INTRODUCTION

Recent reports from ECDC show a 22% rise in new cases of Neisseria gonorrhoeae (NG) infection within the UK, presenting a need for accurate diagnosis of cases (ECDC, 2018). Nucleic acid amplification testing have been shown to be highly accurate and specific for the detection of NG from swab and urine samples. However, developing an External Quality Assessment (EQA) program on any diagnostic molecular target is challenging. EQA reports from 2010-2013 showed that participants targeting 16S rRNA of NG reported false negative results for NG positive samples. With more and more laboratories using this assay in question (Figure 1) this issue needed a solution. It was assumed that the problem of not detecting the target was due to the degradation of the rRNA.

AIM AND OBJECTIVES

Aim: This study aimed to determine the NG 16S rRNA degradation rate at different storage conditions.

Objectives:
1. To prepare an NG positive simulated urine bulk.
2. Dispensed samples to be stored at different storage conditions
3. To determine the RNA degradation rate using qRT-PCR
4. To determine design criteria for simulated urine EQA samples

METHODS

Sample Preparation:
A clinical bacterial isolate of NG (referred to as neat) was diluted 4500x in simulated urine to make a bulk. Aliquots were made from this bulk and were stored at -20°C, +4°C, RT and at +37°C.

Nucleic acid extraction
Every 2 weeks (over a 12 week period), 400μL specimens underwent total nucleic acid extraction via the MagNa Pure Compact automated extraction platform. The eluate was stored at -80°C.

RNA quantification and reverse transcription (RT)
Eluates underwent RNA and DNA quantification by spectrophotometer and thereafter DNA was removed by enzymatic digestion. RT was performed by using the Quantitect reverse transcription assay (Qiagen).

Amplification:
SYBR Green I (Qiagen) was used for PCR amplification and for the detection of the target sequence. Specific primers based on Boel et al. 2005 (Figure 3) were used.

PCR conditions:

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Initial Denaturation</th>
<th>Final Annealing</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>95°C 20s</td>
<td>50°C 30s</td>
<td>68°C 30s</td>
</tr>
<tr>
<td>RNA</td>
<td>95°C 20s</td>
<td>60°C 30s</td>
<td>72°C 30s</td>
</tr>
</tbody>
</table>

RESULTS

Figure 4. PCR optimization
The original neat samples were used for PCR optimization. Fluorescence was detected in processed water tube which was due to primer dimer formation.

Figure 5. RNA degradation rate results
To determine the cause of the primer dimer formation RT was carried out by using the forward primer separately. Processed water was run as a negative control. Melting curve analysis confirmed the presence of primer dimers when the the reverse primer was used. There were no primer dimers when the forward primer was used for RT.

Figure 6. New primer and probe design
New primers (forward primer in blue and reverse primer in purple) and a probe (5’ FAM BHQ1, red) were designed by using Primer3. The expected amplicon size was 221 base pairs.

DISCUSSION AND FUTURE WORK

- 45μL of simulated urine bulk was prepared and 90 specimens were dispensed in 0.5μL aliquots.
- 22 specimens were stored at each storage conditions and were used for PCR analysis.
- The RNA degradation was determined and we found that the RNA was only stable if stored at -20°C and +4°C.
- EQA samples must be prepared from freshly cultured NG to achieve a higher initial RNA concentration. Based on the RNA degradation rate, the minimum quantity of RNA must be determined guaranteeing that the RNA will be detectable over the distribution period.

Future work:
- Optimize PCR conditions with the newly designed primers and probe.
- Samples need to be stored beyond the 12 week period if stored at -20°C and +4°C to determine long term RNA stability.
- UK NEQAS will dispatch a simulated urine sample based on this study findings in the next molecular detection scheme for Neisseria gonorrhoeae. Results from participants will be analysed and compared with in-house test results.

ACKNOWLEDGEMENTS

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REFERENCES