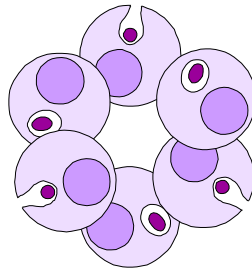


Chlamydia Screening Study



CLASS

Chlamydia Screening Study

Laboratory Protocol

Contents

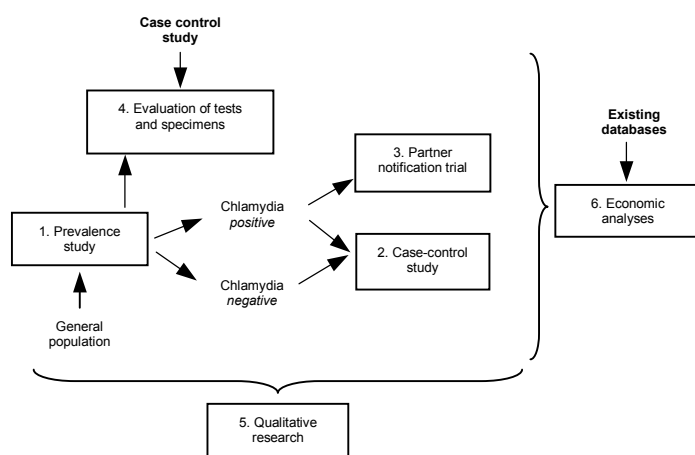
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1. PROTOCOL FOR LABORATORY STUDIES

1.1 Overview Of Class And Background To Lab Studies

The Chlamydia Screening Study (CLaSS) consists of a number of inter-linked studies (Figure 1), most with a laboratory component. This document gives a brief overview and describes the protocols for the laboratory work to be performed within CLaSS, including the objectives, methodologies and the plan of investigation for the evaluation of diagnostic tests.

Figure 1 – Links between component studies



A cross-sectional prevalence study (1) to identify cases with genital Chlamydial infection in a general community sample of men and women aged 16-39 years in Avon and the West Midlands is at the centre of the proposal. Clinical specimens collected from participants in the prevalence study and from positive subjects who return for the Case control study will be used in the evaluation of diagnostic tests (4).

Individuals with chlamydial infection will enter a *randomised controlled trial* (3) comparing two different partner notification strategies. A *case-control study* (2) will compare Chlamydia cases and non-infected controls to identify demographic and behavioural risk factors for genital Chlamydia infections. *Qualitative research methods* (5) will be used to examine expectations, experiences and preferences among asymptomatic women, and women and men involved in screening and partner notification procedures. The results from these studies will inform *cost-effectiveness analyses* (6) of different screening strategies. Existing databases (mainly from Sweden) will also be interrogated to inform economic analyses.

The *Evaluation of diagnostic tests* will be carried out in the Public Health Laboratory Services (PHLS) of Bristol and Birmingham. This document expands on the original protocol submitted to the HTA and takes account of results of the comparison of Nucleic Acid Amplification Assays (NAAs) tests with an Enzyme Linked Immunoassay (EIA), which was ongoing in Bristol's PHLS at the time of the original proposal.

2. STUDY OBJECTIVES

- To identify people with and without genital *Chlamydia trachomatis* infection in the general population for inclusion in the case-control and partner notification studies of ClaSS.
- To compare the performance of two of the leading NAA tests in terms of sensitivity and ease of the overall process.
- To compare the sensitivity of the NAA tests with EIA in the general population.
- To examine the pooling of specimens to reduce costs.
- To study the stability of specimens.

2.1 Choice Of Tests

2.1.1 Two types of assays have been chosen as potentially suitable screening tests for identifying people with genital chlamydia infection in the general population

- a) Nucleic acid amplification assays
- b) Enzyme-linked immunoassays

This choice is based on the results of the Bristol PHLS evaluation of the sensitivity of different assays (Paul, I.. *et al.* 2001). The evaluation of two NAAs will allow comparison of their labour requirements and robustness in a routine laboratory context.

2.1.2 There are two NAA platforms:

- a) Roche Cobas PCR Polymerase Chain Reaction (Appendix 1)
- b) Becton Dickinson ProbeTec Strand Displacement Amplification (SDA) (Appendix 2)

These are available in both laboratories and are semi-automated at present. Both platforms can potentially be upgraded to be fully automated. Internal controls allow information on the presence of inhibitors in samples to be generated automatically.

2.1.3 There is one Enzyme Linked Immunoassay in the study:

- a) Dako PC PCE Chlamydia Enzyme Linked Immunoassay (Appendix 3)

2.2 Comparisons Between EIA And NAAs

2.2.1 Major aspects that require consideration in the context of a screening test evaluation include:

- a) Test sensitivity
- b) Test stability
- c) Labour requirements (including throughput of samples)
- d) Use of laboratory space
- e) Costs

2.2.2 At present, EIA reagent costs are about 28% of the best price for a NAA. EIA is also less labour intensive and requires less laboratory space. In the recent Bristol PHLS study which was carried out on samples from a GUM clinic, the Dako PC system had 90% sensitivity relative to Cobas PCR using vulvo-vaginal swabs. (Paul,I. et al. 2001) Specimen stability may be better for EIA than for the NAAs and this may counterbalance the lower sensitivity in vulvo-vaginal samples. EIA does not perform well in female urine (Paul,I. et al. 2001, Caul, E. et al. 1988).

3. OVERVIEW OF LAB STUDIES

3.1 Objective 1

3.1.2 A general community sample of men and women aged 16-39 years in Avon and the West Midlands will be offered testing for chlamydia. Potential study participants will be randomly selected from their GP surgery list to take part in the prevalence study. Along with a letter explaining the study, potential participants will receive a study pack by post. The pack will contain a urine bottle and, for women, a vulvo-vaginal swab. Detailed instructions on how to collect the samples will be included (See Prevalence Survey Protocol). Participants are invited to return their samples by post to the PHLS to be tested for *Chlamydia trachomatis* (see Prevalence Survey Protocol).

3.2 Objective 2

3.2.1 Comparison of EIA sensitivity with both molecular methods, in a low prevalence setting will be made by comparing:

- a) Vulvo-vaginal swabs by EIA with vulvo-vaginal swabs by PCR (in Bristol)

- b) Vulvo-vaginal swabs by EIA with vulvo-vaginal swabs by SDA (in Birmingham)
- c) Male urine by EIA with male urine by PCR (in Bristol)

Note: There will be no comparison of SDA with EIA on male urine in Birmingham as this is precluded by volume requirements or of female urine at either site as sensitivity of EIA has been shown to be low in female urine.

3.2.2. An assessment of the reproducibility and robustness of the two molecular methods will be conducted through a Specimen Exchange Programme. This will involve:

The comparison of results from urine and vulvo-vaginal samples from the prevalence study in a routine laboratory setting using SDA and PCR.

3.2.3 In addition a small study will examine the possibility of using EIA to screen male urine. This will involve:

Assessing the feasibility of re-using urine samples which have been treated with UPP (SDA) in the Dako PC EIA.

The specimen exchange programme will also supply internal quality control data.

3.3 Objective 3

3.3.1 *Specimen Pooling* will be performed to quantify the optimum number of samples which can be pooled, taking account of:

- a) Assay test sensitivity
- b) Prevalence of *Chlamydia trachomatis*
- b) Cost effectiveness

3.4 Objective 4

3.4.1 *Specimen Stability* will be assessed by examining the decline in sensitivity of tests on urine samples and vulvo-vaginal swabs held at the laboratory in ambient temperature (recorded on collection sheet) over a range of time (24 and 48 hours) Time '0' is taken to be the time the sample was collected by the patient. For logistic reasons, this study will utilise specimens from the GUM clinic.

4. STUDY SAMPLES

4.1. Specimen Collection

4.1.2 Specimen collection is by postal survey, complying with European directives on regulations covering the transport of biological specimens. These directives have implications for the transport of samples by Royal Mail. All study participants are asked to collect an early morning samples of urine and females a vulvo-vaginal swab (See Prevalence Survey protocol). Samples collection time will be recorded on the prevalence questionnaire along with the date of last menstrual period for females. Samples will then be returned in the study pack and the time of arrival logged at the PHLS.

