospital for Women and Children, Madras.

Ninety two patients with NGU on their first visit to the Hospital were screened for the genital mycoplasmas and ureaplasmas. A case was considered as NGU clinically based on the presentation of a patient with purulent, mucoid, mucopurulent or watery discharge and with a varying degree of dysuria. Microbiologically, NGU was diagnosed based on the presence of 5 or more pus cells per high power field microscopy from the urethral swabs and centrifuged deposit of urine. Gonorrhoea was ruled out by culture. None of the patients received any antibiotic treatment during the preceding month. Also, 28 patients with NGU were followed up and specimens were collected 10 or more days later during their subsequent visit to the Hospital.

Eighteen cases of prostatitis were screened for genital mycoplasmas and ureaplasmas. Patients with prostatitis presented
with 'prostatic pain' and any one or a combination of symptoms that include urgency, frequency, dysuria and painful ejaculation.

Fifty one cases of PID were screened for the genital mycoplasmas and ureaplasmas. PID was clinically diagnosed in patients who presented with a combination of symptoms of abdominal tenderness, severe abdominal tenderness, abdominal rebound tenderness, Pyrexia over 38°C, rigors, malaise, anorexia, and vomiting. Those patients who did not receive any antibiotic treatment during the preceding month alone were included in the study. Also, 20 patients with PID were followed up and specimens were collected 10 or more days later during their subsequent visit to the Hospital.

Forty eight patients with post partum fever and 27 patients with post abortal fever were screened to ascertain the role of mycoplasmas and ureaplasmas in these infections. Patients with a temperature of 100.4°F (38°C) or more were only included in the study. Since fever during the 24 hours after delivery is usually not considered with in the classical definition of post partum fever, patients who were febrile after 24 hours of delivery or abortion were only included in the study.

Tubal aspirates from 25 cases of infertility were obtained by Hysteroscopy and screened for the genital mycoplasmas and ureaplasmas. Hysteroscopy was performed in patients with a history of one year or more of infertility.
Thirty two patients with vaginitis and cervicitis and 6 patients with abscess of Bartholin's gland were screened for the genital mycoplasmas and ureaplasmas. Patients with vaginitis and cervicitis presented with discharge and with the symptoms of vulvar pruritis, burning dyspareunia, erythema and oedema of the labia minora.

Sixty three cases of sexually transmitted disease were also screened for mycoplasmas and ureaplasmas. This includes 20 cases of gonorrhoea, 17 cases of syphilis, 9 cases of genital Herpes and 17 cases of mixed infections which includes a combination of 2 or more infections such as gonorrhoea, syphilis, genital herpes, genital warts and chancroid. Gonorrhoea was confirmed by culture and syphilis was diagnosed clinically, dark field microscopy and by VDRL test.

B. Controls

Seventy three age matched males who never had any history of ureteritis and who did not receive any antibiotics during the preceding month formed the control population. This was represented by patients who attended the general hospital for treatment of other than genital infections.

Sixty one age matched women who did not receive any antibiotics during the preceding month formed the control population for females. 27 women who were afebrile during and after 24 hours of delivery formed the control population for the post partum
fever cases. 20 women who had abortions and were afebrile during and after 24 hours of abortion formed the control population for post abortal fever cases.

2. MEDIA

A. Transport Media

a. Cary and Blair Transport Medium and Stuart Transport Medium

These dehydrated media from M/s. Hi Media Laboratories Pvt. Ltd., India was used and prepared according to the direction of the manufacturers.

b. PPLO Transport Media

This was prepared according to Velleca et al. (1980), but slightly modified and was found to maintain the viability of genital mycoplasmas. Oxoid PPLO base medium was used and 70 ml of it was prepared according to the directions of the manufacturers. The pH was adjusted to 6.2 and autoclaved at 121°C for 15 min., cooled and the following sterile supplements were added.

a. Normal Horse serum ...
   ... 20 ml
b. 25 % yeast extract ...
   ... 10 ml
c. Penicillin G. (1,00,000 u/ml) ...
   ... 2.0 ml
d. Polymyxin B sulphate (5000 μ/ml) ...
   ... -1.0 ml
e. Amphotericin (5000 μ/ml) ...
   ... - 0.1 ml
f. 0.4 % phenol red ...
   ... 0.3 ml
B. Growth Media

a. Blood Agar:

This was prepared by using standard procedure (Cruickshank et al., 1975).

b. MacConkey Agar, Sabouraud Dextrose Agar and Brain Heart Infusion Broth:

These dehydrated media from M/s. Hi Media Laboratories Pvt. Ltd., were used and prepared according to the directions of the manufacturers.

c. Bile Broth:

This was prepared as follows:

- Ox bile ... 90 ml
- Glycerol ... 10 ml
- Trypticase (peptone) ... 2 g

The trypticase was dissolved in the broth, filtered and autoclaved at 121°C for 15 minutes.

d. Chacko-Nair Medium:

It was prepared as follows:

1. Trypsin Digested Beef Extract

To 100 gms of minced beef free from fat 500 ml of distilled water was added and was mixed thoroughly in a waring blender.
15 ml of 1 N NaOH was added and kept in water bath at 75°C for 10 minutes and then cooled to 40°C. To this 0.2 gms of trypsin powder was added and this was placed in an incubator at 37°C for 5 hours (shaking at every 1 hour interval). The trypsin digestion was stopped with 0.7 ml of glacial acetic acid and this was boiled for 10 minutes. This was left at +4°C overnight and was filtered the following morrow through a piece of gauge.

ii. 500 ml of 1 % NaCl was prepared in a separate flask.

iii. Equal amounts of (i) and (ii) were mixed and 1 gm of glucose and 5 gms of Disodium; mono hydrogen phosphate (Na₂HPO₄) were added. The pH was adjusted to 7.2. This was distributed in 200 ml and 4 gms of agar was added to this, dissolved and autoclaved at 10 pounds for 15 minutes and stored at +4°C.

For the preparation of complete medium the basal medium was melted and maintained at 56°C in a water bath and the following were added per 200 ml.

Vancomycin ... 500 micrograms
Polymyxin ... 1000 micrograms
Mycostatin ... 2000 units.

