**Is It Useful To Treat *Blastocystis* sp.? A double-blind placebo-controlled randomised trial.**

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Supplementary document

**Intestinal Protozoa real-time PCR Panel**

## **Background**

The intestinal protozoa real-time PCR Panel consists of six duplex real-time PCR reactions and one simplex reaction, all based on TaqMan Probe Assays. All assays were validated at the National Reference Centre for Parasitology at SwissTPH, Basel.

## **Methods: assay set-up**

Primer and Probes with their references are mentioned in table 1 as well as reaction set-up in table 2, 3 and 4. The thermoprofile for each reaction was identical and is shown in table 5. All the assays were initially set-up on specific plasmids containing an insert of the corresponding sequence as well as adjacent base pairs and an “AGTC” insert for identification (and contamination control). Details on plasmids, the real-time efficiency and the analytical sensitivity is shown in table 6.

Sensitivity was further tested by at least 5-10 samples (depending on parasite and availability), except for *E. polecki, E. moshkovskii* and *D. fragilis.* For *E. polecki* no confirmed positive case was available at time of validation (only plasmid and literature), for *E. moshkovskii* only 1 culture sample was available (kindly received from Clark Graham, LSTMH) and for *D. fragilis* only 2 confirmed samples were available (kindly received from Lisette van Lieshout, LUMC). Diagnostic sensitivity was found 100% for all assays (table 6). For specificity, a panel of 15-20 FNA from stool samples with parasites including: *Blastocystis* spp*, Endolimax nana, Entamoeba dispar, Entamobea histolytica, Entamoeba hartmanni, Entamoeba coli, Entamoeba invadens, Giardia lamblia, Dientamoeba fragilis, Ascaris lumbricoides, Chilomastix mesnili, Strongyloides stercoralis, Hymenolepis nana, Entamoeba moshkovskii, Enterocytozoon bieneusi, Encephalitozoon* spp.*, Cryptosporidium* *hominis/parvum, Cyclospora cayetanensis, Cystoisospora belli, Iodamoeba bütschlii, Schistosoma mansoni,* and were found to amplify only the corresponding species, except for *Blastocystis. Blastocystis* real-time PCR was found positive in 11 of 24 *Blastocystis* microscopy negativesamples (table 6). However, sequencing of the 18S rRNA gene confirmed the presence of *Blastocystis* in all these 11 samples. Therefore, all assays were 100% specific (table 6).