4.1.3 In the first practice, female participants will be randomised to receive either a single, or double headed vulvo-vaginal swab. The response rate in this practice will be examined to see the differences if any, in the acceptability of different swab types. All vulvo-vaginal samples will be returned as dry specimens. Participants will be asked to provide 25mls of urine. In cases where insufficient sample amounts are returned to the PHLS the default test will be the NAA. Samples will only be processed if accompanied by the signed consent form. Where study participants fail to return a signed consent form, samples will be frozen and a repeat request for signed consent form will be issues to the study participant. Any samples frozen before initial analysis will have the fact recorded on their Laboratory Result Form for the prevalence arm of the study. (Appendix 4). Where a respondent returns an incomplete prevalence questionnaire, i.e. with either the time or date the specimen was collected not recorded, the receiving and processing laboratory personnel will telephone the responded to elicit this information. If a telephone contact number has not been recorded on the return prevalence questionnaire contact will be made by the study office, after being a request from the laboratory. Completion of these data must be signed and dated by the recorder.

4.1.4 See Detailed Prevalence Survey Protocol for subsequent methodological changes and current practice.

4.2. Specimen Storage

4.2.1 Specimens returned by participants in the prevalence study will have urine (males/females) and swabs (females only) tested on arrival at the laboratory. Specimens will be stored at +4 C until the results of the test are known. Where a result is equivocal or in the grey zone, samples will be re-tested, otherwise the specimen will be frozen and stored in aliquots at –20 C. Negative samples will be disposed of after one month unless required for the specimen exchange programme. Positives and discordant samples will be stored indefinitely.

4.2.2 Specimens gathered in the prevalence study will also be used in the specimen exchange study.

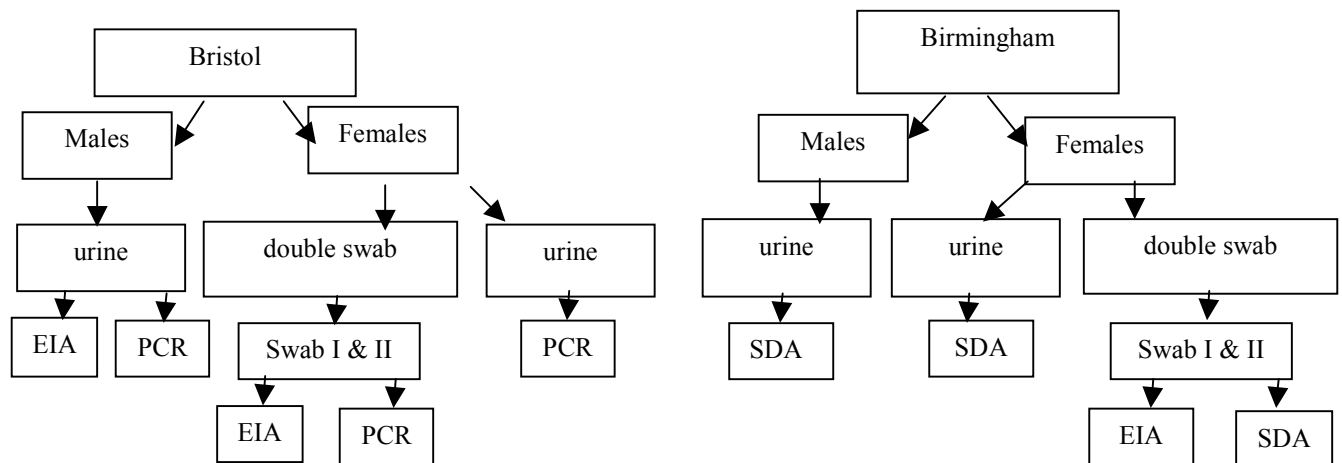
5. PREVALENCE STUDY

5.1 Method Of Sample Processing

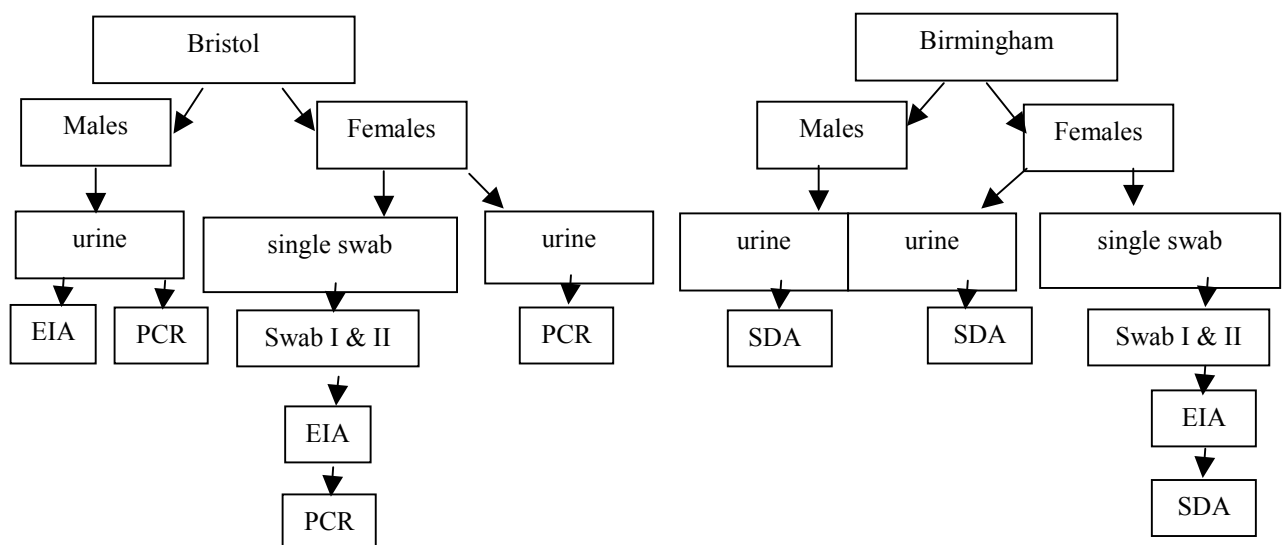
5.2 Diagrammatic illustrations of how samples will be processed at both PHLS are shown below. Flow charts for both double and single headed swabs provide an overview of the proposed method of sample processing. Following evaluation in the first two practices, the study will then be based on the results obtained on the acceptability of the two types of swab.

5.2.1 **Flow Chart 1 and 2** : Overview of processing urine and vulvo-vaginal samples at the Bristol and Birmingham PHLS.

Flow Chart 1: Double headed vulvo-vaginal swab, male and female urine.