The contents of one egg (both yellow and white) were added aseptically to 200 ml of medium and distributed into plates.
e. Medium B Broth: Modified Hayflick Medium (Freundt, 1983)

This was slightly modified and was found to support excellent growth of arginine metabolizing mycoplasmas. The complete medium was prepared as follows:

90 ml of Heart infusion broth obtained from M/s. Hi Media Laboratories Pvt. Ltd, was prepared according to the directions of the manufacturer. The pH was adjusted to 7.4, autoclaved at 121°C for 15 minutes and the following sterile supplements were added:

a. Normal Horse serum ... 20 ml
b. 25 % yeast extract ... 10 ml
c. 1 % thallium acetate ... 0.5 ml
d. 30 % L-arginine HCl ... 0.66 ml
e. Penicillin (1,00,000 u/ml) ... 1.0 ml
f. 0.4 % phenol red .. 0.3 ml

f. Medium Bagar:

This was essentially the same as that of medium B Broth (Freundt, 1983) but differed from it by the inclusion of 1.15 % of purified agar and the omission of phenol red indicator.

g. Arginine Broth Medium (Velleca et al., 1980)

This was slightly modified and prepared as follows:
Seventy ml of oxoid base PPLO broth was prepared according to the directions of the manufacturers, the pH was adjusted to 7.4 and autoclaved. The following sterile supplements were added to the basal medium.

a. Normal horse serum ... 20 ml  
b. 25 % yeast extract ... 10 ml  
c. 30 % L-arginine HCl ... 0.66 ml  
d. Penicillin (1,00,000 u/ml) ... 1.0 ml  
e. Polymyxin (5,000 µ/ml) ... 0.1 ml  
f. Amphotericin (5,000 µ/ml) ... 0.1 ml  
g. Erythromycin (10,000 µ/ml)... 1.0 ml  
h. 1 % Thallium acetate ... 0.5 ml  
i. 0.4 % phenol red ... 0.3 ml

h. Arginine Agar Medium:

This was essentially the same as that of Arginine broth medium (Velleca et al., 1980) but differed from it in that it contained PPLO agar without crystal violet (Hi Media Laboratories Pvt. Ltd.,) and the indicator phenol red was omitted.

i. Glucose Agar Medium (Velleca et al., 1980)

Glucose agar medium was used for the isolation of glucose metabolizing mycoplasmas. 70 ml of base PPLO agar without crystal violet (Hi Media Laboratories Pvt. Ltd.,) was prepared according
to the directions of the manufactures. The pH was adjusted to 7.8, autoclaved and the following sterile supplements were added:

a. Normal horse serum ... 20 ml
b. 25 % yeast extract ... 10 ml
c. 50 % glucose solution ... 2 ml
d. 1 % Thallium acetate ... 0.5 ml
e. Penicillin (1,00,000 u/ml) ... 2 ml

j. Glucose Broth Medium:

This was prepared similar to glucose agar medium (Velleca et al., 1980) but instead of Hi Media Laboratories Pvt. Ltd., base PPLO agar medium without crystal violet, base oxoid PPLO broth medium was prepared according to the directions of the manufacturers and the sterile supplements were added. 0.3 ml of 0.4 % phenol red was added as an indicator.

k. Standard Fluid Medium 10 B (Shepard and Lunceford, 1978)

This was slightly modified and prepared as follows:

Seventy ml of base oxoid PPLO broth was prepared according to the directions of the manufacturers. The pH was adjusted to 5.5, autoclaved at 121°C for 15 minutes, and the following sterile supplements were added:
a. Normal horse serum ... 20 ml
b. 25 % yeast extract, pH 6.0 ... 10 ml
c. 2 % L-crysteine hydrochloride solution ... 0.5 ml
d. 10 % urea solution ... 0.4 ml
e. CVA Enrichment solution (co-factors vitamins and amino acid) ... 0.5 ml
f. 0.4 % phenol red solution ... 0.3 ml
g. Penicillin (1,00,000 IU/ml) ... 1.0 ml
h. Polymyxin (5,000 IU/ml) ... 0.5 ml
i. Amphotericin (5,000 IU/ml) ... 0.1 ml

1. Ureaplasma differential agar medium (A7)

(Shepard and Lunceford, 1976)

The basal medium for ureaplasma differential agar medium marketed by M/s. Gibco Diagnostics, Madison, Wisconsin, USA was used and the basal medium on reconstitution per 100 ml contained the following ingredients.

Peptone 220 (Yeast caesin polypeptone) ... 1.7 gms
Peptone 140 (Pancreatic digest of caesin) ... 1.7 gms
Peptone 110 (Papaic digest / of Soy protein)
Dextrose ... 0.25 gms
Manganous sulphate (MnSO₄.H₂O) ... 0.02 gms
Sodium chloride ... 0.5 gms
Potassium phosphate Dibasic ... 0.25 gms
Agar ... 1.0 gms
As per the directions of the manufacturers 3.3 gms of basal medium was added to 82.5 ml of distilled water, thoroughly mixed, the pH was adjusted to 5.5, autoclaved at 121°C for 15 minutes, cooled to 52°C in a water bath and the following supplements were added.

a. Normal horse serum ... 20.0 ml
b. 25 % yeast extract ... 1.0 ml
c. CVA enrichment ... 0.5 ml
d. 10 % urea solution ... 1.0 ml
e. 4 % L cysteine hydrochloride solution ... 0.25 ml
f. Penicillin (1,00,000 u/ml) 1.0 ml

m. A5H Medium (Shepard and Lunceford, 1976)

80 ml of Tryptone soyabroth (Hi Media Laboratories Pvt. Ltd.,) was prepared according to the directions of the manufacturers. 1.05 gms of agar was added to this, the pH was adjusted to 5.5, autoclaved at 121°C for 15 minutes, cooled to 60°C and the following sterile supplements were added.

a. Normal horse serum ... 20.0 ml
b. 25 % yeast extract ... 1.0 ml
c. CVA Enrichment ... 0.5 ml
d. 10 % urea solution ... 0.2 ml
e. 4 % L-cysteine HCl ... 0.25 ml
f. Penicillin G-(1,00,000 u/ml) ... 1.0 ml
n. Sterol free medium for Acholeplasmas

This medium was prepared similar to glucose agar medium (Velleca et al., 1980) except that it did not contain horse serum.

