INSTITUTO SUPERIOR TECNICO (IST) - LABORATORIO DE ANÁLISES

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First of all, I would like to express many thanks to the STSM committee for enabling me to participate on this internship and for granting the necessary funds.

Also the host institution and especially Dr. Ricardo Santos and Sílvia Monteiro deserve a large gratitude and many thanks from my side, not only for the opportunity to stay in their laboratory but very importantly for their advices, patience and solicitude with me and my work. It had been a pleasure and unforgettable experience. Thanks.

The aim of the internship

The main aim of this STSM at the Instituto Superior Técnico in Lisbon was a comparison study focused on commonly used quantitative real-time PCR (qPCR), which remains on the top of diagnostic methods for food-borne parasites (FBP) and the novel platform based on digital PCR (dPCR). dPCR may represent a new analytical method useful for improvement of current diagnosis and surveillance status of FBP in Europe since it enables absolute quantification and is claimed to be developed for the detection of low copy number of targets.

The experimental work was based on comparing the detection and quantification performed via qPCR and dPCR on artificially contaminated samples of meat and vegetables. As a model food-borne parasites were chosen *Giardia lamblia (GL)* and *Toxoplasma gondii (TG)*, because both are widespread within the human population and toxoplasmosis is also often ranked among “neglected parasitic infections", a disease which is undervalued in terms of monitoring, prevention and treatment, although the number of infected people and the severity of the disease pose a significant risk to human health. Both parasites were spiked in defined concentrations to pellets resulting from the rinsing of vegetables and only *TG* to pork meat lysates.

Introduction

Currently, quantitative real-time PCR (qPCR) remains to be a more popular choice for nucleic acid quantification. The principle of qPCR is monitoring the process of DNA amplification in "real time" - the PCR equipment (Fig. 1) monitors each reaction in real time using a camera or detector. Basically, the technique links the amplification of DNA to the generation of fluorescence which can simply be detected with a detector during each PCR cycle. Hence, as the number of gene copies increases during the reaction, so the fluorescence increases. The amount of target copies is calculated based on a calibration curve that runs in parallel to the samples.

Fig. 1: 7300 real-time PCR system (Applied Biosystems)

Since the parasitical detection in food stuffs requires high sensitivity for detecting targets that have a low-copy number, the technique of our choice are hydrolysis probes. Hydrolysis probes are fluorescently labelled DNA oligonucleotides, designed to bind downstream of one of the primers during the PCR reaction and to give a fluorescent signal during the reaction. The 5' end of the probe is labelled with a fluorescent reporter molecule – concretely FAM or HEX/VIC. These reporters emit light at different wave lengths and can be read through separate detecting channels. On the 3' end of the probe is a quencher molecule, effectively quenches the output from the reporter. Therefore, when the reporter and quencher are physically close to one another the overall level of fluorescent output is low. During the PCR the probe binds downstream of the primer and is cleaved by the polymerase enzyme during the reaction later on. By cleaving the probe the reporter and quencher are separated which means that the quencher no longer has its effect over the reporter and the level of fluorescence increases. With every cycle of PCR more probe is cleaved and more fluorescence is generated.

Fig. 2: Digital PCR chip

On the other hand, digital PCR (dPCR) is a method based on single molecule amplification. The principle of dPCR is partitioning of each sample into many individual qPCR reactions on the sealed chip (Fig. 2), where some of these contain the target molecule (positive) and some don't (negative). After PCR amplification, each chip representing one sample is read and analysed in QuantStudio 3D reader (Fig. 3). The fraction of negative results is used to generate an absolute quantification for the exact number of target molecules in the sample, without reference to standards or endogenous controls. This arrangement enables the precise measurement due to the high number of partitions allowing simultaneous template amplification. Therefore, dPCR should be able to detect minute amounts of genetic material with performance surpassing widely used qPCR.

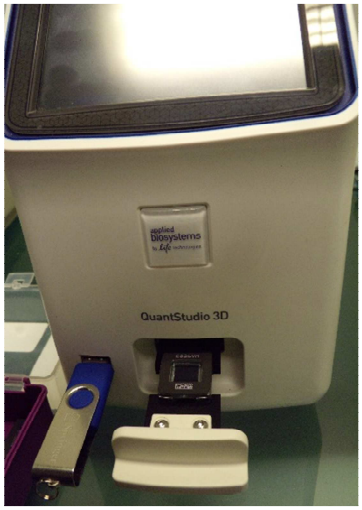

Fig. 3: QuantStudio 3D Digital PCR System (Applied Biosystems)

Target genes

As a target for molecular detection of *Giardia lamblia (GL)* was chosen a partial sequence of species-specific *β-giardin* gene. Product of *β-giardin* is involved in the formation of the adhesive disc, which enables attachment of *Giardia* on epithelial cells of the intestinal mucosa. Gene occurs in the genome in single copy.

The molecular detection of *TG* is based on two multi-copy target genes. The first target is a partial sequence of the gene for Repetitive protein B1 and the second one is localised within the sequence of *Repetitive element 529*. Gene *B1* occurs in *TG* genome in 35 copies, while *529-rep* in about 200-300 copies.