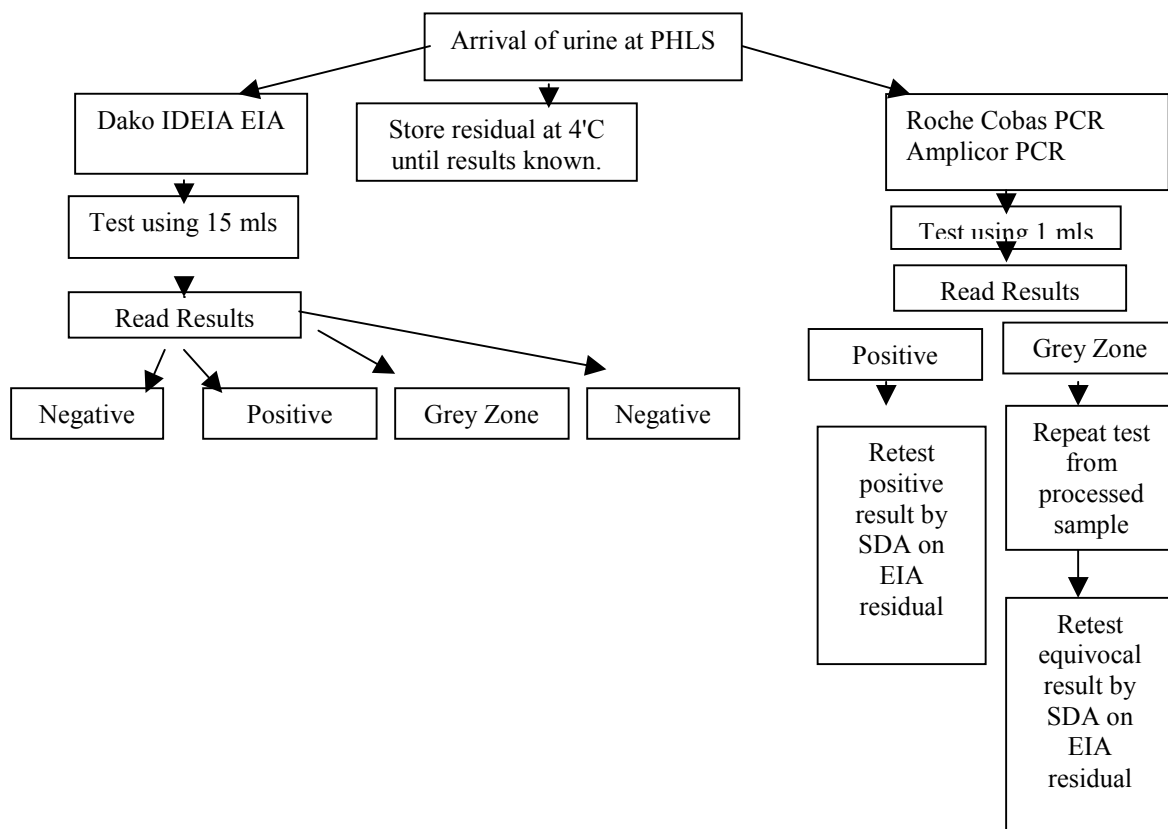
Flow Chart 2: Single headed vulvo-vaginal swab, male and female urine



6. PROCESSING OF SAMPLES

A brief outline of how samples are to be processed follows. For full details on these procedures please refer to the manufacturers' protocols (Appendix 1,2 &3).

6.1 Bristol Male Urine Sample Processing



6.1.1 Polymerase Chain Reaction

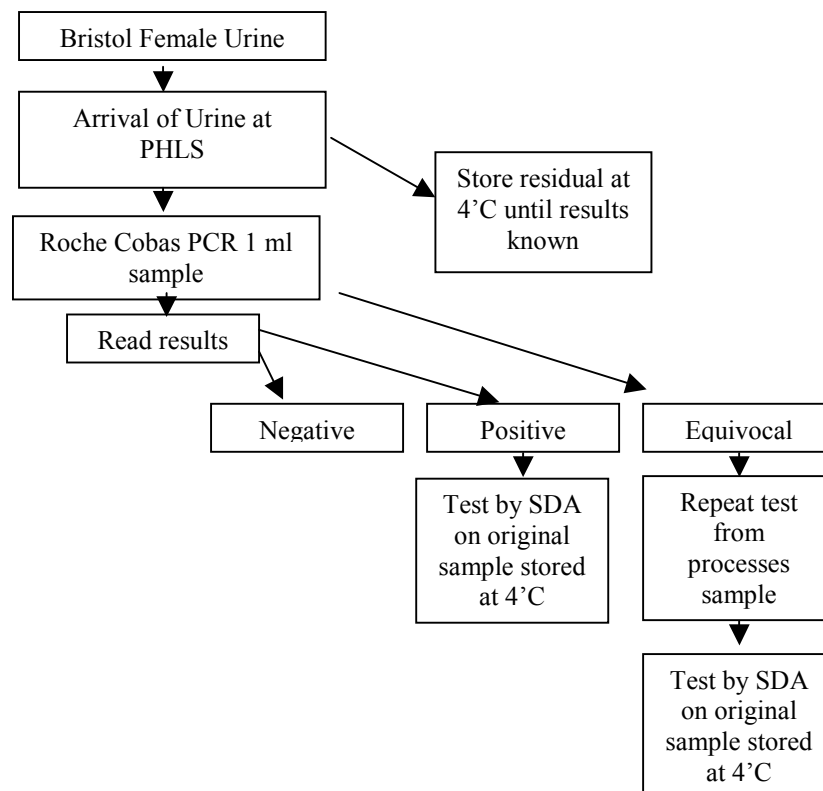
- Remove 1ml of urine into LIP tube.
- Carry out Polymerase Chain Reaction, urine specimens according to kit insert .
- Follow manufacturer recommendations re. equivocal and inhibited specimens
- Enter results on Laboratory Form.

6.1.2 Dako PC EIA Enzyme Linked Immunoassay

- a) Pour 15mls of urine into labelled universal container for EIA.
- b) Store surplus at +4°C until results available ensuring that at least 1 ml is available for LightCycler™ ref. test PCR retesting (reduce volume for EIA if necessary, record if done on less than 15 mls).
- c) Centrifuge urine in the universal and carry out EIA according to Bristol SOP.
- c) Enter results on Laboratory Form.
- e) Store surplus urine and residual urine pellet in EIA buffer at -20°C

6.2 Bristol Female Urine Sample Processing

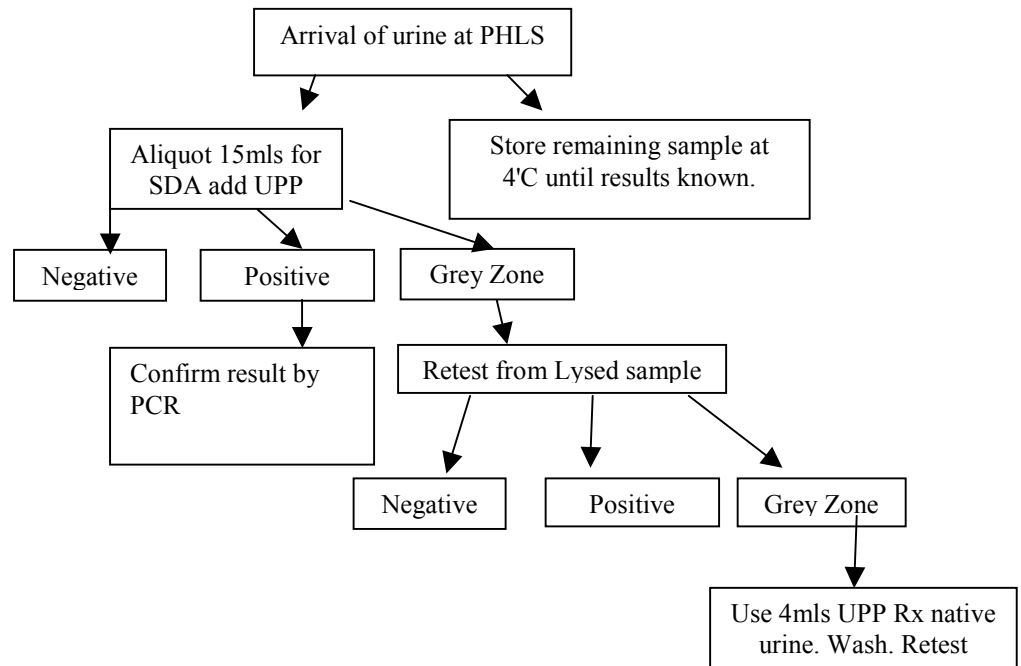
PROCESSING OF FEMALE URINE SAMPLES AT BRISTOL:



6.2.1 Polymerase Chain Reaction

- a) Remove 1ml of urine into LIP tube PCR
- b) Carry out PCR on urine specimens according to kit insert
- c) Follow manufacturer recommendations re. equivocal and inhibited specimens
- d) Enter results on Laboratory Form.