C. Media for Biochemical Tests

a. U-9 urease colour test fluid medium
   (Shepard and Lunceford, 1970)

   Tryptone broth (Hi Media) ... 0.75 gms
   Sodium chloride (NaCl) ... 0.5 gms
   Monobasic potassium phosphate (KH₂PO₄) ... 0.02 gms

   The ingredients were dissolved, the pH was adjusted to 5.5, autoclaved at 121°C for 15 minutes and the following sterile supplements were added:

   a. Normal horse serum ... 4.0 ml
   b. 10 % urea solution ... 0.5 ml
   c. 0.4 % phenol red ... 0.3 ml
   d. Penicillin (1,00,000 u/ml) ... 1.0 ml

b. Glucose fermentation media for Acholeplasmas

   Ninety five ml of oxoid base PPLO broth medium was prepared according to the directions of the manufacturers. The pH was adjusted to 7.8, autoclaved at 121°C for 15 minutes and the following sterile supplements were added:
a. 25 % yeast extract ... 5.0 ml
b. 50 % glucose solution ... 1.2 ml
c. 1 % thallium acetate ... 0.5 ml
d. Penicillin (1,000,000 u/ml) ... 0.5 ml
e. 0.4 % phenol red ... 0.3 ml

D. Supplements

The supplements used in different media were prepared as follows:

a. Normal Horse Serum

Horse serum was obtained by bleeding the horses at King Institute of Preventive Medicine, Madras. Each batch was tested for sterility and ensured not to contain any inhibitors against mycoplasmas. It was dispensed in 20 ml volumes and stored at -20°C.

b. 25 % yeast extract

250 gms of active bakers yeast was added to 1 litre of sterile distilled water, boiled for 10 minutes while mixing it with a glass rod and cooled. This was centrifuged and the sediment was discarded. The pH of the supernatant was adjusted to 6.0 or 7.2 depending on the requirement, autoclaved at 115°C for 15 minutes dispensed in screw capped test tubes and stored at -20°C.
c. 1 % Thallium acetate

One gm of Thallium acetate (Aldrich) was added to 100 ml of sterile distilled water and dissolved. This was sterilized in a seitz filter, dispensed in 1 ml amounts and stored at -20°C.

d. 2 % and 4 % L-cysteine hydrochloride solution

Two gms of L-cysteine hydrochloride (Hi Media Laboratories Pvt. Ltd.,) or 4 gms of L-cysteine HCl was added to 100 ml of distilled water to constitute 2 % or 4 % L-cysteine HCl. It was sterilized by seitz filtration, dispensed in 0.5 ml volumes and stored at -20°C.

e. 30 % L-arginine hydrochloride

This was prepared by the addition of 6 gms of L-arginine HCl (Hi Media Laboratories Pvt. Ltd.,) in 20 ml of sterile distilled water, dissolved, filtered in a seitz filter and stored at -20°C.

f. 10 % urea solution

2.5 gms of urea was dissolved in 25 ml of sterile distilled water, sterilized by seitz filtration, dispensed in 0.5 ml amounts and stored at -20°C.

g. CVA Enrichment

This was procured from M/s. Gibco Diagnostics, Madison, Wisconsin, USA and consists of co-factors, vitamins and amino acids
in dehydrated form. This was reconstituted with the reconstituting fluid supplied by the manufacturers.

h. 50 % glucose solution

10 gms of glucose (Hi Media Laboratories Pvt. Ltd.,) was added to 20 ml of sterile distilled water, filtered in a seitz filter, dispensed in 2 ml volumes and stored at -20°C.

i. 0.4 % phenol red solution

0.4 gms of phenol red (Hi Media Laboratories Pvt. Ltd.) was added to 10 ml of 0.1 N NaOH and to this 20 ml of distilled water was added and dissolved. 10 ml of 0.1 N HCl was added to neutralize the NaOH and the solution was made upto 100 ml in a volumetric flask and autoclaved at 121°C for 15 minutes.

j. Benzyl penicillin, amphotericin, polymyxin, vancomycin, mycostatin and Erythromycin were obtained from M/s. Hi Media Laboratories Pvt. Ltd., and the desired concentration of the antibiotics was prepared in sterile distilled water as per the direction of the manufacturers.

3. Specimens

The specimens collected in males with NGU, prostatitis, in patients with sexually transmitted diseases and in male controls include four cotton tipped urethral swabs and urine. In patients with prostatitis, prostatic fluid on massage was collected after the collection of the urethral swab. The swabs were transported
to the Laboratory in Cary and Blair's Transport medium for the isolation of bacteria and fungi, in PPLO transport medium for the isolation of mycoplasmas of ureaplasmas and in Stuart's transport medium for the isolation of gonococcus. A wet preparation of the urethral smears was made to detect T. vaginalis and candida. A grams stain of the urethral exudate was carried out for gonococcus and candida. 5 ml of blood was collected, serum separated and stored in a deep freezer at -20°C for serology.

The specimens collected in PID and cervicitis include two cervical cotton swabs, one charcoal impregnated cervical swab and urine. In cases of infertility two vaginal cotton swabs, one charcoal impregnated cervical swab and urine was collected. The swabs were transported to the Laboratory in Cary and Blair's transport medium, PPLO transport medium and the charcoal impregnated cotton swab in Stuart's transport medium. A wet preparation was carried out for the detection of T. vaginalis and candida. Also, Tubal aspirates were collected by hysteroscopy in cases of infertility and the specimen was transported to the Laboratory in PPLO transport medium and in Stuart's broth base. 5 ml of blood was collected, serum separated and stored at -20°C for serological studies.

In cases of vaginitis, four vaginal swabs of which one was a charcoal impregnated cotton swab and urine were collected. In cases of abscess of Bartholin's gland two cotton swabs from
the abscess and one charcoal impregnated swab from the cervical canal and urine were collected. In female patients with sexually transmitted diseases, urine and three vaginal swabs were collected. In suspected cases of gonorrhoea a charcoal impregnated cotton swab was also collected at the site of the lesion or from the cervical canal in the absence of an obvious lesion. In female controls urine, three vaginal swabs and one charcoal impregnated swab from the cervical canal were collected. 5 ml of blood was collected, serum separated and stored at −20°C for serological studies.

The cotton tipped swabs were transported to the laboratory in a Carry and Blair transport medium and in PPLO transport medium. The charcoal impregnated swab was transported to the Laboratory in a Stuart transport medium. Also, a wet preparation was carried out for the detection of T. vaginalis and Candida.

Twenty ml of blood was collected in patients with post partum fever, post abortal fever, control population for post partum fever and post abortal fever and 15 ml of it was inoculated into 6 different media at bed side. 5 ml of clotted blood was transported to the laboratory, serum separated and stored at −20°C for serological examination. Three vaginal swabs and urine were collected and the swabs were transported to the laboratory in Cary and Blair transport medium and in PPLO transport medium. A wet preparation was carried out for the detection of T. vaginalis and candida.
4. Processing of specimens

A. Microscopy

In males a grams stained preparation of the uncentrifuged urine, prostatic massage and a wet preparation of centrifuged deposit of urine and prostatic massage was examined. In females a grams stain of uncentrifuged urine was examined. In patients with infertility a smear of tubal aspirate was examined by grams stain.

