Assessment of multiplex real-time PCR against nested PCRs and DFA for the detection of Giardia, Cryptosporidium and *Entamoeba histolytica* in sewage

CM Vassalos*, A Charlett ¹, E Vassalou ², A Biba ³, G Dounias ², G Tzanakaki ², A Mavridou ⁴, G Spanakos³

¹ Public Health England; ² National School of Public Health, Athens; ³ Hellenic Centre for Disease Control & Prevention; ⁴ Technological Educational Institute of Athens

* European Programme for Public Health Microbiology, ECDC
Introduction

- Giardia Cryptosporidium and *Entamoeba histolytica*
  - Intestinal protozoa
  - Waterborne outbreaks worldwide

- Sewage
  - Re-use (irrigation)
  - Dispersion of intestinal protozoa

- Detection of intestinal protozoa in sewage
  - Microscopy
  - Molecular tools are increasingly applied
  - No reference method
Aim

- To verify the possibility of using multiplex real-time PCR as an alternative to
  - Direct immunofluorescence assay
  - Nested PCRs

- for the detection of Giardia, Cryptosporidium and *E. histolytica* in sewage
Sewage sampling

Sewage samples \((n=73)\)

### Characteristics of sewage treatment plants

<table>
<thead>
<tr>
<th>STP</th>
<th>Coordinates</th>
<th>Area</th>
<th>Served population (peak)</th>
<th>Capacity_{max} (m^3/day)</th>
<th>Decontamination</th>
<th>Further treatment</th>
<th>Irrigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>38°23'40.2&quot;N 22°55'49.62&quot;E</td>
<td>Rural</td>
<td>25,000</td>
<td>5,000</td>
<td>Yes</td>
<td>No</td>
<td>Crops</td>
</tr>
<tr>
<td>R2</td>
<td>38°28'48.001&quot;N 22°35'3.72&quot;E</td>
<td>Rural</td>
<td>4,500</td>
<td>2,200</td>
<td>Yes</td>
<td>No</td>
<td>Gardens</td>
</tr>
<tr>
<td>U</td>
<td>38°14'47.902&quot;N 21°44'4.466&quot;E</td>
<td>Urban</td>
<td>199,572</td>
<td>43,075</td>
<td>Yes</td>
<td>Sand filtration</td>
<td>Urban parks</td>
</tr>
</tbody>
</table>
Sample processing

Sample collection
- 2-L sample at each sampling site

Concentration
- Alum flocculation

Storage
- 200 μL aliquots of final pellet at -80 °C

DNA extraction
- QIAamp DNA Mini Kit

Molecular Protozoa detection
- Nested PCRs
- Multiplex Real-time PCR
- RIDA®GENE Parasitic Stool Panel II

Direct Immunofluorescence
- Merifluor®Cryptosporidium/Giardia
## Assessment of multiplex real-time PCR

### Multiplex real-time PCR

<table>
<thead>
<tr>
<th>Protozoa</th>
<th>Simultaneous detection</th>
<th>Internal control (inhibition detection)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giardia</td>
<td></td>
<td></td>
<td>Quantitative (Ct-values)</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Direct immunofluorescence microscopy vs Nested PCRs

<table>
<thead>
<tr>
<th>Detection</th>
<th>Results</th>
<th>Published 2-step PCR protocols</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giardia</td>
<td>No of (oo)cysts</td>
<td>Giardia, Cryptosporidium, <em>E. histolytica</em></td>
<td>Pos/Neg</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
<td><a href="2002">Read</a>, <a href="2003">Nichols</a>, <a href="2000">Evangelopoulos</a></td>
<td></td>
</tr>
</tbody>
</table>
## Multiplex real-time PCR validation

### Analytical methodology

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Statistic</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Validity</strong></td>
<td>Sensitivity</td>
<td>$Se = \frac{TP}{TP+FN}$</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>$Sp = \frac{TN}{TN+FP}$</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>ROC AUC</td>
<td>$Se = f(1-Sp)$</td>
</tr>
<tr>
<td><strong>Reliability</strong></td>
<td>(% Agreement</td>
<td>(Concordance of results/No of samples) x 100</td>
</tr>
<tr>
<td></td>
<td>Kappa coefficient</td>
<td>$\kappa = 1-((1-p_o)/(1-p_e))$</td>
</tr>
</tbody>
</table>
## Results

### Results of multiplex real-time PCR

<table>
<thead>
<tr>
<th>Protozoa</th>
<th>Ct-values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detected</td>
<td>Undetermined</td>
</tr>
<tr>
<td>Giardia</td>
<td>50</td>
<td>18</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td>2</td>
<td>66</td>
</tr>
</tbody>
</table>

| Inhibition    | 5         |
## Cryptosporidium detection in sewage

### Comparison of multiplex real-time PCR with direct immunofluorescence

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>DFA</th>
<th>Validity</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>Pos</td>
<td>0</td>
<td>0</td>
<td>0.95</td>
</tr>
<tr>
<td>Neg</td>
<td>4</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>64</td>
<td>68</td>
</tr>
</tbody>
</table>