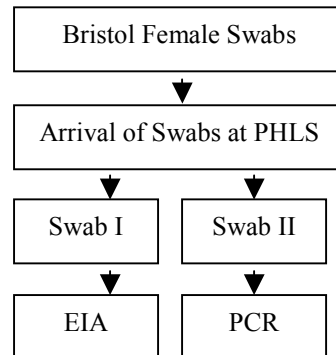
6.3 Birmingham Male And Female Urine Sample Processing



6.3.1 Becton Dickinson ProbTec Strand Displacement Amplification

- a) Aliquot urine sample into two containers
- b) Add UPP bag to the 15ml urine container
- c) Store remainder at +4°C until results available
- d) Carry out SDA on samples according to kit insert.
- e) Follow manufacturer recommendations re. equivocal and inhibited specimens
- f) Enter results on the Laboratory Results Form (Appendix 4).
- g) Store residual urine at -20°C, make separate aliquot of 2mls if the specimen is to be part of the specimen exchange
- h) Store residual male UPP treated urine for EIA at -20°C.

6.4 Bristol Female Double Headed Swab Sample Processing



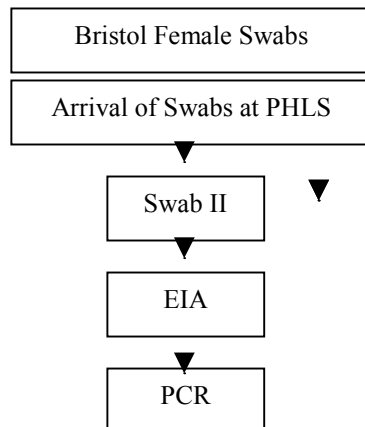
6.4.1 Dako PC Enzyme Linked Immunoassay (EIA)

- Using gloves, break off one of the swabs into a LIP tube
- Add EIA specimen diluent, elute by vortexing and
- carry out Dako PCE protocol as per Bristol SOP
- Record result on Laboratory Form
- Store swab eluate residual at -20°C

6.4.2 Roche Cobas Polymerase Chain Reaction (PCR)

- Using gloves, break off second swab into LIP tube.
- Place PCR swab elution buffer (2SP, 1 ml) into LIP tube and elute by vortexing.
- Transfer 100 μl aliquot of eluted swab buffer into PCR preparation tube and proceed with protocol as per kit insert.
- Follow manufacturer recommendations re. equivocal and inhibited specimens
- Retain residual specimen at 4°C until results known.
- Enter results on Laboratory Form.

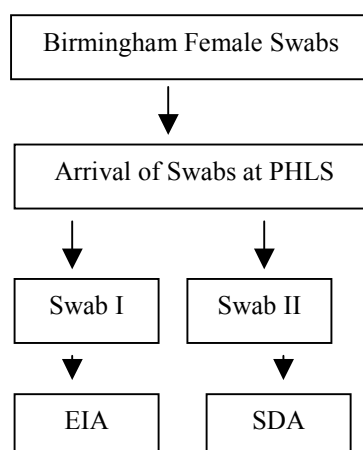
6.5 Bristol Female Single Headed Swab Sample Processing



6.5.1 Roche Cobas Polymerase Chain Reaction (PCR) for single swab.

- a) After the completion of the EIA test take 100µl of the EIA swab eluate into a numbered microcentrifuge tube containing 100µl of Roche lysis buffer.
- b) Incubate for 10 min at room temperature (RT) and proceed with normal protocol as per test insert.

6.6 Birmingham Female Double Headed Swab Sample Processing



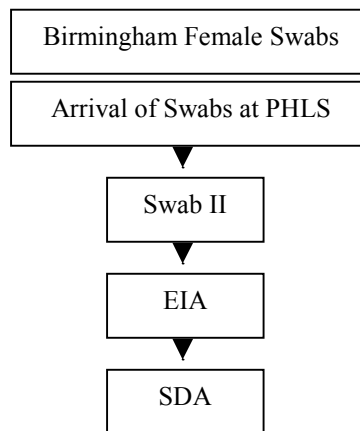
6.6.1 Dako PC EIA Enzyme Linked Immunoassay

- a) Using disposable gloves, break off one of the swabs into a LIP tube.
- b) Add EIA specimen diluent, elute by vortexing and carry out Dako PCE protocol according to manufacturer's insert (Appendix 3).
- c) Record result on Laboratory Form.
- d) Store residue of swab eluate at -20°C if not required for confirmatory testing.

6.6.2 Becton Dickinson ProbTec Strand Displacement Amplification (SDA)

- a) Using gloves, place the swab into the pre-filled (2ml) swab sample diluent tube.
- b) Mix by swirling the swab in the diluent for 5-10 seconds. Express the swab along the inside of the tube. Remove swab.
- c) Proceed with protocol as per kit insert.
- d) Follow manufacturer recommendations re. equivocal and inhibited specimens
- e) Enter results on Laboratory Form (Appendix 4).

6.7 Birmingham Female Single Headed Swab Sample Processing



6.7.1 Becton Dickinson Probitec Strand Displacement Amplification (SDA)

- a) After the completion of the EIA, return to residual swab eluate in EIA buffer and remove store 110µl in a labelled microcentrifuge tube at 4°C (for confirmatory PCR if required).
- b) Take 0.2ml of the EIA eluate and add to 2mls of BD lysis buffer in a capped lysis tube.
- c) Proceed with SDA lysis step as per the manufacturer's protocol (Appendix 2).

7. SPECIMEN EXCHANGE STUDY

7.1 Objectives

Method comparison/investigation

- a) To compare the reproducibility and robustness of the Becton Dickinson Probitec ET (SDA) with the Roche Cobas PCR.
- b) To evaluate the feasibility of using UPP treated urine with Dako's PC Enzyme Linked Immunoassay. (This is particularly relevant to screening males as it would allow EIA to be used as a primary screen and SDA as a confirmatory assay)

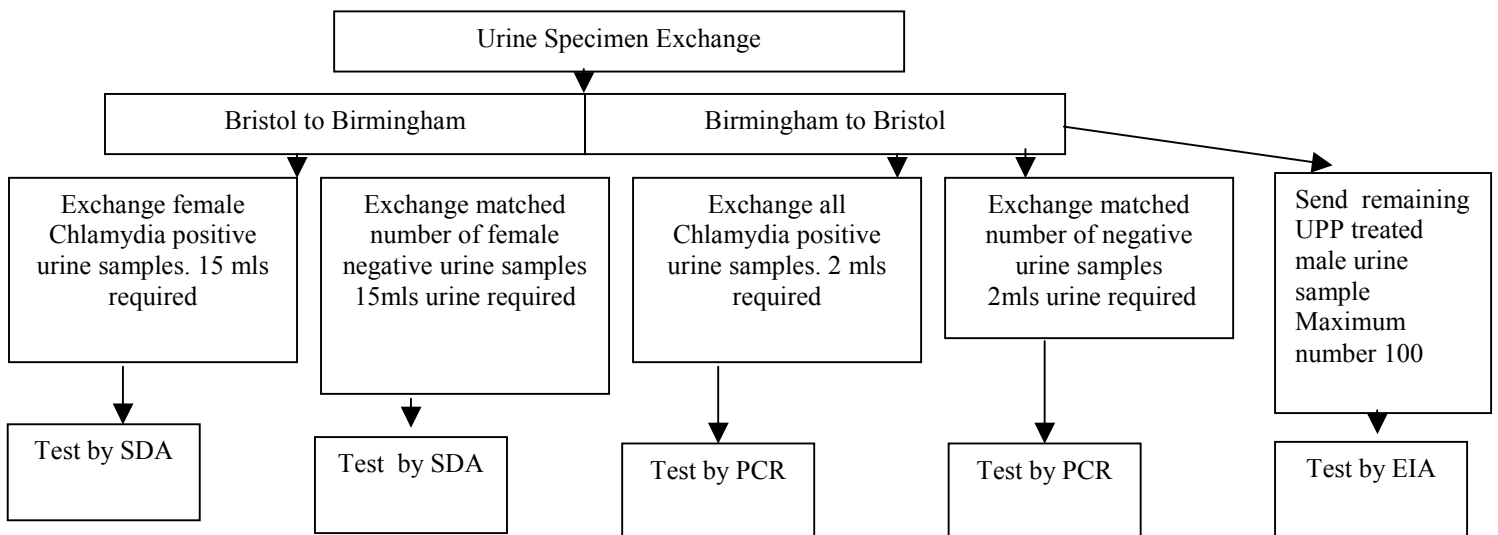
Quality Assurance.

