Deliverable D-JRP-PARADISE-WP2.1

Protocol for 18S rDNA-based amplicon sequencing for detection of relevant FBPs

JRP19-ET1.1-PARADISE

Responsible Partners: P13-SSI

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PROTOCOL FOR 18S RDNA-BASED AMPLICON SEQUENCING FOR DETECTION OF RELEVANT FBPS

BACKGROUND

This is a public deliverable of One Health EJP Joint Research Project:
PARADISE: Parasite Detection, Isolation and Evaluation

Work Package:
JRP-PARADISE-WP2 NGS-based genomics and metagenomics

Task:
JRP-PARADISE-WP2-T3 Experimental amplicon-based and shotgun metagenomics for detection of foodborne parasites

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PARADISE aims to deliver informative typing schemes and innovative detection strategies applicable to food matrices for both parasites. Using NGS technologies (genomics and metagenomics), the project will generate much needed data that will enrich our understanding of the epidemiology and genomics of these organisms, and provide the basis on which improved strain-typing schemes will be developed and rigorously tested. In parallel, strategies (nanobodies, aptamers, use of hybridization probes) to enrich for the target pathogens in different matrices will also be developed and tested. Furthermore, PARADISE will engage in multicentre studies to validate the newly developed methods, testing their applicability across the spectrum of relevant matrices in an unprecedented effort at the EU level. These new methodologies will form the basis for integrated approaches aimed at controlling FBPs in the European food chain.

WP2 of PARADISE aims at 1) generating novel genome data from selected isolates of *C. parvum* and *G. duodenalis* (WP2-T1); 2) using in silico approaches and a referenced database to interrogate available metagenomes to detect foodborne parasites (WP2-T2); and 3) using amplicon-based and shotgun metagenomics approaches to detect target parasites using spiked food matrices (WP2-T3).

This deliverable briefly sums up the protocol for 18S rDNA-based amplicon sequencing for detection of relevant FBPs.

GOAL

To optimise a platform based on amplicon-based next-generation sequencing for detecting parasites, with special emphasis on foodborne parasites.

METHOD DESCRIPTION

The principle of the method relies on amplification of ribosomal DNA from eukaryotic organisms (18S). The method is part of a 16S/18S platform.

Ribosomal gene amplification and sequencing

18S rRNA gene segments are amplified by PCR using a total of three primer sets G3F1/G3R1 (5'-GCCAGCAGCCGCGGTAATTC-3' / 5'-ACATTCTTGGCAAATGCTTTCGCAG-3'), G4F3/G4R3 (5'-CAGCCGCGGTAATTCCAGCTC-3' / 5'-GGTGGTGCCCTTCCGTCAAT-3') and G6F1/G6R1 (5'-TGGAGGGCAAGTCTGGTGCC-3' / 5'-ACGGTATCTGATCGTCTTCGATCCC-3'). G3 and G6 primers target the hyper-variable regions V3-V4 of the 18S rRNA gene, and G4 primers target V3-V5. 18S rDNA is amplified in a 25 µL volume, using the REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA) with 0.4 µM of each primer and 2 µL of template. PCR cycling conditions include initial denaturation at 95 °C for 3 min, 20 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 sec, and a final elongation at 72 °C for 4 min. PCR products are prepared for sequencing by a second PCR (adaptor PCR [PCR2]), using the same PCR protocol as described above. This PCR attaches an adaptor A, an index barcode, and a forward sequencing primer site to the 5' end of the amplicons and an adaptor B, an index barcode, and a reverse sequencing primer site (RSP) to the 3' end of the amplicons. DNA is quantified using the Quant-ITTM dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific), and PCR2 products are pooled in equimolar amounts across samples. Undesirable DNA amplicons are removed from the pooled amplicon library by Agencourt AMPure XP bead (Beckman Coulter) purification. The resulting AMPure beads-purified pooled amplicon library is diluted to its final concentration of 11.5 pM DNA in 0.001 N NaOH and used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA 29122, USA). The library is sequenced with the 500 rxn MiSeq Reagent Kit V2 in a 2 x 250 nt setup (Illumina Inc., San Diego, CA 29122, USA).

Sequence-based identification of parasite taxa

Data output is analysed with BION (http://box.com/bion), a recently developed pipeline that accepts raw sequence data and includes steps for de-multiplexing, primer-extraction, sampling, sequence- and quality-based trimming and filtering, de-replication, clustering, chimera-checking, reference data similarities and taxonomic mapping and formatting. Non-overlapping paired reads are allowed for analysis. The filtered abundance matrix is analysed in R using the phyloseq (v.1.26.0) and ggplot (v.3.1.0) packages.

EVALUATION

The method has been applied to genomic DNA extracted from various matrices such as faeces, other clinical sample types (EDTA blood, spinal fluid, skin/cornea biopsies/scrapings, etc), food products, and environmental samples.

The method has already been used in various studies, including the ones included in the reference list. In PARADISE-project, the method is further optimised and validated for detection of parasites, in particular foodborne parasites.

Specific challenges/limitations of the approach that are addressed include the following:

- The platform has very low sensitivity for the detection of some flagellates including *Giardia* and also microsporidia. Primer adjustments are tested during the first and second years of PARADISE, with the aim to increase the likelihood of successful amplification and sequencing of *Giardia*-specific DNA.

- During the first year of PARADISE, the platform was tested for its ability to identify Anisakidae to species level. It was concluded that the current setup cannot do this. Further work may include an algorithm for decision-making on when the platform should be followed by application of more specific molecular methods.

References

- Differentiation of Blastocystis and parasitic archamoebids encountered in untreated wastewater samples by amplicon-based next-generation sequencing. Stensvold CR, Lebbad M, Hansen A, Beser J, Belkessa S, O'Brien Andersen L, Clark CG. Parasite Epidemiol Control. 2019 Dec 21;9:e00131. doi: 10.1016/j.parepi.2019.e00131. eCollection 2020 May. PMID: 31909230
- Detection and identification of Acanthamoeba and other non-viral causes of infectious keratitis in corneal scrapings by real-time PCR and next-generation sequencing-based 16S-18S gene analysis. Holmgaard DB, Barnadas C, Mirbarati SH, Andersen LO, Nielsen HV, Stensvold CR. J Clin Microbiol. 2020 Nov 25:JCM.02224-20. doi: 10.1128/JCM.02224-20. Online ahead of print. PMID: 33239372