The STSM report

Title: Different diagnostic methods (detection and molecular typing) for protozoan foodborne parasites (*Cryptosporidium* spp. and *Giardia duodenalis*) in animal (cattle, pig, sheep) fecal samples.

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Aim and Objectives

The aim of STSM was to learn different diagnostic methods (detection and molecular typing) for protozoan foodborne parasites (*Cryptosporidium* spp., *Giardia duodenalis*) in animal (cattle, pig, sheep) fecal samples as well as to analyze the samples previously collected within post-doctoral research "One health multidisciplinary approaches for epidemiology and prevention of selected parasitic zoonozes (OMEPPAZ)". The STSM aim was especially relevant to my professional expertise in trouble shooting of molecular methods and molecular typing of *Cryptosporidium* spp.

Description of work

During the STSM the following methods were learned and discussed:

- fluorescent microscopy for antibody labeled cyst/oocyst detection,
- DNA extraction from fecal samples,
- PCR for species speciation,
- PCR for subtyping,
- Sanger sequencing, sequence analyses.

1) The fluorescent microscopy for antibody labeled cyst/oocyst detection was prepared with AquaGlo kit. This kit AquaGlo is designed to detect the cyst and oocyst stages of *Giardia duodenalis* and *Cryptosporidium* spp. in particulates isolated

from water and other environmental samples utilizing the principle of direct immunofluorescence.

Samples for the fluorescent microscopy was previously prepared at the Institute BIOR with saturated NaCl flotation method which has previously been described as highly efficient at recovering oocysts/cysts from fecal samples (Kuczynska and Shelton, 1999). For the flotation 1 g of fecal samples was used and after continuous flotation and centrifugation steps it resulted in 2ml of concentrated material which was used for further analyses.

The preparation of the samples for the fluorescent microscopy was prepared as follows: 10μL of the thoroughly suspended faecal material was added to Teflon printed 12 mm, 3 well slide (Immuno-Cell, Mechelen, Germany) after a tenfold dilution, dried on the well and fixated by submerging the slide in acetone. The material was thereafter stained with FITC-labeled anti-Cryptosporidium/Giardia mAbs (AquaGlo, Waterborne, Inc., USA) for 30 minutes in moisture chamber, before rinsing the antibody-solution off with PBS. For enumeration, brightly stained oocysts with typical morphology were counted in all well at 200 x magnification. Each detected cysts /oocysts represents 200 cysts/oocysts per one gram of feaces.

2) DNA was extracted using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, approximately 250 μg of sample material (pellets) was homogenized and lysed using the supplied PowerBead Tubes and a buffer dissolving humic acids along with solution C1, facilitating complete cell lysis. Bead beating was performed using MP FastPrep-24 5G (MP Biomedicals, Solon, United States) for 2 x 60 seconds at 2400 RPM. After a brief centrifugation, the supernatant was collected and mixed with inhibitor removal solution C2 that precipitates non-DNA substances. Following an additional wash with a second inhibitor removal, the collected supernatant was mixed with a high concentration salt solution (C4). This to enable binding of DNA to the silica membrane used when washing the DNA with ethanol. Finally, after all residual ethanol had been removed through centrifugation, the DNA was released from the silica membrane with the sterile elution buffer included in the kit.

3) *Cryptosporidium* specific 18S nested PCR. For the first amplification reaction contained 1x AllTaq Master Mix (QIAGEN, Hilden, Germany) and 10 μM of forward and reverse primer (5'-TTCTAGAGCTAATACATCCG-3' and 5'-

CCCATTTCCTTCGAAACA GGA-3') in a total volume of 20 µL. Subsequent to an initial denaturation for 2 minutes at 95° C, 40 cycles ensued, comprised of 95° C for 10 sec, 55° C for 15 sec and 72° C for 30 sec, followed by a final extension at 72° C for 5 minutes. For the second amplification reaction, 2 µL from the first reaction was used as DNA template and added to a reaction mix identical to the first and secondary forward and reverse primers (5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3') were used. The reaction conditions were also the same as in the first reaction, with the exception that it consisted of 40 cycles with an elongation at 72° C for 20 sec. Amplified products were detected by UV transillumination after electrophoretic size separation on a 1% agarose gel.

