ApicoWplexa virtual Meeting, February 18, 2021:

Diagnosis of apicomplexan parasites

Abstracts

Keynote 1:

Molecular tools for the detection and genotyping of *Cryptosporidium* spp. and their use in epidemiological surveys

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PCR-based methods coupled to Sanger sequencing constitute powerful tools in epidemiological investigations, allowing not only the identification of pathogens with high sensitivity but also the assessment of their molecular diversity. This information is essential to ascertain transmission dynamic and zoonotic potential. In the case of the diarrhoea-causing enteric protozoan parasite *Cryptosporidium* spp., detection and molecular subtyping to a large extent has relied on amplification of partial fragments of the multiple-copy small subunit ribosomal ribonucleic acid (*ssu* rRNA) and the single- copy 60-kDa glycoprotein (*gp60*) genes of the parasite. The first part of this presentation will be devoted to the description of the diagnostic

and genotyping algorithm based on the *ssu* rRNA and *gp60* genes currently in place at the Spanish National Centre for Microbiology. Its suitability, advantages and limitations will be thoroughly discussed. To demonstrate the practical applicability of this approach, we will present data from recent collaborative work conducted in highly endemic Mozambique in which large populations of children with and without diarrhoea were screened for the presence of *Cryptosporidium* spp. and other enteric protist species. Consequently, five *Cryptosporidium* species including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, and *C. viatorum* were detected in the investigated paediatric populations. A high intra-species genetic variability was observed within *C. hominis* (subtype families Ia, Ib, Id, Ie, and If) and *C. parvum* (subtype families IIb, IIc, IIe, and IIi). The epidemiological and public health implications of these findings will be discussed.

Keynote 2:

Molecular diagnosis of *Sarcocystis* species in intermediate host, definitive host, and environmental samples

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Members of the genus *Sarcocystis* are worldwide distributed parasites of mammals, birds and reptiles. Research has been mainly focused on muscular sarcocystosis in economically important animals, such as cattle, sheep, goats, and pigs. Although several *Sarcocystis* species were newly described in these hosts and some taxonomic inaccuracies have been resolved, there are still diagnostic issues due to co-infections and low infection rate of some *Sarcocystis* spp. It is needed to develop methods for the identification of *Sarcocystis* species in definitive hosts under natural conditions. Studies on the diversity of *Sarcocystis* spp. in certain intermediate and definitive hosts are limited by legal restrictions and conservation policies. Therefore, environmental DNA samples could be a great source for epidemiological investigations of *Sarcocystis*. The objective of the present work was to create and optimize molecular-based method for the identification of *Sarcocystis* species in intermediate host, definitive host, and environmental samples. The obtained results provide strong evidence showing that predators of the family Mustelidae can serve as definitive host of *Sarcocystis* spp. using cattle as intermediate host and reveals gaps in cattle carrion and beef waste management. Also, it is currently being optimized to diagnose *Sarcocystis* species in different environmental samples such as water, soil, and fodder. Our

studies of water contamination by sporocysts are important in uncovering sources of infectious diseases in food-producing animals.

Short presentations:

Short presentation 1:

Selection, validation and SOP development of a molecular detection method for identification of *Toxoplasma gondii* oocysts in leafy-green vegetables

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Toxoplasma gondii is an important zoonotic pathogen worldwide with up to 60% of acquired toxoplasmosis cases being associated to foodborne transmission. In addition to the well-recognized role of meat of infected animals as a source, human infection has been also associated to the consumption of unwashed raw fresh produce contaminated with the environmentally resistant *T. gondii* oocysts. However, estimation of the relative importance of fresh produce as an infection source has been hampered by the lack of specific regulations and standards, and outbreaks have been rarely detected and their investigation have been limited. Due to a high sensitivity and applicability for high-throughput testing, molecular methods appear the most suitable to address food safety risk assessment challenges typical for foodborne protozoan parasites. Despite several molecular detection methods for *T. gondii* oocyst contamination have been described, a widely applicable method for fresh produce remains to be defined. One of the aims of the TOXOSOURCES project is to fill the knowledge gap concerning the relevance of fresh produce contamination by *T. gondii* oocysts. For this, we defined a feasibility plan to establish a standard operating procedure (SOP) to be implemented among consortium partners and applied in a multicenter pilot survey. First, to provide a solid basis for SOP design, an extensive literature

review and multi-attribute assessment of the molecular methods described and currently used to detect *T. gondii* oocysts was conducted (Slana et al., 2021 https://doi.org/10.3390/microorganisms9010167). Building on the results, comparative experimental work was conducted at two partner institutes for all the key analytical steps necessary for the SOP development (i.e. oocyst recovery from fresh produce, DNA extraction, and detection by PCR). As oocysts of *T. gondii* are highly resistant to environmental conditions and do not multiply in the environment, oocyst recovery from the sample is the first and key step to enable successful detection. Molecular detection must then rely on efficient DNA extraction from the robust oocysts, together with a reduction of possible contaminants that could inhibit the DNA amplification. Finally, amplification must be specific and sensitive to detect DNA from low numbers of oocysts, ideally a single oocyst. By the experimental work, key characteristics of the method, such as sensitivity, reproducibility, repeatability, ease of handling, availability and costs of reagents and equipment, hands on and turn-around times, were evaluated. Video tutorials were compiled to facilitate the implementation of the SOP. The final SOP will be a public deliverable from the project.

