BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form ([http://bmjopen.bmj.com/site/about/resources/checklist.pdf](http://bmjopen.bmj.com/site/about/resources/checklist.pdf)) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below. Some articles will have been accepted based in part or entirely on reviews undertaken for other BMJ Group journals. These will be reproduced where possible.

### ARTICLE DETAILS

<table>
<thead>
<tr>
<th>TITLE (PROVISIONAL)</th>
<th>Unusually low prevalence of Mycoplasma genitalium in urine samples from infertile men and healthy controls – a prevalence study</th>
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<tbody>
<tr>
<td>AUTHORS</td>
<td>Tripkovic, Vesna (proxy) (contact); Plecko, Vanda; Zele-Starcevic, Lidija; Skerlev, Mihael; Ljubojevic-Hadzavdic, Suzana; Plesko, Sanja; Marekovic, Ivana; Jensen, Jørgen</td>
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### VERSION 1 - REVIEW

| REVIEWER            | Asem A. Shehabi  
Department of Pathology-Microbiology, Faculty of Medicine, The Jordan University, Amman, Jordan. |
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<td>REVIEW RETURNED</td>
<td>24-Apr-2014</td>
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**GENERAL COMMENTS**

I recommend the authors to include more recent published studies on the relation between M.genitalium and infertility. Any additive discussion would enhance the importance of the reported study's findings.  
Example of new published study:  
Eman A. Abusaraha, Ziad M.Awwadb, Ekatherina Charvalosc, and Asem A. Shehabi. Molecular Detection of potential sexually transmitted pathogens in semen and urine specimens of infertile and fertile males. Diagnostic Microbiology and Infectious Disease, 77 (2013) 283–286

| REVIEWER            | Marko Potočnik  
Dept. of Dermatovenerology, University Medical centre Ljubljana, Ljubljana, slovenia |
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<td>REVIEW RETURNED</td>
<td>05-May-2014</td>
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The reviewer completed the checklist but made no further comments.

| REVIEWER            | Helle Friis Svenstrup  
Awapatent A/S  
Denmark |
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<td>REVIEW RETURNED</td>
<td>30-Jun-2014</td>
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**GENERAL COMMENTS**

This study investigates the prevalence of Mycoplasma genitalium (MG) in urine samples from infertile men and healthy controls in Croatia. The number of positive samples were low (2/145 – 1.4%) but the PCR method used (e.g. using MgPa 1-3 primers) is very
suitable for the identification of MG since a high sensitivity and specificity can be achieved using this primer set. In addition, this PCR method has been used in many clinical studies and thus, makes the studies more comparable. Also, importantly, internal controls were used to exclude PCR inhibition in the samples. The positive samples were confirmed with a different PCR test, which in addition gave information on resistance to azithromycin. The study is based on small numbers of participants but the study is relevant since previous literature suggests that mycoplasmas may cause infertility in men and women but only few studies on this specific topic are available.

I do have a few questions to the study below:

This clinical study is well designed and excludes the most prevalent other STIs. Furthermore, clinical information regarding Tubal Factor Infertility (TFI) in the female partner was also included in the study. None of the female partners had TFI – however, how was this diagnosed, e.g. which method was used to measure tubal occlusion or the lack thereof?

It was not clear from the study how much urine in total was taken from the patients and how it was stored until testing for MG, CT, NG and TV? Also what was the original volume of urine used for MG PCR (and the other tests)?

Also, it is not clear, which PCR instrument was used in the Croatian lab since the reference (7) (on page 6, l. 8) is a reference for the Danish lab where all samples were re-tested and confirmed. Was the bacterial load of MG measured? This information would be of interest since the patients are asymptomatic.

The two MG positive sample from infertile men - did the men have the same type/level of abnormal semen?

Among the 13 excluded patients – how many of these were CT, NG and TV positive and co-infected? – and did you test for MG in these samples too?

Were there any co-infections of MG, Ureaplasma and M. hominis in the samples included for the study? Did you perform as specific test for the Ureaplasma sp?, e.g. U. parvum and U. urealyticum? As stated in the article Ureaplasma and M. hominis may be considered as part of the normal flora but there may be a difference in the pathogenicity of Up and Uu.

In general, the level of STI infections in men and women attending fertility work-up is low since the number of current sexual partners usually is low. I think the expected prevalence of Mycoplasma genitalium should be discussed more with more comparisons to the literature. Since the data on MG is scarce, it may be interesting and most relevant to make a comparison to the prevalence of Chlamydia in infertile men.

Spelling errors: Abstract p. 2 l. 4 “than” instead of “then”

2. Reviewer 1 “to include more recent published studies on the relation between M. genitalium and infertility”.

Answer: The role of M. genitalium (MG) in male infertility is not known yet, due to the small number of well-designed studies. We tried to design the study in which all other potential infective causes of infertility were excluded. In recent studies the prevalence of MG in infertile men were almost similar: 3,2% was in the study of Abusaraha et al (2013), and 4,8% in the study from Gdoura et al (2008).