4) Out of the PCR products showing positive bands on the gel, 5 µL was mixed with 0.5 µL Exonuclease I (Exo I, 20 u/ µL) and 1 µL SAP (Fermentas FastAP Thermosensitive Alkaline Phosphatese 1u/ µL) and incubated at 37 ° C for 15 minutes, followed by 85° C for an additional 15 min.

5) The purified PCR-products (1 µL) was added to a sequencing mix consisting of 14 µL molecular grade water and 2 µL of 10 µM of forward and reverse secondary primers (separately), with a total reaction volume of 17 µL. The products was filled in the sequencing plates and sent to SciLifeLab (UPPSALA, SNP/SEQ platform) for sequencing.

6) Obtained sequences were analysed using open access program BioEdit and sequences were blasted at NCBI database for *Cryptosporidium* spp. differentiation.

7) Samples which were containing *C. parvum* were used for gp60 nested PCR for subtyping. For the first amplification reaction contained 1x AllTaq Master Mix (QIAGEN, Hilden, Germany) and 10 µM of forward and reverse primer (5'-ATAGTCTCCGCTGTATTC-3' and 5'-GCAGAGGAACCAGCATC-3') in a total volume of 20 µL. Subsequent to an initial denaturation for 2 minutes at 95° C, 40 cycles ensued, comprised of 95° C for 10 sec, 50° C for 15 sec and 72° C for 30 sec, followed by a final extension at 72° C for 5 minutes. For the second amplification reaction, 2 µL from the first reaction was used as DNA template and added to a

reaction mix identical to the first and secondary forward and reverse primers (5'-TCCGCTGTATTCTCAGCC-3' and 5'-GAGATATATCTTGGTGCG-3') were used. The reaction conditions were also the same as in the first reaction, with the exception that it consisted of 40 cycles with an annealing at 52° C for 15 sec. Amplified products were detected by UV transillumination after electrophoretic size separation on a 1.5% agarose gel.

8) Out of the PCR products showing positive bands on the gel further PCR product purification and sequencing was done as described previously, with the exception that for the sequencing gp60 specific forward and reverse primers were used. Obtained sequences were analyzed using open access program BioEdit and gp60 subtypes were named based on the number of serine-coding trinucleotide repeats.

Main results

1) In total 100 samples (cattle and pig) were tested with the fluorescent microscopy for the presence of *Giardia duodenalis* cysts.

2) DNA was successfully extracted from around 160 concentrated fecal samples and from those 78 samples were subjected to *Cryptosporidium* specific 18S nested PCR. From those 42 samples showed good positive result and were further analysed by Sanger sequencing. The following *Cryptosporidium* species were differentiated: *C. parvum, C. andersoni, C. ryanae and C. bovis*. *C. parvum* positive samples were further analysed with specific gp60 nested PCR for subtyping and the *C. parvum* subtype IIaA15G1R1 was found.

3) The sensitivity of used enzyme for nested PCR should be improved and optimized. During the STSM main ideas for optimization were discussed.

Future collaboration possibilities with the host institution

The opportunity to work with Karin Troell, who is very experienced in mentioned field, and STSM gave an exceptional opportunity for horizontal exchange of experience and a good practices for my personal development.

The methods learned during this STSM will be further used within my postdoctoral research "One health multidisciplinary approaches for epidemiology and prevention of selected parasitic zoonozes (OMEPPAZ)" and continuous work on method optimization is ongoing with the help of Karin Troell and her expertise.

Future plans, including potential **future publications**

After analyzing all of the samples, the results will be published together with the experts from host institution.

Outputs produced (e.g. academic paper, funding application, new dataset etc.)

Preliminary results were presented as a poster presentation at the Final meeting of COST FA1408 12th to 14th of February 2019, Oeiras, Portugal.

References

Kuczynska, E., & Shelton, D. R. (1999). Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures, and soils. Applied and environmental microbiology, 65(7), 2820-6.