Pelvic inflammatory disease (PID) is a key issue facing women's reproductive health and has a multifactorial aetiology. Although Chlamydia trachomatis causes a substantial proportion of cases, serological evidence has associated Mycoplasma genitalium with PID. Previous investigations have been hampered by the lack of simple, accurate diagnostic methods, but polymerase chain reaction (PCR) assays are now available. A recent Kenyan study suggested an association between M genitalium and acute endometritis.

Here, samples from a case-control study undertaken in England were used to evaluate the relationship between M genitalium, C trachomatis, and PID.

**METHODS**

A case-control methodology was used, which included women aged 16–46 years of age. Patients were derived from a larger case-control study of risk factors associated with PID. A PCR test for M genitalium that had been developed by the Public Health Laboratory Service became available halfway through the study, and was included in the protocol. Detailed data on sexual behaviour, demographic factors, and reproductive health were collected as part of the larger PID case-control study, but could not be used here because of the small number of M genitalium cases detected and the small overall sample size.

PID cases were diagnosed using the clinical criteria suggested by Hager et al.—that is, lower abdominal pain, adnexal tenderness, and tenderness with motion of the cervix and uterus. This is the standard definition used in departments of genitourinary medicine. Controls were women attending obstetrics and gynaecology for bilateral tubal sterilization. The same buffer was then used to elute nucleic acid from the column a second time. Amplification was performed by in-house PCR using the LightCycler instrument.

The following primers were used to target the 16S rRNA gene of M genitalium: 16SFG2: 5’ CCT TAT CGT TAG TTA CAT TGA CTA A 3’ and 16SRC: 5’ TGA CAT GGC CTT CCA ATA AA 3’.

Reactions were set up for the LightCycler consisting of (final concentrations in 10 µL reactions): 500 nM each primer, 500 µM each dNTP, 50 mM TRIS-HCl pH 8.3, 5 mM MgCl2, and 0.65 U Platinum Taq polymerase (prediluted in 1 µl 2.5 mg/ml BSA to prevent denaturation). This mix was dispensed and made up to 10 µl using the QIAamp extracts. The LightCycler instrument (Roche Molecular Systems) was programmed as follows: after initial denaturation of 1 minute at 95°C, 50 reactions were carried out using the microimmunofluorescence (MIF) test. Results of the MIF test were interpreted as follows: C trachomatis titre = 16, no evidence of infection; titre ≥ 32, evidence of infection; titre of C trachomatis ≥ titre of C pneumoniae > titre of C trachomatis, cross reaction unlikely; titre of C pneumoniae > titre of C trachomatis, indeterminate result.

The M genitalium PCR test used the following protocol. A volume of 200 µl of swab eluate was processed using the QIAamp DNA mini kit (Qiagen Ltd) blood and body fluid protocol according to the manufacturer’s instructions. For swabs in LCx medium or transport medium 1 µg calf thymus DNA was added as a carrier before extraction. Swabs transported to the laboratory were incubated at room temperature in 500 µl phosphate buffered saline for 1 hour and vortex mixed. A volume of 200 µl of expressed fluid was processed using the QIAamp DNA mini kit swab protocol without carrier DNA. Nucleic acid was eluted from QIAamp columns using 50 µl of buffer AE which was incubated on the column for 5 minutes. The same buffer was then used to elute nucleic acid from the column a second time. Amplification was performed by in-house PCR using the LightCycler instrument.

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Blood samples were also taken to assess serological evidence of chlamydial infection using the microimmunofluorescence (MIF) test. Results of the MIF test were interpreted as follows: C trachomatis titre = 16, no evidence of infection; titre ≥ 32, evidence of infection; titre of C trachomatis ≥ titre of C pneumoniae > titre of C trachomatis, cross reaction unlikely; titre of C pneumoniae > titre of C trachomatis, indeterminate result.

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cycles were performed consisting of 0 second at 95°C, 0 seconds at 53°C, and 15 seconds at 72°C (program type: melting curves). After each cycle a single fluorescence reading was taken. The results were ascertained using a melt cycle at 0.2°C per second with continuous fluorescence readings. A positive specimen was judged to have a Tm within plus or minus 1°C of the positive control. This was approximately 88.5°C. Positive results were confirmed using a hemi-nested block based PCR.

Statistical analysis was undertaken using the Fisher’s exact test (Stata 6). Cases and controls were compared in terms of age using the Mann-Whitney test.

RESULTS
A total of 82 women were included in the study, 45 with a clinical diagnosis of PID and 37 from patients undergoing tubal ligation. The median age of the cases was 25 (range 16–43), whereas that of the controls was 34 (range 21–45). Cases were significantly younger than the controls (p<0.001).

Evidence of M genitalium infection was found in 13% (6/45) of the cases compared to none of the controls, 27% (12/45) of the cases had C trachomatis infection (LCR with or without serology) compared to none of the controls, and 16% (7/45) of the cases had only serological evidence of C trachomatis infection compared to 5% (2/37) of controls (table 1). BV was not detected in any of the cases and controls. Two cases had co-infections: one with C trachomatis and N gonorrhoeae, the other between M genitalium and C trachomatis (this patient also had serological evidence of C trachomatis infection). The remaining five M genitalium infected patients had no serological evidence of C trachomatis infection. Of the patients with M genitalium infection, half were over 30 years old. Cases were more likely to present with M genitalium and/or C trachomatis than controls (p<0.001).