- c) To provide a system of internal Quality Assurance (QA) for molecular diagnosis by confirming the results found in the sister PHL with the 'alternative' molecular test.

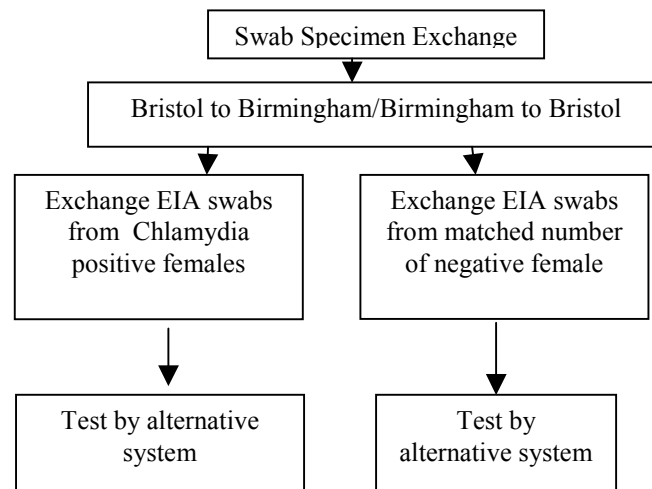
- d) Comparison of results with a coded panel of *Chlamydia trachomatis* DNA. Dilutions of DNA with known levels of plasmid target will be submitted to both laboratories, thus providing external QA for molecular diagnosis. This use of purified DNA will also give a direct assessment the absolute sensitivity of the tests. The coded panel of DNAs will be provided by the GUIRL and a protocol for this EQA exercise is given in Appendix 5.

(At present there is no alternative UK EQA scheme that we can join that provides *Chlamydia trachomatis* specimens with levels of target suitable for molecular diagnostic methods. However, the GUIRL is in discussion with the National External Quality Assurance Scheme (UK NEQUAS) about assisting in the development of such a panel and, in the event of one becoming available, we will certainly avail ourselves of it)

Flow Chart 6 Urine Specimen Exchange between the two PHLs



Flow Chart 7 Swab Specimen Exchange between the two PHLS



7.2 Sample Exchange.

Samples for exchange should be those from male and female patients identified as positive and also from those females with a discordant result i.e. from a positive swab and negative urine, or vice versa. Untreated urine and EIA samples should be exchanged together with samples from two negative patients from the same test run of the same sex identified in the laboratory. Also those individual samples giving equivocal results which cannot be resolved should be exchanged. Before shipment samples should be blinded so that their status is unknown. A record is kept for future comparison of results using Specimen Exchange Sheet 1 and 2 (Appendix 6). This is a double sided sheet which allows data on specimens from the sending laboratory to be recorded on Side 1, and data on specimens of the receiving laboratory to be recorded on Side 2.

- a) The sending laboratory identifies specimen types to be exchanged according to the protocol.
- b) The sender lists these on Specimen Exchange Sheet 1 & 2 Side 1 (Appendix 6) recording all relevant data.
- c) An exchange number e.g. Bristol = 1001, 1002, 1003 etc. and Birmingham 2001, 2002, 2003 etc. is randomly assigned to each individual specimen i.e. a different number for swab and urine samples in the case of a female.
- d) Exchange numbers are now listed sequentially to match order of samples.
- e) Specimen Exchange Sheet 1 & 2 is sent to the receiving lab as a worksheet to accompany the specimens, a photocopy is retained by the issuing laboratory.
- f) The receiving lab will enter date/time arrived on Specimen Exchange Sheet 1 & 2 Side 2 and their test result.

- g) The receiving laboratory will also complete an individual sheet for each specimen (Individual Specimen Exchange Sheet 3, Appendix 6.).
- h) Both of the completed sheets will be returned to the project office when testing has been completed.
- i) Photocopies can be retained by the laboratory if required.

Transport of samples will take place between the two sites on dry ice in batches of approximately 50 samples

8. SPECIMEN STABILITY STUDY.

Introduction

The investigation of specimen stability is an important aspect of the project since it will show whether the use of postal return of specimens to the laboratory compromises the sensitivity of the assays used for diagnosis. The specimen type that has been mostly investigated in this regard is first catch urine (FCU). A study, performed in Bristol, showed that holding FCU at ambient temperature reduced the sensitivity of detection significantly (Caul et al. 1997). Few data have appeared since but it is notable that the major manufacturers of both LCR and PCR DNA amplification kits for the detection of *C. trachomatis* recommend refrigerated transport for urine specimens. The BD Probetec SDA assay uses a different approach in which a 'urine preservation pouch' (UPP) is added to the urine. Both these requirements limit the settings in which screening specimens can be taken.

Several groups have shown that vulvo/vaginal swabs give sensitivities of detection as good as or better than FCU and the latter result was found in Bristol in a study performed as a preliminary to this project (Paul, I et al. 2001). No work that addresses the problem of stability of vulvo/vaginal swabs has been published although one preliminary report at a conference last year suggested that stability was good. Moreover, there is no stability data for FCU published for the BD SDA assay. The study outlined below is designed to gain preliminary data in both these areas.

8.1. OBJECTIVE

- To assess the effect of exposure to ambient temperature on sample stability for FCU and vulvo/vaginal swabs using both the PCR and SDA assays.

8.1.1. Source of specimens

The logistics of performing this study require a steady supply of positive specimens. These will be obtained from women who have been found to be chlamydia-positive during routine screening at the Bristol GUM clinic.

All patients attending the clinic are currently screened for *Chlamydia*. Women attending day clinics will be asked to provide 25mls of FCU and a double headed vulvo-vaginal swab in addition to the normal cervical swab that is taken during their routine examination. The time of specimen collection will be recorded and the specimens will both be stored at ambient temperature during transport to the laboratory. Specimens from day clinics routinely reach the laboratory on the same day they are taken. (The extra specimens for this arm of the study are the

same as we used for the vulvo/vaginal swabs study referred to above. Paul, I. *et al.* 2001)

8.1.2. Tests to be investigated

The two tests investigated will be the molecular assays used for case finding (PCR and SDA).

8.1.3. Length of storage period

The important variable for this study is the length of time the specimen is exposed to ambient temperature prior to assay. Preliminary assessment of the time that participant's specimens take to reach the laboratory by post shows that the majority (76%) reach the laboratory by around 24 hours while a further 20% take 48 hours to reach the laboratory. We will investigate stability over these two periods with a few specimens being left for longer periods (see below)

Currently, routine specimens from day clinics are transported to the laboratory on the day they are taken and an EIA assay result is available for the around early afternoon the next day, barring weekends. In this study, experimental specimens will be assayed only after a patient has been shown to be infected by a confirmed EIA assay as explained below.

8.1.4. Conditions of storage for trial

On receipt in the laboratory specimens will be held at ambient temperature rather than being refrigerated as is our normal practice for specimens destined for molecular assays.

The laboratory ambient temperature will be recorded for each experimental batch using a maximum/minimum thermometer and the local outside ambient temperature, to which specimens have been exposed during transport, will also be recorded.

A provisional EIA result for the patient will be available from the testing of the routine cervical swab at around 24 hours after the swab was taken. The specimens from patients who have a positive or grey zone result will then be divided into two. One aliquots of urine will be frozen immediately at -20°C as a urine pellet and the swabs will be frozen 'dry' at -20°C . The other specimens will be held at room temperature for a further 24 hours and then also frozen at -20°C . (For the BD assay, urine will be treated with UPP for two hours prior to storage). For specimens arriving on a Friday, it will not always be possible to arrange for the 48hr specimen to be stored, these will be left over the weekend and will provide some data on specimens exposed to ambient temperature for 96 hours.