B. Culture

The swabs collected in males and females and transported in Cary and Blair transport medium was incubated onto blood agar and Sabouraud Dextrose agar. uncentrifuged urine from males and females was inoculated onto blood agar and was cross streaked on MacConkey agar with a calibrated loop for colony count. The prostatic massage fluid from males was inoculated onto blood agar, MacConkey agar, Sabouraud Dextrose agar and Chacko-Nair Medium. The swabs collected in males and females and transported in Stuart transport media was inoculated onto Chacko-Nair Medium for the isolation of gonococcus. Urine was used for the isolation of gonococcus in males where there was no discharge of pus. The tubal aspirates collected and transported in Stuart's broth base was used for the inoculation of blood agar, MacConkey agar and Chacko-Nair medium. The swabs transported in PPLO transport medium in males and females, the centrifuged deposit of urine in males and females,
FIGURE 2: COLLECTION AND PROCESSING OF SPECIMENS IN NGU, MALE CONTROLS AND IN MALE SEXUALLY TRANSMITTED DISEASE CASES

Urethral swabs
  - Grams stain and wet preparation
  - Carry and Blair Transport medium
  - PPLO Transport medium
  - Stuart's Transport Medium
    - Medium B broth
      - Arginine broth
      - Glucose broth
      - Standard fluid medium 10-B
    - Chacko-Nair Medium
      - Grams stain blood agar
      - MacConkey agar

Urine
  - Uncentrifuged
  - Centrifuged deposit
    - Wet preparation
      - Medium B broth
      - Arginine B broth
      - Arginine broth
      - Glucose broth
      - Standard fluid medium 10-B
      - Medium B agar
      - Arginine agar
      - Glucose agar
      - Ureaplasma differential agar (A7)

Blood
  - Serum
FIGURE 3: COLLECTION AND PROCESSING OF SPECIMENS IN PATIENTS WITH PROSTATITIS

Urethral swab
- Grams stains and wet preparation
  - Cary and Blair Transport medium
    - Blood agar
    - Sabouraud Dextrose agar
  - PPLO Transport medium
    - Medium B broth
    - Arginine broth
    - Glucose broth
    - Standard fluid medium 10-B
    - Medium B agar
    - Arginine agar
    - Glucose agar
    - Ureaplasma Differential Agar (A7)
  - Stuart's Transport medium
    - Chacko-Nair medium

Prostatic message
- Grams stain and wet preparation
  - Blood agar
  - MacConkey agar
  - Sabouraud Dextrose agar
  - Chacko-Nair medium
    - Medium B broth
    - Arginine broth
    - Glucose broth
    - Standard fluid medium 10-B
    - Medium B agar
    - B agar
    - Arginine agar
    - Glucose agar
    - Ureaplasma differential Agar (A7)

Urine
- Uncentrifuged
- Centrifuged deposit
  - Grams stain
  - Blood agar
  - MacConkey agar

Blood
- Serum
  - Medium B broth
  - Arginine broth
  - Glucose broth
  - Standard fluid medium 10-B
  - Medium B agar
  - Arginine agar
  - Glucose agar
  - Ureaplasma differential agar (A7)
FIGURE 4: COLLECTION AND PROCESSING OF SPECIMENS IN PATIENTS WITH PID AND CERVICITIS

- Cervical swabs
  - Wet preparation
  - Cary and Blair Transport medium
    - Blood agar
    - Sabouraud Dextrose agar
  - PPLO Transport medium
    - Medium B broth
    - Arginine broth
    - Glucose broth
    - Standard fluid
    - Medium 10-B
    - Medium B agar
    - Arginine agar
    - Glucose agar
    - Ureaplasma differential Agar (A7)
  - Stuart Transport medium
    - Chacko-Nair medium
    - Grams stain
    - Blood agar
    - MacConkey agar
  - Urine
    - Uncentrifuged
    - Centrifuged deposit
      - Medium B broth
        - Arginine broth
        - Glucose broth
        - Standard fluid
        - Medium 10-B
        - Medium B agar
        - Arginine agar
        - Glucose agar
        - Ureaplasma differential Agar (A7)
  - Blood
    - Serum
FIGURE 5: COLLECTION AND PROCESSING OF SPECIMENS IN PATIENTS WITH INFERTILITY

Tubal aspirates
- Stuart's broth base
- PPLO Transport medium

Blood agar
- MacConkey agar
- Chacko-Nair medium

Medium B broth
- Arginine broth
- Glucose broth
- Standard fluid medium
- Medium 10-B
- B agar
- Arginine agar
- Glucose agar
- Ureaplasma differential agar (A7)

Vaginal swabs
- Wet preparation
- Cary and Blair Transport medium

Blood agar
- Sabouraud Dextrose agar

Wet preparation
- PPLO Transport medium

Cervical swab
- Stuart's Transport medium

Medium B broth
- Arginine broth
- Glucose broth
- Standard fluid medium 10-B
- Medium B agar
- Arginine agar
- Glucose agar
- Ureaplasma differential agar (A7)

Cervical swab
- Chacko Nair medium

Grams stain
- Blood agar
- MacConkey agar

Urine
- Uncentrifuged
- Centrifuged deposit

Medium B broth
- Arginine broth
- Glucose broth
- Standard fluid medium 10-B
- Medium B agar
- Arginine agar
- Glucose agar
- Ureaplasma differential agar (A7)

Blood
- Serum
FIGURE 6: COLLECTION AND PROCESSING OF SPECIMENS IN FEMALE CONTROLS, PATIENTS WITH VAGINITIS AND FEMALE PATIENTS WITH SEXUALLY TRANSMITTED DISEASE

Vaginal swab
  - Wet preparation
  - PPLO Transport medium
    - Cary and Blair Transport medium
      - Blood agar
        - Sabouraud Dextrose agar
      - Medium B broth
        - Arginine broth
        - Glucose broth
        - Standard fluid medium 10-B
        - Medium B agar
        - Arginine agar
        - Glucose agar
        - Ureaplasma differential agar (A7)

Cervical swab
  - Stuarts Transport medium
    - Chacko-Nair medium

Urine
  - Uncentrifuged
    - Grams stain
      - Blood agar
      - MacConkey agar
    - Centrifuged deposit
      - Medium B broth
      - Arginine broth
      - Glucose broth
      - Standard fluid medium 10-B
      - Medium B agar
      - Arginine agar
      - Glucose agar
      - ureaplasma differential agar (A7)

Blood
  - Serum
FIGURE 7: COLLECTION AND PROCESSING OF SPECIMENS FROM PATIENTS WITH ABSCESS OF BARTHOLIN'S GLAND

Abscess of Bartholin's gland:
- Cary and Blair Transport medium
  - Grams stain
  - Blood agar
  - MacConkey agar
- PPLO Transport medium
  - Medium B broth
  - Arginine broth
  - Glucose broth
  - Standard fluid
  - Medium 10-B
  - Medium B agar
  - Arginine agar
  - Glucose agar
  - Ureaplasma differential agar (A7)