### Comparison of multiplex real-time PCR with nested PCR

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>Nested PCR</th>
<th>Validity</th>
<th>Reliability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>(%)</td>
</tr>
<tr>
<td>Pos</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Neg</td>
<td>10</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>56</td>
<td>68</td>
</tr>
</tbody>
</table>
Cryptosporidium detection in sewage

Multiplex real-time PCR against

- Direct immunofluorescence:
  - Chance agreement.
  - Cryptosporidium oocysts-like bodies (e.g. algae) can be seen in direct immunofluorescence

- Nested PCR:
  - Fair agreement.
  - Multiplex real-time PCR that we employed detected a limited number of Cryptosporidium species compared to nested PCR
  - Multiplex real-time PCR detected Cryptosporidium species of Public Health importance
**E. histolytica detection in sewage**

### Comparison of multiplex real-time PCR with direct immunofluorescence

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>(\kappa)</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DFA for E. histolytica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Comparison of multiplex real-time PCR with nested PCR

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>Nested PCR</th>
<th>Validity</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos</td>
<td>Neg</td>
<td>Total</td>
</tr>
<tr>
<td>Pos</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Neg</td>
<td>2</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>64</td>
<td>68</td>
</tr>
</tbody>
</table>
E. histolytica detection in sewage

Multiplex real-time PCR against

- Nested PCR:
  - Substantial agreement
  - Multiplex real-time PCR possibly had lower sensitivity than nested PCR
  - Or false positive results were obtained with nested PCR*

* In nested PCR that we used, [50- cycles x2] were run
Cutoff Ct-value for Giardia in sewage

$$Pr(\text{nPCR +ve} \mid \text{Ct}) = f(\text{Ct}) = \frac{1}{1+e^{-(c+\beta \cdot \text{Ct})}}$$

Ct-value of $$\frac{c}{-\beta} = \frac{19.963}{0.531} = 37.6$$
## Comparison of multiplex real-time PCR with direct immunofluorescence

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>Direct immunofluorescence</th>
<th>Validity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>59</td>
</tr>
</tbody>
</table>

Giardia detection in sewage
**Giardia detection in sewage**

Comparison of multiplex real-time PCR with direct immunofluorescence

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplex real-time PCR</td>
<td>93</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>DFA</td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

**AUC:** 0.762

**Accuracy:** Good
## Giardia detection in sewage

### Comparison of multiplex real-time PCR with nested PCR

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>Nested PCR</th>
<th>Validity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>36</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>25</td>
</tr>
</tbody>
</table>

This table compares the results of multiplex real-time PCR with nested PCR in detecting Giardia in sewage. The sensitivity of the multiplex real-time PCR is 0.84, and the specificity is 0.80.
Comparison of multiplex real-time PCR with nested PCR

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplex real-time PCR</td>
<td>36</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Validity</td>
<td>0.84</td>
<td>0.80</td>
<td>0.82</td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>Specificity</td>
<td></td>
<td></td>
<td>0.80</td>
</tr>
</tbody>
</table>

Giardia detection in sewage

AUC: 0.921

Accuracy: Excellent
## Reliability of multiplex real-time PCR

### Comparison of multiplex real-time PCR with DFA

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>DFA</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>

### Comparison of multiplex real-time PCR with nested PCR

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
<th>Nested PCR</th>
<th>Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>36</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>25</td>
</tr>
</tbody>
</table>
Multiplex real-time PCR against

- Direct immunofluorescence:
  - Good accuracy.
  - Slight agreement
  - Only in samples with high Giardia load - reflected by low Ct-values-, Giardia cysts were seen in direct immunofluorescence

- Nested PCR:
  - Excellent accuracy.
  - Substantial agreement
  - In samples with Ct-values between 35 and 37.6 (cutoff), nested PCR gave negative results
To sum up

<table>
<thead>
<tr>
<th>Direct immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experienced microscopists are required to detect Giardia cysts and Cryptosporidium oocysts</td>
</tr>
<tr>
<td>Unable to discriminate between protozoan (oo)cysts and (oo)cyst-like bodies</td>
</tr>
<tr>
<td>Time-consuming</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nested PCRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three classic nested PCRs (3x2) are usually required for detection of the three parasites</td>
</tr>
<tr>
<td>Electrophoresis is necessary for PCR-product visualization</td>
</tr>
<tr>
<td>Time-consuming</td>
</tr>
</tbody>
</table>

while

<table>
<thead>
<tr>
<th>Multiplex real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>High output system</td>
</tr>
<tr>
<td>Quantification</td>
</tr>
<tr>
<td>Fast and effortless</td>
</tr>
</tbody>
</table>
At least for *Giardia* presence in sewage

- For *Cryptosporidium*, *E. histolytica*:
  Further studies in settings with higher protozoan load
Multiplex real-time PCR has a potential to be an efficient alternative to direct immunofluorescence or nested PCRs for Giardia, Cryptosporidium and Entamoeba histolytica in sewage.

Conclusion

To minimize the public health risk posed by sewage reuse regarding Giardia, Cryptosporidium and *Entamoeba histolytica* in sewage.
Thank you
Ευχαριστώ
Thank you
Ευχαριστώ