Only specimens from patients that are subsequently confirmed as Chlamydia-positive will be used. All other specimens will be discarded. When a sufficient batch of specimens from positive patients has been accumulated, they will be assayed as normal.

8.1.5. Size of the trial and interpretation of results.

With no pre-existing data for the stability of vulvo-vaginal swabs it is difficult to predict the size of trial necessary to achieve statistically significant results. We intend to collect data for 50 positive patients for each of the assays in the first instance.

We have a substantial dataset, on the frequency of positive urine and vulvo-vaginal swab specimens by the Cobas assay in patients with a positive EIA result at the cervix. This is based on 238 positive patients from our recent head to head trial of EIA and PCR (Paul, I. *et al.* 2001), The vulvo/vaginal swabs from a large subset of the patients were also assayed by the BD assay. This data was obtained from specimens assayed as soon as they arrived in the laboratory. We will use this data as a comparator to ascertain if there has been a statistically significant drop in numbers detected at 24 and 48 hours.

8.1.6. Detailed description of specimen handling for the two assays and specimen types.

Double headed Vulvo/vaginal swabs and 25ml first catch urine specimens will be marked with:

1. The patient identifier
2. Time specimen was taken
3. A coloured spot project identifier.

Request forms for the patient will also be marked with the project spot identifier.

The maximum/minimum thermometer for the laboratory should be adjusted at the end of each working day and the outside temperature noted.

1. Urine specimens for assay by the Cobas PCR assay.

- a. On receipt in the laboratory, project specimens will be placed in a designated box at ambient temperature and the ambient readings for the laboratory recorded on the data sheet.
- b. When the EIA results become available, the urine specimens from patients with positive or grey zone will be identified.
- c. 1 ml will be removed to a labelled eppendorf tube and spun at 12,500-16,000g for 5 minutes.

- d. The supernatant will be carefully removed and the pellet stored at -20°C and the time and the ambient temperature readings for the laboratory recorded on the data sheet.
- e. After a further 24 hours a further 1 ml aliquot is spun and stored for the assay as in c and d above.
- f. If the EIA result is confirmed, the pellets will be assayed by the Roche Cobas assay as per the manufacturer's instructions and the results recorded on the data sheet.

2. Vulvo/vaginal swabs specimens for assay by the Cobas PCR assay.

- a. On receipt in the laboratory, project specimens will be placed in a designated box at ambient temperature and the ambient readings for the laboratory recorded on the data sheet.
- b. When the EIA results become available, the double swab specimens from patients with positive or grey zone will be identified.
- c. One swab will be broken off, placed in a labelled LIP tube with one ml of SPG buffer, and stored at -20°C .
- d. The time of storage will be recorded on the data sheet.
- e. After a further 24 hours, the other swab is treated as per c and d.
- f. If the EIA result is confirmed, the swabs will be assayed by the Roche Cobas assay as per the manufacturer's instructions and the results recorded on the data sheet.

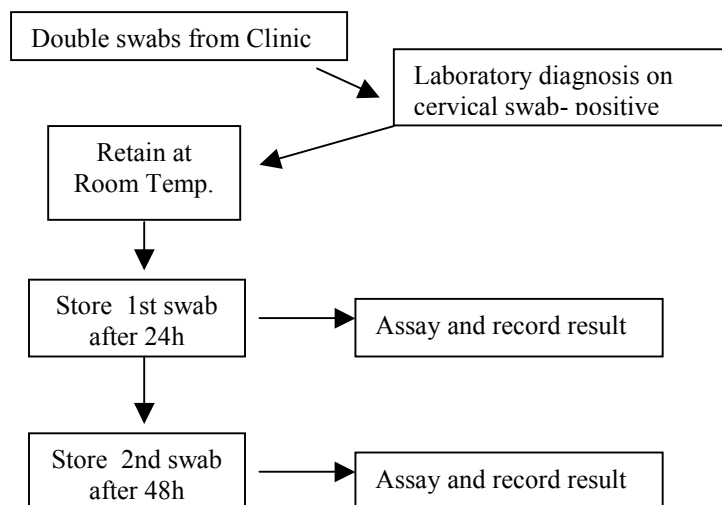
3. Urine specimens for assay by the BD Probetec ET PCR assay.

- a. On receipt in the laboratory, project specimens will be placed in a designated box at ambient temperature and an ambient reading for the laboratory recorded on the data sheet.
- b. When the EIA results become available, the urine specimens from patients with positive or grey zone will be identified.
- c. Half the urine specimen (ca 12 mls) will be decanted into a fresh labelled urine pot. A UPP is to be added and the pot left at room temperature for a further 2 hours.
- d. 4 mls will be removed to a labelled centrifuge tube and spun at 2,000g for 30 minutes
- d. The supernatant will be carefully removed and the pellet stored at -20°C and the time recorded on the data sheet.
- e. After a further 24 hours a further 4 ml aliquot is spun and stored for the assay as in c and d above.
- f. If the EIA result is confirmed, the pellets will be assayed by the BD Probetec assay as per the manufacturer's instructions and the results recorded on the data sheet.

4. Vulvo/vaginal swabs specimens for assay by the Cobas PCR assay

- a. On receipt in the laboratory, project specimens will be placed in a designated box at ambient temperature and the ambient readings for the laboratory recorded on the data sheet.
- b. When the EIA results become available, the double swab specimens from patients with positive or grey zone will be identified.
- c. One swab will be broken off, placed in a labelled Probetec swab tube and stored at -20°C .
- d. The time of storage and the ambient readings for the laboratory will be recorded on the data sheet.
- e. After a further 24 hours, the other swab is treated as per c and d.
- f. If the EIA result is confirmed, the swabs will be assayed by the BD Probetec assay as per the manufacturer's instructions and the results recorded on the data sheet

Flow Chart 7 of Stability Study- Swabs



9. STUDIES OF SPECIMEN POOLING

Specimen pooling is a method of reducing cost of screening by restricting the number of tests performed to find cases. It is especially effective when the condition being tested for has a relatively low prevalence and a highly sensitive assay is available. The necessity to do two rounds of assay adds delays to obtaining the results. However, it has been suggested that chlamydial screening lends itself to pooling because there is not the same urgency to produce results in a screening programme as there is with routine diagnosis and very sensitive tests are available.

There are several factors that determine the success of pooling, among which are:

- The sensitivity of the assay, to allow it to overcome the inevitable dilution effect of pooling.
- The capacity of the assay to accommodate extra specimen input to counteract dilution.
- The distribution of pathogen target levels in the specimens, i.e. the number of specimens that contain such a low level of target that they will be just detected by individual assay but will be below the detection threshold after dilution.
- The frequency and concentration distribution of inhibitors of the assay in the specimens.
- The prevalence of infection which, given sufficient assay sensitivity, will impact on the optimum number of specimens to pool.

There have been several publications (Kacena, K. *et al.* 1998, Peeling, R. *et al.* 1998, Krepel, J. *et al.* 1999) utilising both LCR and PCR that suggest that pooling for screening is indeed an excellent strategy. All the studies have been empirical in nature and have utilised reduced signal/cut-off ratios to compensate for the dilution effect. They have all been performed on high prevalence populations and it is possible (and indeed is often assumed) that the mean target level may be lower in low prevalence populations. Most importantly, all the studies to date have been performed with urine and there has been no investigation of vulvo/vaginal swabs.

Two studies showed no drop in sensitivity due to pooling but, interestingly, the latest study for LCR (Krepel J. *et al.* 1999) does show a slight loss in sensitivity. Unfortunately, this study used re-testing of 'false negative' pools which makes the final results very difficult to interpret. Only two, very recent poster presentations are available on pooling with the BD Probetec SDA assay. Both report a drop in sensitivity by ca. 8% when pools of five were used (McDonald, R. . *et al.* 2001, Wood, B. . *et al.* 2001).