Cervical swab:
- Chacko-Nair medium

Urine:
- Uncentrifuged
  - Grams stain
  - Blood agar
  - MacConkey agar
- Centrifuged
  - Medium B broth
  - Arginine broth
  - Glucose broth
  - Standard fluid
  - Medium 10-B
  - Medium B agar
  - Arginine agar
  - Glucose agar
  - Ureaplasma differential agar (A7)

Blood:
- Serum
FIGURE 8: COLLECTION AND PROCESSING OF SPECIMENS IN PATIENTS WITH POST PARTUM FEVER, POST ABORTAL FEVER, CONTROLS FOR POST PARTUM FEVER AND POST ABORTAL FEVER

Blood

Brain Heart Infusion Broth

Serum

Wet preparation

Gavy and Blair Transport medium

Vaginal swab

PPLO Transport medium

Medium B broth

Arginine broth
Glucose broth
Standard fluid medium 10-B

Blood agar
Sabouraud Dextrose agar

Urine

Uncentrifuged

Grams stain

Blood agar
MacConkey agar

Centrifuged deposit

Medium B broth

Arginine broth
Glucose broth
Standard fluid medium 10-B

Medium B agar
Arginine agar
Glucose agar
Ureaplasma differential Agar (A7)
the prostatic massage fluid in males were inoculated in medium B broth, arginine broth, glucose broth medium, medium B agar, arginine agar and glucose agar for mycoplasmas, standard fluid medium 10-B and ureaplasma differential agar medium (A7) for the ureaplasmas.

Blood from post partum fever, post abortal fever, controls for post partum fever, controls for post abortal fever were inoculated in 2.5 ml volumes per 25 ml of medium at bed side into Brain heart infusion broth and Bile broth for the isolation of bacteria, Medium B broth, arginine broth and glucose broth for the isolation of mycoplasmas and standard fluid medium 10-B for the isolation of ureaplasmas.

5. Examination of Specimens

Blood agar, MacConkey agar and Sabouraud Dextrose agar were incubated at 37°C for 24 hours and 48 hours. A grams stain of the different colonies were made and the bacterial isolates were identified by colony morphology and biochemical tests (Bailey and Scott, 1978). The biochemical tests included sugar fermentation, catalase and oxidase production, motility test, Indole reaction, nitrate reduction, urease test and hydrogen sulphide production, all of which were done using standard techniques as described by Cruickshank et al. (1975).
Suspected colonies of candida from Sabouraud dextrose agar were gram stained and examined for gram positive budding yeast like organisms. The species was identified based on the formation of germ tube, sugar fermentation of dextrose, maltose, sucrose, lactose and sugar assimilation of dextrose, maltose, sucrose, lactose, galactose, raffinose and cellobiase.

Chacko-Nair medium was incubated in a candle jar at 37°C and a grams stain examination of suspected colonies of gonococcus was made and if found to be gram negative diplococci, it was inoculated into sugar media for biochemical reactions (Cruickshank et al., 1975). It was confirmed by performing an oxidase test on the plate (Cruickshank et al., 1975).

Brain Heart infusion broth was incubated at 37°C in a candle jar and bile broth at 37°C. Brain heart infusion broth and bile broth were subcultured onto blood agar and MacConkey agar respectively after 24 hours and the bottles were reincubated. The blood agar and MacConkey agar plates were incubated at 37°C for 24 hours. If there was no growth in plates the broth were subcultured after 48 hours. Bile broth was discarded after 48 hours even if there was no growth on solid media where as brain heart infusion broth was reincubated and subcultured after days 7 and 14 before discarding it as negative. The bacterial isolates were identified by colony morphology and biochemical reactions (Cruickshank et al., 1975).
Medium B broth, Arginine broth medium, glucose broth medium, Medium B agar, arginine agar medium and glucose agar were incubated at 37°C in a candle jar. Standard fluid medium 10-B and ureaplasm differential agar medium (A7) were incubated at 37°C anaerobically in an air tight glass stainless steel jars with room temperature catalyst (Dexopellets) and internal gas generating system. The internal gas generating system (gas pack system) consisted of a polythene bag with sodium borohydride, sodium bicarbonate and citric acid in 700 mg quantities. 10 ml of distilled water was added to the gas pack prior to use to evolve the hydrogen-carbon dioxide (H₂-CO₂) mixture.

In medium B broth and arginine broth the growth was indicated by the change in colour from salmon to red by the arginine metabolizers. In standard fluid medium 10-B the growth of ureaplasmwas indicated by the change in colour from dark yellow to red. In glucose broth medium the growth of glucose metabolizers was indicated by the change of the medium from red to yellow. When there was a growth in the liquid medium it was subcultured onto the corresponding solid medium. If there was no growth in liquid medium it was subcultured on days 3, 7 and 14 before discarding it as negative.

Primary culture of medium B agar, arginine agar medium, glucose agar medium and ureaplasm differential agar medium (A7) were examined for the growth of mycoplasmas and ureaplasmases for a period of 14 days before discarding it as negative.
Mycoplasma colonies exhibited a characteristic fried egg appearance due to a dense centre where the colony has grown into the agar and a less dense periphery where the colony spread along the surface of the agar. When stained with Dienes stain the colonies stained with a dark blue centre and a light blue periphery. *M. hominis* was identified by growth inhibition test with specific antisera (Clyde, 1964).

*U. urealyticum* colonies were smaller when compared to mycoplasma colonies and were round and coarsely granular. *U. urealyticum* was confirmed by their characteristic golden brown colonies on ureaplasma differential agar medium (A7) (Shepard and Lunceford, 1976) and a positive test in the U-9 urease colour test fluid medium (Shepard, 1973). In U-9 urease colour test fluid medium a positive test was indicated by the breakdown of urea by urease a feature, which is unique among the class Mollicutes. Consequently, there was a shift in the pH of the medium as indicated by a change in colour from yellow to red.

Acholeplasma species isolated were confirmed by their characteristic fried egg appearance, by their ability to grow in sterol free media, glucose fermentation and resistance to 1.5 per cent Digitonin and 20 per cent sodium-polyanethol-sulfonate (Freundt *et al.*, 1973).
6. Tests for Identification

A. Reagents

a. Kovac's reagent

Para dimethyl amino benzaldehyde \( \ldots \) 5.0 gms
Amyl alcohol \( \ldots \) 75.0 ml
Hydrochloric acid (Conc.) \( \ldots \) 25.0 ml

b. Nitrate reagent

Solution A - 8 gms of sulphanilic acid in 1,000 ml 1 N acetic acid.