Workload

1st Week: During the first days of my stay I became familiar with the safety regulations and good laboratory practice in the host institution.

The very first task was to prepare the food matrices. As an example of vegetables I bought unworked carrots (from soil). Carrots were scraped off and the trimmings were rinsed in buffer and stomachered, after centrifugation I've obtained pellets.

For the meat lysates, pork meat of pigs from conventional breeding was chosen. A sample of 25 g per animal was cut on the small pieces, digested for 24 hours, stomachered and cooled during the centrifugation to get rid of the fat. Both prepared matrices were tested on qPCR on negativity for both parasites of interest.

For the sample spiking trofozoits of *GL* were cultured, while for *TG*, oocysts obtained from cat's faeces were used. The exact number of trofozoits in fully grown culture of *GL* and isolated oocysts stored in water were counted from cell lysates using qPCR.

2nd Week:

The second week started with sample spikings. A total of 56 samples were prepared - four pellets and four lysates (0,5 ml volume) for each concentration (from 10^6 to 10^0). Since it is known that *GL* is spread mainly in water often used for watering the crops and *TG* oocysts are the source of infection from the soil, pellet samples were spiked with both parasites. Meat lysates made of pork were spiked with *TG,* since the pigs are common intermediate hosts with muscle tissue cysts.

The very next step were DNA isolations. The pellet samples were isolated using PowerSoil® DNA Isolation Kit (MO-BIO) and meat lysate samples with DNeasy Blood & Tissue Kit (Qiagen) with some slight changes within the working protocols. Together with the samples negative controls of isolation were prepared.

First of all, the isolated DNA and the negative controls were tested in duplicates using qPCR with quantified plasmid standards. Reactions for *GL* and *TG* were prepared separately according to certified methodologies for detection and quantification by qPCR in tissue samples *(TG)* and water and foodstuffs of plant origin *(GL)* developed on department of Food

and feed safety, Veterinary Research Institute in Brno (Czech Republic) by RNDr. Michal Slaný, Ph.D. and MVDr. Alena Lorencová, Ph.D.

3rd Week:

The third week was dedicated to the optimization of the dPCR method for the quantification of *TG* and *GL*. Primers and probes concentration, sample volume and thermal profiles were evaluated.

Fig. 4: GeneAmp PCR System 9700 (Applied Biosystems)

Optimal final concentration of primers in reaction were evaluated on 0,5 µM and for all probes on 0,2 µM. Sample volume was estimated on 4 µl. Thermal profile and timing of the individual steps (extension of time in denaturation and annealing step), since the dPCR instrument (Fig. 4) combine both thermocycler and qPCR technologies.

Reaction profile of digital PCR:

95°C	10 min		
95°C	30 s	}	47x
60°C	2 min		
40°C	2 min		

We also found out that the isolated DNA has to be cut before putting on dPCR chip in case of multi-copy target genes of *TG*, otherwise we would not be able to obtain absolute quantification. As the most suitable restriction enzyme was chosen HaeIII, which cuts the sequence in 5´-GG/CC-3´ areas.

4th Week:

During the last week of internship were performed quantifications of the isolated samples by digital PCR using optimized conditions. Data obtained from QuantStudio 3D reader were evaluated in QuantStudio® 3D AnalysisSuite™ Cloud Software within ThermoFisher Scientific websites, where it is possible to check each chip´s surface coverage, precision of analysis and quantification of the target (copies/µl) (Fig. 5).

Fig. 5: QuantStudio 3D AnalysisSuite cloud workspace – chip evaluation (Life Technologies)

Results:

The results from both qPCR and dPCR are still being processed, however, according to the first data obtained, it appears that qPCR is in spite of all assumptions more sensitive than dPCR in detection and quantification of both parasites. The preliminary results show that the concentration of the target in dPCR is more or less one to two grades lower than qPCR in *GL* and about one grade lower in *TG.* The results show also that care should be taken to quantify the standards used in qPCR reactions since an inaccurate quantification will lead to deviation in the final results. It is also of the upmost importance to correctly-optimize the dPCR reactions because the protocols that are implemented in the laboratory and that work with other PCR equipments will not work with the dPCR equipment.

Future perspectives:

The STSM in Lisbon gave me the opportunity to meet and get in touch with some successful and interesting scientists from the food-borne parasitic problematics. Moreover, I have gained invaluable insight into the workings of a diagnostic laboratories concerning food safety, in terms of surveillance and controlling outbreaks.

With the results of these experiments we will prepare at least one journal article concerning the optimization and comparison of different PCR methods for the quantification of food-born parasites.

Moreover, I will to perform a comparison between the dPCR with the chip technology and dPCR based on droplets technology in order to compare sensibilities of the 2 different equipments.

The STSM also contributed to establish the partnership between the host laboratory of Dr. Ricardo Santos and my home laboratory (Dr. Petr Králík and Dr. Michal Slaný), made possible through the collaboration on a COST Action FA1408 project. This partnership will lead to the joint project application and student sharing amongst both laboratories.