We have developed a method to measure the absolute target levels of chlamydial plasmid DNA in specimens which compensates for any variation in the extraction yield between specimens by the inclusion of an internal control. The results obtained with this method clearly indicate that there are significantly fewer vulvo-vaginal swabs which have low levels of DNA target relative to the levels we found in female

urine specimens (Longhurst, Eastick and Herring- unpublished results). A similar result was also reported at a conference last year by Helen Lee and her colleagues. These results suggest that vulvo-vaginal swabs may well be a superior specimen to urine and may give good results with pooled specimens.

9.1. DESIGN OF THE STUDY

In our initial proposal we resolved to utilise GUM clinic specimens to study pooling but the studies published in the interim mean that this approach would add little to what has been established. We thus propose to use studies from the community survey to investigate pooling.

The advantages of this change in design are:

- The pooling will be performed on a low prevalence population
- The specimens used will be fresh rather than stored which is closer to 'real life'. One of the original pooling papers made the point that this was an important aspect (2).
- This design makes the optimum use of extracted specimens, there is no requirement to re-extract specimens.
- Individual specimens and pools will be tested in the same assay run, thus the pooling results will effectively be blinded. If retesting of false negative pools is carried out as per ref. 3, only the first result obtained will be used in the analysis.
- The study will allow vulvo/vaginal swabs to be investigated as well as urine.
- This approach does not require additional specimens from the clinic.

It was our intention to monitor prevalence and investigate assay sensitivity by performing dilution experiments on the first positive specimens we received during the initial 3 months of the study. However, the low rate of specimen return during the early part of the project has meant that the prevalence estimate has wide confidence limits and insufficient specimens have been available to dilute. The dilutions study is critical for deciding the eventual pool size. We have thus decided to proceed using by writing a provisional protocol for two pool sizes instead of selecting a single pool size to give optimum economy. The sizes chosen are loosely based on the confidence intervals for the prevalence estimate obtained over the first four months of the project.

9.2. OVERVIEW OF POOLING STUDY TO BE CONDUCTED IN PARALLEL WITH PREVALENCE STUDY (PROVISIONAL).

The actual values obtained for women aged 16-25 years, which we consider to be the most relevant group for chlamydial screening was 4.6% with 95% confidence intervals of 2.1-8.6%. This contrasts markedly with the much high prevalence of 9.1% overall found by the recent Department of Health-funded pilot studies of opportunistic screening for women in this age group.

Table 3 show the relationship between prevalence and the optimum pool size. Inspection shows that for a prevalence of 4.6% the optimum pool size is 5 whereas for 9.1% the optimum pool size is 4. However, as can be seen from the figure for tests necessary if a pool size of 4 is used with a population prevalence of 4,5% (in italics in the table), the difference in the actual savings in tests made by the use of a non-optimal pool size of 4 is only about 3%. Thus, mindful that the pilot result suggests a higher prevalence than 4.6%, we have selected 4 as one of our pool sizes.

Table 3 – Relationship between prevalence and optimum pool size

Prevalence %	Optimum Pool size	No. of test required per 10 ⁴ specimens*
2	8	2742
2.5	7	3053
3	6	3337
3.5	6	3636
4	6	3839
4.5	5	4056
<i>4.5</i>	<i>4</i>	<i>4182</i>
5	5	4262
5.5	5	4464
6	5	4661
6.5	5	4854
7	4	5019
7.5	4	5179
8	4	5336
8.5	4	5491
9.0	4	5643
10	4	5939
11	4	6226
12.5	3	6634
14	3	6973
*Calculated from the number of pools expected positive plus the tests on individual specimens in those positive pools.		

It is hoped that when screening has been in force for some years the population prevalence will fall and this will make the use of larger pools more cost-effective. Consequently, we also propose to investigate the use of pools of 8. This pool size is also appropriate to the lower of our two confidence intervals. (It is also logistically convenient to use pool sizes that are simple multiples of each other). However, the use of this pool size will only be used if the dilution studies described below show that the assays have sufficient sensitivity to accommodate the lower amounts of DNA target.

9.3. DILUTION STUDIES TO INSTRUCT POOLING STUDY

Dilution experiments with the first 20 positive specimens will be performed to establish that the assays are sensitive enough to accommodate the x8 dilution proposed for the pooling study. The Data Collection sheet is shown in Appendix 8.

Detailed protocol for dilution studies.

1. Dilution of positive urine specimens using the Cobas PCR.

- a. Select five clearly negative urine specimens make a pool of 5x 1ml of urine to act as a diluent.
- b. Add 1.0ml of the negative pool to 4 tubes. Label x2, X4, X8 and X16 plus no. for positive specimen.
- c. Make twofold dilution series using 1.0 ml of the positive specimen urine.
- d. Ignoring the x2 dilution, remove 0,5 ml from each tube and assay by Cobas PCR as per the manufacturer's instructions.
- e. Record the results for the x4,x8 and x16 dilutions on the dilution study data form.

1. Dilution of positive vulvo-vaginal swab specimens using the Cobas PCR.

- a. Select five, clearly negative vulvo-vaginal swab eluates and make a pool of 5x 0.2 ml to act as a diluent.
- b. Add 0.2ml of the negative pool to 4 tubes. Label X2, X4, X8 and X16 plus no. for positive specimen.
- c. Make two-fold dilution series using 0.2 ml of the positive specimen.
- d. Ignoring the x2 dilution, remove 0.1 ml of each dilution and assay by Cobas PCR as per the manufacturer's instructions.
- e. Record the results for the x4,x8 and x16 dilutions on the dilution study data form.

1. Dilution of positive urine specimens using the BD Probetec assay.

N.B. Since there is no recommendation for the long-term storage of UPP-treated urine, this trial will be performed on lysed extracts.

- a. Select residual, lysed pellet from five clearly negative urine specimens and make a pool of 5x 1ml to act as a diluent.
- b. Add 1.0ml of the negative pool to 4 tubes. Label x2, X4, X8 and X16 plus no. for positive specimen.
- c. Make twofold dilution series using 1.0 ml of the positive specimen lysed urine pellet specimen.

- d. Ignoring the x2 dilution, remove 0,2 ml from each tube with the dedicated pipettor and assay by BD Probetec as per the manufacturer's instructions.
- e. Record the results for the x4,x8 and x16 dilutions on the dilution study data form.

1. Dilution of positive vulvo-vaginal swab specimens using the BD Probetec assay.

- a. Select residual, lysed swab eluate from five clearly negative swab specimens and make a pool of 5x 1ml to act as a diluent.
- b. Add 1.0ml of the negative pool to 4 tubes. Label x2, X4, X8 and X16 plus no. for positive specimen.
- c. Make twofold dilution series using 1.0 ml of the positive specimen lysed swab eluate specimen.
- d. Ignoring the x2 dilution, remove 0,2 ml from each tube with the dedicated pipettor and assay by BD Probetec as per the manufacturer's instructions.
- e. Record the results for the x4,x8 and x16 dilutions on the dilution study data form.

Inspection of the results of this study will be used to decide if the assays have sufficient sensitivity for pooling by both 4 and 8. During the remainder of the community study, pooling will be carried out for both urine and vulvo/vaginal swabs at the same time as individual testing. This should generate a comprehensive data set for both assays

9.4. DETAILED PROTOCOL FOR THE POOLING OF SPECIMENS.

Detailed protocol for the pooling of specimens.

a. For pools of 4.

1. Urine specimens by the Cobas assay (Bristol).

Pooled specimens will be generated by mixing 125µl of urine from each of 4 specimens into the appropriate screw cap tube. This pool will be designated with a 'P' number and the constituent specimens noted immediately on the laboratory sheet.

Specimen P will be processed for Cobas as normal in parallel with all the other individual specimens.

The pool result for the Cobas assay will be recorded on the laboratory sheet. The results for the Cobas assay will also be recorded on the sheet for the pool constituents.

If the pool result is positive or equivocal but the pool contains no individual specimens with positive or equivocal results, the individual pool specimens should be re-tested using the lysed specimens.

When there are insufficient specimens to make up a pool of 4, residual urine specimens will be stored at 4⁰C. Subsequently, these specimens can be used to make pools with incoming fresh specimens *providing they are not stored for more than 7 days*.