Solution B - 12 gms of dimethyl alpha naphthyl amine in 1,000 ml 5 N acetic acid.

c. Dienes stain (Stock solution)

Methylene blue \( \ldots \) 2.50 gms
Azure II \( \ldots \) 1.25 gms
Maltose \( \ldots \) 10.00 gms
\( \text{Na CO}_2 \text{ } 3 \) \( \ldots \) 0.25 gms
Distilled water \( \ldots \) 100 ml
B. Tests for bacteria and fungi

a. Fermentation of carbohydrates

Fermentation of carbohydrates by gram negative aerobic bacillus was demonstrated by inoculating liquid media containing 1% of various carbohydrates - glucose, sucrose, lactose, maltose, mannitol, raffinose, rhamnose, salicin, xylose and lactose. One drop of the pH indicator bromothymol blue was added to each tube after 18 hours incubation period.

Cysteine trypctase agar base with glucose, lactose, maltose and sucrose was used for the demonstration of carbohydrate fermentation by gonococcus. The medium was inoculated by making multiple stabs.

b. Catalase test

One drop of 3% hydrogen peroxide ($H_2O_2$) was added to a drop of broth culture on a glass slide and observed for the formation of bubbles.

c. Oxidase test

A 1% solution of tetra methyl para phenyl diamine dihydrochloride was freshly prepared and the reagent was flooded on the colonies in a solid medium. The development of purple colour of the colonies was observed.
d. Motility

Motility was tested by a hanging drop preparation of the broth culture in a carbohydrate free medium.

e. Indole production

Growth in peptone water medium was tested with a few drops of Kovac's reagent for the appearance of a deep pink colour.

f. Nitrate reduction test

Culture was inoculated into peptone broth with 0.2% potassium nitrate. After incubation nitrate reagent was added.

g. Urease test

The culture was inoculated into urea broth. Production of urease was detected by addition of phenol red which turned bright red due to the production of ammonia.

h. Hydrogen sulphide production

A semi solid medium with 10% ferrous chloride was inoculated with a straight wire. Production of hydrogen sulphide resulted in blackening of the medium.

i. Germ tube test

Candida was inoculated into 0.5 ml of human serum in a small test tube and incubated at 37°C for 3 hours. A loopful was placed on a slide and examined for germinating blastospores.
j. Sugar assimilation tests

The candida species in question was inoculated by Pour plate technique in yeast nitrogen base agar medium. Glucose, galactose, maltose, sucrose, lactose, raffinose, cellobiase sugars impregnated on filter paper discs were placed on the medium and the assimilation pattern of the sugars by candida was observed.

C. Tests for mycoplasmas

a. Dienes stain technique

The test plate with mycoplasma colonies was flooded with 0.5 to 1.0 ml of a 1 : 3 dilution of stock dienes stain.

It was immediately rinsed with distilled water into a discarding pan to remove the stain.

The agar was decolourized by adding 1 ml of ethyl alcohol (95 %) for 1 minute. The alcohol was decanted into a discarding pan and the decolourization with ethyl alcohol was repeated once more.

The plate was rinsed with distilled water and allowed to dry.

Mycoplasma colonies with fried egg morphology stained distinctly with dark blue centres and light blue peripheries.
b. Growth inhibition test for M. hominis

One drop of about 10⁵ ccu 50/ml M. hominis broth culture was inoculated onto one half of the 2 inch petri plates with medium B agar. The other half of the medium was inoculated with a different strain so that 2 strains were tested on each plate. Care was taken to ensure that the 2 test strains did not mix up. The inoculum was allowed to run down evenly on the surface of the medium and left at room temperature for 30 minutes enabling the medium to absorb the inoculum. Sterile filter paper discs about 6 mm were saturated with 0.025 ml of an undiluted hyper immune M. hominis antisera and the discs were placed on the inoculated medium. The antisera was supplied by courtesy - FAO/WHO collaborating centre for animal mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark. The plates were incubated at 37°C in a candle jar and were read after 3 days. A zone of inhibition of growth of 1 mm or more was considered that the inoculated organism was specifically inhibited by the specific antiserum.

II. Quantitation

Serial ten fold dilutions of urine specimens from 86 cases with NGU and 67 controls were quantitated in standard fluid medium 10B to determine the titer of U. urealyticum. When there was growth, the last tube that showed a shift in alkaline pH from acidic
pH represented a titer of one 50 % colour changing unit (1 ccu 50/ml) of urine. The standard fluid medium 10 B when positive was sub-cultured onto A7 medium. U. urealyticum was confirmed by their characteristic golden brown colonies on A7 medium (Shepard and Lunceford, 1976) and a positive test in the U-9 urease colour test fluid medium (Shepard and Lunceford, 1970).

III. Characterization of U. urealyticum and M. hominis

1. Sensitivity to 1.5 % Digitonin

The susceptibility of the genital mycoplasmas to 1.5 % digitonin was carried out according to the method of Freundt et al. (1973). A 1.5 % (w/v) of Digitonin (Loba) solution was prepared in ethanol. To dissolve the Digitonin the ethanolic preparation was heated in a water bath at 56°C for 30 minutes. Sterile filter paper discs of about 6.0 mm diameter were impregnated with 0.02 ml of 1.5 % digitonin, dried in 37°C for 48 hours and stored at 4°C until use.

Seventy three strains of U. urealyticum, 65 strains of M. hominis and 8 strains of Acholeplasma species were tested for their sensitivity against 1.5 % Digitonin. A5H agar medium for U. urealyticum, medium B agar for M. hominis and glucose agar medium for Acholeplasmas was used. 0.05 ml of $10^3$ ccu 50/ml of U. urealyticum, M. hominis and Acholeplasma broth culture was allowed to run down evenly on the surface of the medium. The inoculum
was allowed to be absorbed and the filter paper discs were applied gently on the plate. A5H medium was incubated in an anaerobic jar, medium B agar and glucose agar were incubated in a candle jar. The results were read after 2 to 3 days. A zone of inhibition of growth around the disc was considered as a strain to be sensitive.

2. Sensitivity to 5% sodium-polyanethol-sulfonate

The susceptibility of the genital mycoplasmas to sodium-polyanethol-sulfonate was carried out according to the method of Freundt et al. (1973). Sodium-polyanethol-sulfonate was obtained from Loba and a 5% (w/v) aqueous solution was used. Sterile filter paper discs of about 6 mm diameter were impregnated with 0.02 ml of 5% sodium-polyanethol-sulfonate, dried at 37°C for 48 hours and stored at 4°C until use.