Abusaraha EA, Awwadb ZM, Charvalosc E, Shehabi AA. Molecular Detection of potential sexually
transmitted pathogens in semen and urine specimens of infertile and fertile males. Diagnostic Microbiology and Infectious Disease 2013;77: 283–286.

3. Reviewer 2 had no questions nor remarks.

4. Reviewer 3:
   a. Tubal Factor Infertility – how it was diagnosed, which method was used to measure tubal occlusion or the lack thereof?
   Answer: Tubal Factor Infertility in female partners was excluded using hysterosonosalpingography (HSSG).

   b. How much urine in total was taken from the patients and how it was stored until testing for MG, CT, NG and TV? What was the original volume of urine used for MG PCR and the other tests?
   Answer: In total, about 20ml of FVU was taken from the patients; 4-5ml of each sample was used for culture of MH, ureaplasmas and TV. For PCR detection of MG, CT and NG 4-5 ml of each sample was used. Five ml of original FVU were frozen and shipped to Staten Serum Institut in Copenhagen for confirmation by real-time MG PCR. Samples were immediately processed for TV, MH and ureaplasmas. For PCR detection samples were stored at -20°C.

   d. Which PCR instrument was used in the Croatian lab?
   Answer: The PCR was performed in an automated DNA thermal cycler (PCR System 9700, Applied Biosystem)

   e. Was the bacterial load of MG measured?
   Answer: In our laboratory we used conventional in-house PCR (qualitative) and results were confirmed in Staten Serum Institut in Copenhagen, Denmark by real-time PCR. M.genitalium load for two positive samples were 778 c/ml and 6765 c/ml, respectively.

   f. The two MG positive sample from infertile men - did the men have the same type/level of abnormal semen?
   Answer: In the men with M.genitalium load of 778 c/ml was diagnosed oligozoospermia, and in the other (M.genitalium load of 6765 c/ml) was diagnosed asthenozoospermia.

   g. Among the 13 excluded patients – how many of these were CT, NG and TV positive and co-infected? – and did you test for MG in these samples too? –
   Answer: Among the 13 excluded patients one patient had NG infection, in one patient was diagnosed TV, and ten patients were CT positive. These samples were also tested for MG, but all were negative.

   h. Were there any co-infections of MG, Ureaplasma and M. hominis in the samples included for the study?
   Answer: There were coinfections of Ureaplasma spp and M.hominis. In the group of infertile men 43 Ureaplasma spp and 31 M.hominis were found. In twelve samples among infertile men were found coinfections of Ureaplasma spp and M.hominis. In the group of the asymptomatic men coinfection were found in three samples.
   In two samples with positive MG, taken from infertile men, no other pathogens were found.

   i. Did you perform as specific test for the Ureaplasma sp?
   Answer: No, we did not perform specific test for the Ureaplasma spp. Most of the published studies
have reported the prevalence of ureaplasmas in infertile men without discriminating between U. urealyticum and U. parvum. The literature data are not conclusive about the prevalence of Uu and Up. In the study of Abusarah et al. (2013), was found that Up was the most prevalent isolate detected among infertile men (90%).

j. The expected prevalence of Mycoplasma genitalium should be discussed more with more comparisons to the literature. Since the data on MG is scarce, it may be interesting and most relevant to make a comparison to the prevalence of Chlamydia in infertile men.

Answer: MG is well established cause of NGU and it existed a lot of literature data about this topic. The role of MG in infertility is not known and it lacks a lot of well designed studies. We tried to design the study in which all other potential infective causes of infertility were excluded. According to the literature data, the prevalence of MG in men with NGU ranges from 13-42%. However, the prevalence of MG in men with infertility is very low, depending of the study eg. 3.2% as in the study of Abusarah et al. (2013) and 4.8% in the study of Gdoura et al. (2008).


Abusaraha EA, Awwadb ZM, Charvalosc E, Shehabi AA. Molecular Detection of potential sexually transmitted pathogens in semen and urine specimens of infertile and fertile males. Diagnostic Microbiology and Infectious Disease 2013;77: 283–286.

The similar findings are with CT, which is the most often bacterial cause of NGU. C. trachomatis is in women well established cause of tubal factor infertility. In men it causes NGU. Also, it is clearly demonstrated that CT attached to spermatozoa (on the surface and in the nucelous, as well). However, its role in male infertility, as well as the role of MG, is not clear yet. There are significant differences in the prevalence of CT infections in men with infertility and it varies from 0 % to 42.3%, depending of the methodology, type of sample and differences of infection rates in different populations. In the recent published Canadian study (Samplaski et al 2014), the prevalence of CT infection studied on 5588 infertile men, was 0.3%. The author concluded that this low prevalence clearly demonstrates that small proportion of male infertility is caused by CT.


k. Spelling errors: Abstract p. 2 l. 4 “than” instead of “then”
Answer: Corrected.

| REVIEWER | Helle Friis Svenstrup  
| Denmark |
| REVIEW RETURNED | 28-Jul-2014 |

| GENERAL COMMENTS | The authors have responded adequately to comments and questions and implemented the answers into the manuscript, thus, I have no further comments. |