DISCUSSION
This is the first case-control study of PID to test for M genitalium. The results suggest that there is an association between M genitalium and PID, and that M genitalium is not merely a commensal organism detected at the site of an STI infection. These findings are similar to those from studies of M genitalium in men with non-gonococcal urethritis.

There is no standard methodology for sampling the female genital tract for M genitalium. Here, the endocervix was used because it was thought to be the site from which M genitalium migrates to the upper genital tract. The performance of the LightCycler PCR assay was similar to that of the block based assay using the same primers, but detected fivefold less DNA (equivalent to 10 genome copies) of M genitalium in a dilution series. In tests using 28 common micro-organisms, none produced a product with the Tm typical of that from M genitalium. However, the testing methodology may lack sensitivity because many of the specimens had previously been tested for C trachomatis using the LCR test which includes an incubation step at 95°C in a high magnesium buffer. Ancodatal evidence suggests that mycoplasma DNA may be susceptible to degradation, perhaps due to a relatively low guanine and cytosine content. The inclusion of a serological test would have provided evidence of previous exposure to M genitalium but was not carried out because these specialist techniques were not available at the laboratory.

The Hager definition of PID lacks specificity and consequently some patients included in this study may not have had PID. This problem is inherent to all studies of PID. However, the most crucial element in the design of a case-control study is not to include cases in the control group. Here all controls were laparoscoped to ensure that none of the controls had PID.

The multifactorial aetiology of PID is well established but the public health control of PID has largely focused on the control of genital chlamydial infection. The National Strategy for Sexual Health and HIV recently published by the Department of Health (England) emphasised the need to screen young women for genital chlamydial infection. Although this is a welcome development, this study is a timely reminder that chlamydial intervention alone is unlikely to eradicate PID. The antibiotics used to treat genital chlamydial infection may be less effective in the treatment of M genitalium and, since infection has been shown to be largely independent of C trachomatis, it is likely that the epidemiology of these infections may be different.

Effective PID prevention and control rests on improved knowledge of the pathogenesis and epidemiology of the aetiological agents that cause this clinical syndrome. Further studies need to investigate the pathological basis of the relation between M genitalium and PID using samples from women with PID diagnosed using laparoscopy and endometrial biopsy. This study was based on an opportunistic cohort and it was not specifically designed to examine the relation between M genitalium and PID. A bias within the study was the significant difference in age between the case and control groups. However, since half the patients with M genitalium were over the age of 30 years, this source of bias is unlikely to have significantly influenced the study. Nevertheless, results reported here give an interesting insight into the sequelae associated with M genitalium and provide directions for future studies. Little is known about the epidemiology of M genitalium and specifically designed large scale epidemiological investigations are needed to determine the prevalence, incidence and factors associated with this emerging infection. However, before such studies can be undertaken clear guidelines are needed on the diagnosis of M genitalium and the sites that should be sampled.

ACKNOWLEDGEMENTS
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Conflict of interest: none declared.

CONTRIBUTORS
IS initiated and designed the project and undertook the analysis with PR; KE and AH developed the M genitalium PCR and did the testing; HM undertook the LCR and serological testing; KH, RG, and PH recruited cases and controls for the study.

Table 1 Evidence of M genitalium and C trachomatis in cases and controls

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=45)</th>
<th>Controls (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M genitalium</td>
<td>6*</td>
<td>0</td>
</tr>
<tr>
<td>C trachomatis</td>
<td>12††</td>
<td>0</td>
</tr>
<tr>
<td>(ligase chain reaction with or without serological evidence)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C trachomatis</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>(serology only)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>20§‡</td>
<td>35</td>
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</tbody>
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*Includes 1 co-infection with C trachomatis
†Includes 1 co-infection with N gonorrhoeae
‡7 had serological evidence of C trachomatis infection.
§1 had N gonorrhoeae.
ECHO

Quality and use of synovial fluid tests open to question

The use of synovial fluid tests gives cause for concern, despite their obvious diagnostic value in certain joint diseases, suggests a comprehensive literature review of their applications from the University of Bristol.

The review, based on 300 papers out of an initial trawl of 6556, published between 1980 and 2001, confirms that synovial fluid analysis can be of major diagnostic value. It is especially useful in acute arthritis, when a crystal arthropathy or septic arthritis is suspected, and in intercritical gout.

But standards fall well short of optimal for the traditional assays of microbiology, white blood cell counts, and microscopy for pathogenic crystals. And there are “worrying variations” in reported sensitivity, specificity, and reliability, and scant evidence of quality control, it finds.

Furthermore, the use of the newer cytological and biochemical marker assays is based primarily on anecdotal evidence, with no research into their sensitivity, specificity, and reliability, it says. Added to which, the well known difficulties of interpreting the results of biochemical assays restrict their use in clinical medicine.

The authors point out that the poor standards highlighted by the review may be partly due to the fact that synovial fluid analysis is routinely under researched, excluded from routine diagnostic pathology services, and is a victim of low throughput of samples in most units.

More research is urgently needed, say the authors, who conclude that the immediate priority should be to rationalise the use, and improve the quality control, of synovial fluid assays, given their importance in acute arthritis.