Sixty nine strains of U. urealyticum, 61 strains of M. hominis and 8 strains of Acholeplasmas were tested for their sensitivity against 5% sodium-polyanethol-sulfonate. 0.05 ml of $10^3$ cccu 50/ml of U. urealyticum, M. hominis and Acholeplasmas broth culture were inoculated on A5H agar, medium B agar and glucose agar respectively and allowed to be absorbed. The filter paper discs impregnated with 5% sodium-polyanethol-sulfonate was applied gently on the plate and the A5H agar medium was incubated anaerobically, B agar and glucose agar in a candle jar. The results were
read after 2 to 3 days. A zone of inhibition of growth around the disc was considered as a strain to be sensitive.

IV. Effect of physical and chemical agents

1. Effect of Thallium acetate

The effect of Thallium acetate on the growth of 52 strains of U. urealyticum and 55 strains of M. hominis was tested by an agar dilution technique. ASH agar medium and medium B agar were used for U. urealyticum and M. hominis respectively. Thallium acetate was prepared in distilled water at a concentration ten times that desired in the final test. The media were prepared and one part of the ten fold concentration of Thallium acetate was added to nine parts of the medium to obtain the desired concentrations of thallium acetate. The media was poured in petri plates and allowed to set. The final concentration of Thallium acetate used in the media was 0.0005 %, 0.001 %, 0.002 %, 0.005 %, 0.01%, 0.02 %, 0.04 %, 0.1 %, 0.2 % and 1.0 %. The plates were stored at 4°C until use.

A concentration of $10^4$ ccu 50/ml of U. urealyticum and M. hominis were used. The organisms were inoculated as a spot on the medium, allowed to dry before incubation. A control plate without thallium acetate was also inoculated. The results were read after 2 to 3 days of incubation. A strain was considered resistant when there was an uniform growth with typical colonial morphology.
2. Effect of ether

Forty three strains of U. urealyticum and 45 strains of M. hominis were treated with 20 % and 40 % (v/v) diethyl ether. The pretreatment titer of the organisms were recorded by a serial ten fold dilutions. Diethyl ether was added at a final concentration of 20 % and 40 % (v/v) to the broth culture and it was kept at +4°C. The broth culture was titrated after 12 hours and 24 hours of post ether treatment.

3. Effect of temperature

Forty three strains of U. urealyticum and 47 strains of M. hominis were exposed at 56°C for 5 minutes and 10 minutes. The organisms were titrated in broth prior to and after exposure at 56°C for 5 minutes and 10 minutes.

V. Serology

Antibody response in patients with different genital infections of U. urealyticum and M. hominis was estimated by metabolic inhibition test and IHA test. 5 ml of blood from patients with different genital infections and controls was collected and paired samples in those cases possible was also collected. The blood was allowed to clot, serum separated and stored at -20°C.
1. Metabolic Inhibition test

A. Patients

Metabolic inhibition test was carried out according to the modified method of Lina Deodhar et al. (1986) in 304 acute sera, 131 convalescent sera from male and female patients with different genital infections and from 164 male and female controls against U. urealyticum - 960 and N-56 strains. 120 acute and 44 convalescent of the above sera were also tested against U. urealyticum strain isolated from the corresponding patient.

Metabolic inhibition test was also carried out in 278 acute sera, 121 convalescent sera from male and female patients and from 155 male and female controls against M. hominis PG 21 and P-18 strains. 120 acute sera and 34 convalescent of the above sera were also tested against M. hominis strain isolated from the corresponding patient.

B. Antigens

The antigens used in metabolic inhibition test were U. urealyticum type strain - 960, M. hominis type strain PG-21 (Courtesy - FAO/WHO collaborating centre for Animal Mycoplasmas, Denmark), U. urealyticum N-56 a strain isolated from a case of NGU from whom no other organism was recovered and M. hominis P-18 a strain isolated from a case of PID. Also, U. urealyticum strains and M. hominis strains isolated from the same patient were used
to test against the sera of the same patient. The organisms were
cloned thrice so as to obtain a pure culture before use in the
test. The organisms were grown on solid media and a agar block
with well isolated single colony was transferred into broth culture
and incubated. After 24 hours the broth culture was titrated by
serial ten fold dilutions and the organism from the highest dilution
was plated onto solid media. The procedure was repeated thrice
so as to ensure a pure growth of the organism. After the organisms
were cloned thrice it was titrated in ten fold dilutions and a concentra-
tion of $10^3$ ccu 50/ml was used in the metabolic inhibition test.

C. Test

The patients serum was inactivated at 56°C for 30 minutes
and the test was carried out in test tubes. 0.2 ml of inactivated
serum was serially diluted in two fold dilutions starting with a
dilution of 1 : 2 and upto 1 : 512 in standard fluid medium 10B
broth for U. urealyticum and medium B broth for M. hominis.
A standard dose of 0.4 ml at a concentration of $10^3$ ccu 50/ml
of the organism was used as antigen. To this 0.8 ml of broth
was added. A serum control, broth control and mycoplasma control
were also put up and the tubes were incubated at 37°C.

The test was read when there was a change of 0.5 pH
units in mycoplasma controls. This was indicated by a change
of colour from yellow to salmon for U. urealyticum in standard
fluid medium 10 B broth and from salmon to purple in medium B broth for M. hominis. The change in pH was determined by comparing the colour change with the medium containing phenol red adjusted to known pH values. The highest dilution of serum that inhibited a pH change by 50 % or more was considered as the metabolic inhibition titer. If the controls have changed 0.5 pH units, a change of less than 0.25 pH unit was considered that this dilution contained sufficient antibody to inhibit mycoplasma growth.

2. Indirect haemagglutination test

A. Patients

IHA (Velleca et al., 1980) was carried out in 297 acute sera, 126 convalescent sera of patients with different genital infections and 159 male and female controls against U. urealyticum antigens. IHA was also carried out in 290 acute sera, 122 convalescent sera of patients with different genital infections and 159 male and female controls against M. hominis antigens.