**Table 1. Primer and Probe sequences with reference literature**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Primer** | **Type** | **Probe label** | **Sequenz von 5` zu 3`** | **Size of product (bp)** | **Reference** |
| **Duplex 1: *Entamoeba dispar* and *E. histolytica* 18S qPCR** | | | | |  |  |
|  | Ent\_F | Forward |  | AGGATTGGATGAAATTCAGATGTACA | 153 | Inspired by Troll 1997 |
|  | Ent\_R | Reverse |  | TAAGTTTCAGCCTTGTGACCATAC |  |  |
| *E. dispar* | ED\_P | Probe 1 | HEX-BHQ1 | TGAAGAAACATTGTTTCTAAATCCAAGT |  |  |
| *E. histolytica* | EH\_P | Probe 2 | FAM-BHQ1 | AGAGAAGCATTGTTTCTAGATCTGA |  |  |
|  |  |  |  |  |  |  |
| **Duplex 2: *Cryptosporidium* spp.18S qPCR** | | | | |  |  |
|  | Cry\_F | Forward |  | ACATGGATAACCGTGGTAATTCT | 186 | Inspired by Mary 2013 |
|  | Cry\_R | Reverse |  | CAATACCCTACCGTCTAAAGCTG |  |  |
| *Cryptosporidium* spp. | CryPan\_P | Probe 1 | HEX-BHQ1 | GTGACATATCATTCAAGTTTCTGACCT |  |  |
| *C. hominis/C. parvum* | CryHP\_P | Probe 2 | FAM-BHQ1 | ACTCGACTTTATGGAAGGGTTGTAT |  |  |
|  |  |  |  |  |  |  |
| **Duplex 3: *Microsporidium* sp.18S qPCR** | | | | |  |  |
|  | Mic\_F | Forward |  | CAGGTTGATTCTGCCTGAC |  | Inspired by Notermans 2005 |
| *Encephalitozoon* spp. | Mic\_R1 | Reverse |  | CTATCACTGAGCCGTCCG | 97 |  |
| *E. bieneusi* | Mic\_R2 | Reverse |  | CAACATTACTGAGCCGTTCG | 98 |  |
| *E. bieneusi* | MicEBV\_P | Probe 1 | FAM-BHQ1 | GTCTCT**R**AGATTAAGCCATGCA |  |  |
| *Encephalitozoon* spp. | MicENC\_P | Probe 2 | HEX-BHQ1 | TTCTCTGGG**R**CTAAGCCATGC |  |  |
|  |  |  |  |  |  |  |
| **Duplex 4: *E. moshkovskii* and *E. polecki* 18S qPCR** | | | | |  |  |
| *E. moshkovskii* | EM\_F | Forward 1 |  | TGGTTAGTAAAGTACAAGGATAGCTTT | 187 | Inspired by Verweij 2003 |
|  | EM\_R | Reverse 1 |  | GATCAGAAATTC TCATTGGTTACTTGT |  |  |
|  | EM\_P | Probe 1 | FAM-BHQ1 | AGTCGGCCACTCTCTTCACG |  |  |
| *E. polecki* | EP\_F | Forward 2 |  | AACTGTTTTAAATATCTGACCTATCAACT | 91 | Inspired by Stensvold 2011 |
|  | EP\_R | Reverse 2 |  | GAAATCAAACCCTTATTTCTCGTTAC |  |  |
|  | EP\_P | Probe 2 | HEX-BHQ1 | GTATGATAGAGGCATACC**Y**AAGTGATAACG |  |  |
|  |  |  |  |  |  |  |
| **Duplex 5: *D. fragilis* and *G. lamblia* 18S qPCR** | | | | |  |  |
| *D. fragilis* | DF\_F | Forward 1 |  | CAACGGATGTCTTGGCTCTT | 109 | Modified from Verweij 2007 |
|  | DF\_R | Reverse 1 |  | AATACGCAATGTGCATTCAAAG |  |  |
|  | DF\_P | Probe 1 | HEX-MGB-EQ | CAATTCTAGCCGCTTAT |  |  |
| *G. lamblia* | GL\_F | Forward 2 |  | GACGGCTCAGGAC**R**ACGGT | 76 | Modified from Verweij 2003 |
|  | GL\_R | Reverse 2 |  | GCTGCGTCACGCTGCTC |  |  |
|  | GL\_P | Probe 2 | FAM-BHQ1 | CCCGCGGCGGTCCCTGCTAG |  |  |
|  |  |  |  |  |  |  |
| **Duplex 6: *Cyclospora cayetanensis* and *Cystoisospora belli* 18S qPCR** | | | | |  |  |
| *C. cayetanensis* | CC\_F | Forward 1 |  | CAGTTTCGAGGTAGTGACGAG | 98 | Inspired by Verweij 2003 |
|  | CC\_R | Reverse 1 |  | TCCAATTGTTACTCTGGAAGGAT |  |  |
|  | CC\_P | Probe 1 | HEX-BHQ1 | TGCTTTGTAATTGGAATGATAGGAATT |  |  |
| *C. belli* | CB\_F | Forward 2 |  | ATATTCCCTGCAGCATGTCTGTTT | 90 | Ten Hove 2008 |
|  | CB\_R | Reverse 2 |  | CCACACGCGTATTCCAGAGA |  |  |
|  | CB\_P | Probe 2 | FAM-BHQ1 | CAAGTTCTGCTCACGCGCTTCTGG |  |  |
|  |  |  |  |  |  |  |
| **Simplex 7: *Blastocystis* sp. 18S qPCR** | | | | |  |  |
| *Blastocystis* sp. | BH\_F | Forward |  | GGTCCGGTGAACACTTTGGATTT | 119 | Stensvold 2012 |
|  | BH\_R | Reverse |  | CCTACGGAAACCTTGTTACGACTTCA |  |  |
|  | BH\_P | Probe | FAM-MGB-Q5 | TCGTGTAAATCTTACCATTTAGAGGA |  |  |

**Table 2. Reaction of Duplex 1, 2 and 3**

|  |  |  |
| --- | --- | --- |
| **Reagents** | **Concentration** | **Reaction** |
| H2O |  | 4 µl |
| GeneExpression Mastermix (Thermofisher) | 2x | 12.5 µl |
| Primer F+R | 10µM each | 2 µl |
| Probe P1 | 10µM | 0.75 µl |
| Probe P2 | 10µM | 0.75 µl |
| **DNA** |  | **5 µl** |
| **Total** |  | **25 µl** |

**Table 3. Reaction of Duplex 4, 5 and 6**

|  |  |  |
| --- | --- | --- |
| **Reagents** | **Concentration** | **Reaction** |
| H2O |  | 4.6 µl |
| GeneExpression Mastermix (Thermofisher) | 2x | 12.5 µl |
| Primer F1+R1 | 50µM each | 0.7 µl |
| Primer F2+R2 | 50µM each | 0.7 µl |
| Probe P1 | 10µM | 0.75 µl |
| Probe P2 | 10µM | 0.75 µl |
| **DNA** |  | **5 µl** |
| **Total** |  | **25 µl** |

**Table 4. Reaction of Simplex 7**

|  |  |  |
| --- | --- | --- |
| **Reagents** | **Concentration** | **Reaction** |
| H2O |  | 5 µl |
| GeneExpression Mastermix (Thermofisher) | 2x | 12.5 µl |
| Primer F+R | 10µM each | 2 µl |
| Probe P | 10µM | 0.5 µl |
| **DNA** |  | **5 µl** |
| **Total** |  | **25 µl** |

**Table 5. Thermoprofile for all reactions**

|  |  |  |  |
| --- | --- | --- | --- |
| **Stage** | **Temperatur** | **Zeit** | **Zyklen** |
| 1 | 50°C | 2 min. | 1 |
| 2 | 95°C | 10 min. | 1 |
| 3 | 95°C | 15 sec. | 40 |
| 58°C | 1 min. |