2. Vulvo/vaginal swab specimens by the Cobas assay.

Pooled specimens will be generated by mixing 25µl of swab eluate in 2SP from each of 4 specimens. This pool will be designated with a 'P' number and the constituent specimens noted immediately on the laboratory sheet.

Specimen P will be processed for Cobas normal in parallel with all the other individual specimens.

The pool result for the Cobas assay will be recorded on the laboratory sheet. The results for the Cobas assay will also be recorded on the sheet for the pool constituents.

If the pool result is positive or equivocal but the pool contains no individual specimens with positive or equivocal results, the individual pool specimens should be re-tested using the lysed specimens.

When there are insufficient specimens to make up a pool of 4, residual swab specimen will be stored at 4⁰C. Subsequently, these specimens can be used to make pools with incoming fresh swab specimens *providing they are not stored for more than 7 days*

3. Urine specimens by the BD Probetec assay (Birmingham)

Pooled specimens will be generated by mixing 1000µl of urine from each of 4 specimens. This pool will be designated with a 'P' number and have a suffix to denote the pool size. The individual record sheet will show the number of the pool containing that sample.

The pools will be processed by SDA as normal in parallel with all the other individual specimens.

The pool result will be recorded on the specimen pooling sheet (Appendix 9). This sheet shows the date pooled and tick boxes indicating the following possible results for the pool: inhibitory, positive, negative,

GZ=grey zone. Tick boxes also record concordance of the pool result with those of the individual samples. ie

A Yes = concordance

B No, pool +ive = positive pool, but no positive individual samples

C No, pool –ive = negative pool, but contains positive individual samples

In the event of B as shown above, all individual samples in that pool will be re-tested. If the individual samples remain negative on re-test this will be scored as a 'false positive'.

In the event of C as shown above, the assay will be scored as a 'false-negative'

If retesting is required, results will be recorded on the reverse of the pooling sheet.

When there are insufficient specimens to make up a pool of 4, residual urine specimens will be stored at 4⁰C. Subsequently, these specimens can be used to make pools with incoming fresh specimens *providing they are not stored for more than 6 days*

4. vulvo/vaginal swab specimens by the Probetec assay. (Birmingham)

Pooled specimens will be generated by mixing 500µl of lysed swab extract from each of 4 specimens. This pool will be designated with a 'P' number and the constituent specimens noted immediately on the laboratory sheet.

Specimen P will be assayed by Probetec as normal in parallel with all the other individual specimens.

The pool result for the Probetec assay will be recorded on the laboratory sheet. The results for the Probetec assay will also be recorded on the sheet for each of the pool constituents.

If the pool result is positive or equivocal but the pool contains no individual specimens with positive or equivocal results, the individual pool specimens should be re-tested using the lysed specimens (maximum storage –5 days at 4⁰C, re-lyse).

When there are insufficient specimens to make up a pool of 4, residual lysed swab specimens will be stored at 4⁰C. Subsequently, these specimens can be used to make pools with incoming fresh specimens *providing they are not stored for more than 5 days.*

b. For Pools of 81. Urine specimens by the Cobas assay (Bristol).

Prepare pool by mixing 100µl from the residual prepared lysate of two consecutive pools of four urine specimens. If there are insufficient specimens to allow a pool of 8 to be prepared lysates stored at 4⁰C can be used *providing they are not stored for more than 7 days.*

Enter 50 µl into the assay and proceed as for pools of 4 above but using reporting sheet for pools of 8.

2. Vulvo/vaginal swab specimens by the Cobas assay.

Prepare pool by mixing 100µl from the residual prepared lysate of two consecutive pools of four swabs. If there are insufficient specimens to allow a pool of 8 to be prepared lysates stored at 4⁰C can be used *providing they are not stored for more than 7 days.*

Enter 50 µl into the assay and proceed as for pools of 4 above above but using reporting sheet for pools of 8.

3. Urine specimens by the BD Probetec assay (Birmingham)

Prepare pool by mixing 250µl from the residual prepared lysate of two consecutive pools of four urine specimens. If there are insufficient specimens to allow a pool of 8 to be prepared lysates stored at 4⁰C can be used *providing they are not stored for more than 5 days.*

Enter 150µl into the assay (priming) and proceed as for pools of 4 above above but using reporting sheet for pools of 8.

4. vulvo/vaginal swab specimens by the Probetec assay. (Birmingham)

Prepare pool by mixing 100µl from the residual prepared lysate of two consecutive pools of four swabs. If there are insufficient specimens to allow a pool of 8 to be prepared lysates stored at 4⁰C can be used *providing they are not stored for more than 5 days.*

Enter 50 µl into the assay and proceed as for pools of 4 above above but using reporting sheet for pools of 8.

The reporting sheets for the pooling are shown in Appendix 9.

10.0 -INTERPRETATION OF DIAGNOSTIC RESULTS

Female specimens				
<u>Molecular Result¹</u>		<u>EIA result</u>		
urine	Vvs	Vvs	<u>Comment</u>	<u>Report</u>
+	+	+		+
+	+	GZ		+
+	+	- ²		+
- ³	+	+	accepting the EIA as confirmation	+
- ³	+	GZ	accepting the EIA as confirmation	+
- ³	+	- ⁴	if positive on EIA vulvo/vaginal swab with alternative molecular test	+
+	-	+	accepting the EIA as confirmation	+
+	-	GZ ²	accepting the EIA as confirmation.	+
+ ⁴	-	- ²	if +ve retest with alternative molecular test ⁴ on urine	+
- ³	-	+ ⁴	if positive on EIA vulvo/vaginal swab with alternative molecular test	+
- ³	-	GZ ⁴	if positive on EIA vulvo/vaginal swab with alternative molecular test.	+
-	-	-		-
¹ . Either initial result or second result on an inhibitory or equivocal specimen. If two equivocal results use alternative molecular test (SDA, Cobas or Reference PCR)				
² Test EIA vulvo/vaginal swab residue by reference PCR.				
³ Test urine by reference PCR if volumes allow (requires 1ml).				
⁴ . Perform confirmation using SDA in Bristol or Cobas PCR in Birmingham. For vulvo/vaginal swabs Cobas PCR can be performed on EIA extract, for SDA the EIA extract must be spun down prior to the test. (<i>Both manufacturers have approved these modifications</i>).				

Male specimens			
Bristol			
EIA	PCR ^{1.}	Comment	Report
+	+		+
GZ	+		+
-	+ ²	if Ref PCR on 2 nd aliquot is +ve	+
+	- ²	if Ref PCR on 2 nd aliquot is +ve	+
GZ	- ²	if Ref PCR on 2 nd aliquot is +ve	+
-	-		-
Birmingham			
	SDA ^{1.}		
	+ ³	if +ve with Cobas PCR,(If –ve with Cobas, send for Ref PCR but report -ve.)	+
	-	-	
^{1.} Either initial result or second result on an inhibitory or equivocal specimen. If two equivocal results use alternative molecular test (SDA, Cobas or Reference PCR)			
² Test urine by reference PCR if volumes allow (requires 1ml) otherwise use EIA residue.			
^{3.} Perform confirmation using Cobas PCR on fresh aliquot. (<i>Both manufacturers have approved these modifications</i>).			

9.4 RECORDING AND TRANSFER OF TEST RESULTS

- The results from the machine output are recorded on the Laboratory report form (Appendix 4) in black or blue pen. Only the unique identification number identifies the participant.
- According to the reporting criteria set out above in section 10, the participants are identified as positive or negative and the result recorded in the appropriate box in the report form. Positive results are written in red pen.
- Forms are then checked by a senior member of the laboratory staff to ensure that the results and the final diagnosis are correctly entered on the form and are consistent.
- The forms are then returned to the co-ordination office together with the consent forms from the original specimen pack
-

Note regarding specimen storage

With the number of specimens being tested it may prove impossible to store the residue of all specimens. However, all residues will be stored for at least 1 month after testing. All positive specimen residues and at least an equal number of negative specimens will be retained until the end of the project.

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