B. Antigens

U. urealyticum type strain - 960 and N-82 an U. urealyticum strain isolated from a patient with NGU from whom no other organism was isolated were used as ureaplasma antigens. M. hominis type strain PG-21 and P28 a M. hominis strain isolated from a case of PID were used as M. hominis antigens. The strains were cloned thrice before preparing the antigens.
U. urealyticum was grown on A5H medium and M. hominis was grown on medium B agar. The agar was cut aseptically into small sections and U. urealyticum was inoculated in standard fluid medium 10 B broth and M. hominis in medium B broth in 250 ml volumes. When there was growth, 50 ml of broth culture was used to inoculate into five 500 ml of fresh broth culture and incubated. After the growth of the organism in broth culture, it was centrifuged for two hours at 24,000 g. The pellet was resuspended in PBS pH 7.2 equal to one half of the original volume of broth. This was centrifuged again at 24,000 g for two hours. The pellet was resuspended in a volume of PBS containing 12.5 per cent sucrose equal to 1/20 the original volume of broth. This was sonicated at 10 KC for 30 minutes, preserved with the addition of merthiolate at a final concentration of 1 : 10,000.

c. Test

Formalinized tanned sheep red blood cells were used in the test. The cells were tanned with different dilutions of tannic acid and it was found tanning the cells with 1 : 20,000 dilution gave the best results. Sheep RBC were sensitized with the optimal dilution of the antigen. It was found by IHA block titration the optimal dilution of the antigen to be used was 1 : 4 for U. urealyticum, 1 : 6 for M. hominis for sensitization of sheep RBC. Formalinized, tanned, sensitized sheep RBC at a concentration of 1.0 per cent were used in the test.
The test was carried out in Linbrow microtiter plates. 0.05 ml of normal horse serum diluent was added to serum dilution wells number 2 to 9 and to the cell control wells. 0.05 ml of 1:10 dilution of patients serum was added each to the first well and the second well. The serum was serially diluted from second well to 9th well so as to get a dilution of 1:10 to 1:5,120. 0.025 ml of sensitized sheep RBC were added to all the wells and to the cell control well. A positive control serum, a negative control serum and a PBS buffer control was also put up in each plate as described for the test serum. The plates were covered and incubated at room temperature for 4 hours.

The test was read when there was no agglutination in the cell control well, negative sera row and PBS control row as indicated by the button formation. A positive test was indicated by a mat formation of RBC due to haemagglutination. The highest dilution of serum that gave a clear positive haemagglutination pattern with the sensitized sheep RBC was considered the end point dilution of the serum.

VI. Serotyping

Forty strains of U. urealyticum isolated from cases of NGU and 13 strains of U. urealyticum isolated from male controls
were serotyped by agar growth inhibition method (Shepard and Lunceford, 1978). The organisms were cloned thrice to ensure the purity of the organisms. The agar growth inhibition test was carried out in A5H agar medium. Prior to performing the test, the moisture in the plates was dried by placing the plates in an incubator. Two drops of U. urealyticum at a concentration of $10^3$ ccu 50/ml was inoculated and the inoculum was uniformly spread with the tip of the pipette. It was air dried for 30 minutes so as to facilitate the absorption of the inoculum. After the antigen inoculum was dried, 8 wells each of about 2 mm diameter were cut and U. urealyticum serotypes 1 to 8 antisera were placed in the wells with a pasteur pipette. The antisera was supplied courtesy, FAO/WHO collaborating centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark. The filled wells were allowed to stand at room temperature for 30 minutes. The plates were incubated in an anaerobic jar with an internal gas generating system of hydrogen and carbon dioxide and the results were read after 24 hours. The serotype was recorded if a zone of inhibition by a specific antiserum was detected. If there was no zone of inhibition the plates were reincubated and examined twice during the day and the following day.

VII. Antibiotic susceptibility testing

Sixty eight strains of U. urealyticum and 59 strains of M. hominis were tested for their antibiotic susceptibility pattern
by agar dilution technique. Chloramphenicol, tetracycline, Erythromycin, kanamycin, polymyxin, B-sulphate, Lincomycin, gentamycin, vancomycin, ampicillin and streptomycin were incorporated at a final concentration of 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32, 64, 128, 256, 512 micrograms/ml in A5H medium and B agar. Cultures of \textit{U. urealyticum} and \textit{M. hominis} were initiated in broth medium. One drop of $10^3$ ccu 50/ml of \textit{U. urealyticum} and \textit{M. hominis} were spot inoculated on A5H medium and B agar respectively. A5H medium was incubated anaerobically and B agar aerobically and the results were read after 1 to 2 days.

\textbf{VIII. Experimental infections in animals}

1. \textit{Experimental infection} of \textit{U. urealyticum} in monkeys

The ability of \textit{U. urealyticum} to cause uretheritis in an experimental model was observed by inoculating intrauretherally in Macaca radiata monkeys. 9 male Macaca radiata monkeys ranging in weight from 2.2 kilograms to 4.3 kilograms were used in the study. The animals were sedated with 28 mgs/kg body weight of sodium pentobarbitone and 3 urethral swabs were collected from each animal. One swab was used for gram staining and two swabs were inoculated into blood agar, MacConkey agar, medium B broth, arginine broth, standard fluid medium 10-B broth, glucose broth, medium B agar, arginine agar, glucose agar, and ureaplasma differential agar medium (A7).
Three strains of U. urealyticum isolated from the patients with NGU were cloned thrice and each strain was inoculated in a group of 3 monkeys. Each sedated animal was intrauretherally inoculated with a catheter with 0.3 ml of U. urealyticum at a concentration of $10^6$ ccu 50/ml.

The inoculated monkeys were examined and urethral swabs and urethral scrapings with a bacteriological loop were taken from each animal on days 3, 7, 14, 21, 28, 35 and 42. The swab was used for grams staining and the scrapings from the loop was inoculated into blood agar, MacConkey agar, medium B broth, arginine broth, standard fluid medium 10-B, glucose broth, medium B agar, arginine agar, ureaplasma differential agar (A7) and glucose agar.

2. Experimental infection of M. hominis in rats

The ability of M. hominis to cause infection in an experimental animal was observed by inoculation of M. hominis into the uterine horns of rats. 3 groups of healthy wister strain rats maintained at King Institute, Guindy, Madras were used in the study. The rats were anaesthetized with chloroform, the ventral surface was shaved and an incision was made on the ventral surface of the abdominal wall. 3 strains of M. hominis which were isolated from PID were cloned thrice and 0.2 ml of $10^7$ ccu 50/ml was inoculated directly into the uterine horns. Each strain of M. hominis was inoculated into a group of 5 rats. A fourth group of 5 rats
formed the control and received only the uninoculated broth. The abdomen was sutured up and the animals withstood the experiment uneventfully.

Specimens were collected from the genital tract on days 3, 5 and 7 with a bacteriological straight wire and inoculated into media for the isolation of M. hominis. The animals were sacrificed on the 7th day and the uterine horns and uterus from the study group and the control group were collected for histopathology. Thin sections of the organs were made and were stained with haematoxylin and eosin for histopathological examination.

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