**Table 6. Plasmids and real-time PCR efficiency, sensitivity and specificity**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Assay | NCBI sequence of plasmid or Patient | AGTC tag | Efficiency [%] | Analytical sensitivity [plasmids/ul] | Sensitivity (n) [%] | Specificity (n) [%] |
| *E. dispar* | Patient 13381 | No | 96.4 | 10 | >99.9 (16/16) | >99.9 (0/29) |
| *E. histolytica* | Patient 17261 | No | 101.8 | 10 | >99.9 (10/10) | >99.9 (0/29) |
| *Cryptospiridium spp.* | AF222998.1 | Yes | 96.2 | 1 | >99.9 (3/3) | >99.9 (0/31) |
| *C. hominis/parvum* | AF222998.1 | Yes | 95.1 | 1 | >99.9 (3/3) | >99.9 (0/31) |
| *E. hellen* | Patient P1082 | No | 91.4 | 1 | 85 (17/20) | >99.9 (0/32 |
| *E. bieneusi* | Patient MA11-2082 | No | 100.6 | 1 | >99.9 (4/4) | >99.9 (0/32) |
| *E. moshkovskii* | SNAKE-I3 | No | 91.5 | 1 | ND | >99.9 (0/22) |
| *E. polecki (E. chattoni)* | AF149912.1 | Yes | 100.8 | 1 | ND | >99.9 (0/31) |
| *D. fragilis* | DQ233442 | Yes | 100.1 | 1 | >99.9 (2/2)4 | >99.9 (0/14) |
| *G. lamblia* | KT048492.1 | Yes | 103.7 | 1 | >99.9 (2/2) | >99.9 (0/17) |
| *C. cayetanensis* | AF111183.1 | Yes | 99.8 | 1 | >99.9 (2/2) | >99.9 (0/18) |
| *C. belli* | DQ06059 | Yes | 101.9 | 1 | >99.9 (5/5) | >99.9 (0/20) |
| *B. hominis* | KY610205.1 | Yes | 92.1 | 10 | 90.5 (57/63) | >99.9 (0/36)5 |

**1** Culture material originate from the Swiss TPH Diagnostic Center.

2 Culture material was kindly received from F. Grimm at the Institut für Parasitologie in Zürich, Switzerland.

3 Culture material was kindly received from Clark Graham at the LSTMH in London, England.

4 Confirmed cases kindly received from L. Lieshout, LUMC in Leiden, The Netherlands.

5 Real-time PCR cut-off at 35 CT. Samples with CT >35 were methodologically not possible to be confirmed by sequencing during validation process.

## **DNA Extraction and sample analysis by real-time PCR**

A stool aliquot of approximately 1g of each patient at baseline and follow-up was frozen at -80°C till molecular analysis. Thawed samples were then extracted using a modified protocol of the Maxwell RSC Blood Kit (Promega). In brief, 100mg stool were added to 600µl lysis buffer (kit), vigorously vortexed and incubated for 5min at 95°C. After short spin down, 40µl Proteinase K was added before incubation at 56°C for 20min. The sample was then centrifuged at 13000rpm for 2min and 300µl of the supernatant was added to the Maxwell RSC blood cartridge. 300µl H2O were also added to the well 1 of the cartridge and the Maxwell RSC blood protocol was run. The DNA was eluted in 100µl Elution Buffer.

All samples from the present study were run on duplicate for each of the mentioned assays above. Inhibition of reaction was tested for amplification for each sample separately by adding 2µl of 103 copies/µl *E. dispar* and *E. histolytica* plasmid to the reaction. 6/52 samples showed an inhibited amplification. DNA extraction of these samples was repeated according to Barda *et al.* (2018).

## ***Blastocystis* spp subtype differentiation.**

Subtypes of *Blastocystis* spp were defined by sequencing of the 18S rRNA gene according to Scicluna *et al.* (2006) with minor modification in the reaction set-up and thermoprofile (table 7 and 8). For PCR, DNA samples were diluted 1:10. Sequencing products were then aligned against reference phylogeny of Stensvold *et al.* (2007) to determine the subtype groups.

**Table 7. Reaction set-up for PCR**

|  |  |
| --- | --- |
| **Reagents** | **volume** |
| H2O | 8.5 µl |
| Qiagen HotStarTaq Plus Master mix Buffer 2x | 12.5 µl |
| Primer BH\_SciF 10 µM | 2 µl |
| Primer BH\_SciR 10 µM | 2 µl |
| **DNA 1:10** | **4 µl** |
| Total | 25 µl |

**Table 8. Thermoprofile for PCR**

|  |  |  |  |
| --- | --- | --- | --- |
| **Stage** | **Temperature** | **Time** | **Cycle** |
| 1 | 95°C | 5 min. | 1 |
| 2 | 94°C | 40 sec. |  |
| 58°C | 1 min. | 40 |
|  | 72°C | 40 sec. |  |
| 3 | 72°C | 10 min. | 1 |
| 4 | 16°C | konstant | 1 |

## **Literature**

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