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Short presentation 2:

A comparative study of the most widely used serological tests in the diagnosis of *Toxoplasma gondii* infection in small ruminants

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Toxoplasma gondii can infect any warm-blooded animal and is a major foodborne zoonotic pathogen worldwide. *Toxoplasma* is also an important cause of reproductive failure in both humans and small ruminants and is responsible for significant economic losses in the livestock industry. The availability of accurate serological diagnostic techniques is key for monitoring, controlling and preventing the disease. Therefore, the objective of this study was to compare the diagnostic performance of the most frequently used commercial ELISA tests (ID Screen® Toxoplasmosis Indirect

Multispecies -ID Screen-, IDEXX Toxotest Ab -IDEXX-, PrioCHECK® Toxoplasma Ab SR -PrioCHECK- and Pigtype® Toxoplasma Ab from Indical Bioscience/ Qiagen -Qiagen-) for the detection of antibodies against *T. gondii* in sheep. In addition, a new lyophilized tachyzoites based- ELISA (Tg SALUVET ELISA 2.0) was developed and included in the comparative study along with a Western Blot assay (WB).
Tg SALUVET ELISA 2.0 was validated with sera from naturally infected goats (n= 434) and WB was considered as the reference test. Next, for the comparative study, the panel sera analyzed by all serological tests included sera from *T. gondii* experimentally (n=124) and naturally (n=241) infected sheep. The analytical specificity of all tests was determined using sera from *Neospora caninum* experimentally infected sheep (n=26).
Tg SALUVET ELISA 2.0 showed a high performance with an area under the curve (AUC) of 0.99 for the cut-off defined (RIPC≥ 32.71, Se= 99.3%, Sp= 99.3%). When using the sera panel composed of *T. gondii* experimentally infected sheep, all techniques showed perfect agreement ($k \geq 0.93$) with high AUC (≥ 0.99) and Se and Sp values for the cut-offs suggested by the manufacturers (ID Screen: Se= 100% and Sp= 100%; IDEXX: Se= 94% and Sp= 100%; PrioCHECK: Se= 100% and Sp= 99%; Qiagen: Se= 94% and Sp= 100%). However, cross-reactivity with antibodies against *N. caninum* was recorded in all commercial ELISAs. The highest number of cross-reactive sera were observed in ID Screen and PrioCHECK (n=14), followed by Qiagen (n=11) and IDEXX (n=6). The results supported the use of Western Blot as a reference specific test and that Tg SALUVET ELISA 2.0 is an accurate technique that could be used in both goats and sheep. Data on the comparative study with sera from *T. gondii* naturally infected sheep will be presented and the need for ELISAs cut-offs readjustment will be discussed in order to reduce cross reactions and obtain comparable data among tests. Cross reactivity is a crucial issue when validating serological techniques since there is an increased evidence of the relevance of *N. caninum* infection in sheep and the coexistence of *T. gondii* and *N. caninum* infections in small ruminant flocks.

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Short presentation 3:

Well, you don't see that every day – Molecular diagnostics of ApiCOWplexan parasites

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Apicomplexan parasites have a world-wide distribution affecting a wide variety of host species and infecting many different tissue types. Often many of the tissue dwelling forms of the parasites (within a species) are also difficult to distinguish morphologically. These factors can make reaching a diagnosis difficult, based purely on microscopy (histology). A tandem approach using both histological morphology and molecular techniques (PCR, sequencing) can be useful. In this presentation we describe three cases, where this dual approach has been used to clearly identify the parasites associated with the infections. The first cases involved the conjunctival infection of a captive inland bearded dragon (*Pogona vitticeps*). Histopathological examination revealed the conjunctival mucosa (eyelid) to be markedly hyperplastic. Attached to the apical border of the conjunctival epithelial cells were spherical organisms, which were histologically compatible with *Cryptosporidium* sp. DNA was extracted from ocular tissues and an 18S rRNA gene PCR revealed that the parasites present in the ocular tissues were *Cryptosporidium avium*, a species of *Cryptosporidium* not often associated with reptiles nor with ocular infections. The second case involved a young suckler calf presenting with cholangiohepatopathy / cholangiohepatitis, where coccidial bodies (including oocysts) were found to be present in multiple bile ducts. Initial screening of DNA (using the 18S rRNA gene) from both FFPE and frozen liver samples indicated the presence of an *Eimeria* sp. Further investigation using the ITS1 locus and the *coxI* gene confirmed the presence of *E. cylindrica*. This is a species of *Eimeria* that had not previously been reported in the UK and is not usually associated with infectious hepatitis. The third and final case involves bovine skeletal muscle. The carcass from an apparently healthy Aberdeen Angus cow was passed fit for human consumption by a meat inspector at the abattoir. Subsequently, a butcher condemned the carcass on finding multiple large “green lesions” spread throughout the striated muscle. Histological examination of the lesions demonstrated Eosinophilic myositis. DNA extracted from these lesions was tested with pan-Protozoan 18S rRNA gene primers and demonstrated 100% sequence identity to *Sarcocystis cruzi*, this finding was confirmed with the amplification of portions of both the ITS1 locus and the *coxI* gene, which also showed 100% sequence identity to *S. cruzi*, a species of *Sarcocystis* not previously reported in the UK. All three of these cases show that a combined molecular and histological approach can greatly aid diagnosis of protozoan infections.