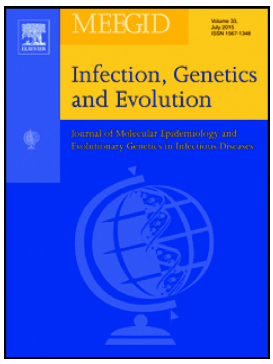

A validated method to identify Echinococcus granulosus sensu lato at species level

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A validated method to identify *Echinococcus granulosus sensu lato* at species level

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ABSTRACT

The zoonotic tapeworm *Echinococcus granulosus sensu lato* (*s.l.*) represents a species complex encompassing multiple causative agents of cystic echinococcosis, a neglected tropical disease affecting more than one million people in the world. At least eight genotypes, grouped in five species, are currently recognized within this species complex, and they differ in terms of relative public health impact. Here we present a molecular method that first identifies the common *E. granulosus sensu stricto* (*s.s.*) (genotypes G1 and G3) based on a PCR-RFLP assay, and can further identify the remaining species based on a multiplex PCR assay. We demonstrate the applicability of the method to DNA extracted from parasitic cyst material of human and animal origin, preserved in ethanol or frozen. The method has been developed and validated at the European Union Reference Laboratory for Parasites (EURLP), according to the ISO/IE 17025.

[1] * Equal contribution

Keywords:

Echinococcus granulosus s.l.; PCR-RFLP; Multiplex PCR; ISO/IEC 17025

1. INTRODUCTION

Cystic echinococcosis (CE) is a chronic and cosmopolitan parasitic zoonosis caused by the tapeworm *Echinococcus granulosus sensu lato* (*s.l.*). This cestode can affect a considerable variety of intermediate and definitive hosts. Sheep and other ungulates, infected by the larval stage (fluid-filled cysts) act as intermediate hosts. Domestic dogs and other canids act as definitive hosts and complete the cycle harbouring the adult worms in the intestine and spreading infective eggs through faeces in the environment. Humans may act as dead-end hosts (Casulli et al., 2019), and ingested eggs develop into fluid-filled cysts, mainly in the liver and/or lungs, but also in other organs or tissue such as bones, brain, kidney, heart and spleen (Yagmur and Akbulut, 2012). Estimates of the global burden for human CE account for 1 million lost disability-adjusted life years (DALYs) each year when accounting for underreporting (Budke et al., 2006). World Health Organization (WHO) acknowledges CE as a significant global public health problem, and has included it among the Neglected Tropical Diseases (NTDs) to be prioritized for control (WHO, 2020a). In Europe, CE is prevalent in pastoral and rural communities, even in medium-high income countries where it should be managed as an orphan disease (Casulli, 2020; Tamarozzi et al., 2018). Further, livestock CE causes condemnation of infected organs and a reduced yield and quality of production, which weights significantly on the economic burden of the disease (Budke et al., 2006; Kere et al., 2019; WHO, 2020b).

Causative agents of CE are members of the *E. granulosus s.l.* complex, which comprised biological strains/genotypes with distinct host-adapted life cycles, geographic distribution, infectivity, immune response and, possibly, pathogenicity. They are currently grouped as *E. granulosus sensu stricto* (*s.s.*) (genotypes G1 and G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6/G7 and G8/G10) and *E. felidis* (Casulli et al., 2020; Hüttner et al., 2008; Kinkar et al., 2018a and 2018b; Nakao et al., 2007; Wen et al., 2019). Of these, *E. granulosus s.s.*, *E. ortleppi* and *E. canadensis* are known agents of human CE, with *E. granulosus s.s.* accounting for the majority of the human CE worldwide (Alvarez Rojas et al., 2014). Only recently, *E. equinus* has been related to one human CE case for the first time (Kim et al., 2020). The taxonomic status, particularly that of *E. canadensis*, is still disputed (Lymbery, 2017; Romig et al., 2015), although *E. canadensis* genotypes G6/G7 and *E. canadensis* genotypes G8 and G10, respectively, have been proved to be phylogenetically distinct and probably representing distinct species (Laurimäe et al., 2018a). Taking into account this diversity, the development of genotype/species-specific molecular tools is not only of epidemiological, but also of public health importance, as it can support the implementation of targeted control programs and prophylaxis (Alvarez Rojaz et al., 2014; Budke et al., 2017; Lymbery and Thompson, 2012).

The purpose of the present study was to develop and validate a PCR-based method for the rapid identification of *E. granulosus s.s.* (G1/G3), and the subsequent, simultaneous detection of *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6/G7) and *E. canadensis* (G8/G10).

2. MATERIALS AND METHODS

2.1 Reference parasites and DNA

The reference material (parasites and DNA) used to develop and validate the method was provided by the WHO Collaborating Centre for the Epidemiology, Detection and Control of Cystic and Alveolar Echinococcosis (Rome, Italy; https://apps.who.int/whocc/Detail.aspx?cc_ref=ITA-107&tor=echino&).

For Phase 1 (DNA extraction, Figure 1), the reference material consisted in cyst germinal layers or protoscoleces of *Echinococcus granulosus s.l.*: *E. granulosus s.s.*, *E. equinus*, *E. ortleppi*, *E. canadensis* G6/G7 and *E. canadensis* G8/G10, which were preserved in 70% ethanol. For Phases 2-4 (PCR-RFLP and multiplex PCR, mPCR, Figure 1) the reference material consisted in DNA of *E. granulosus s.l.*: *E. granulosus s.s.*, *E. equinus*, *E. ortleppi*, *E. canadensis* G6/G7 and *E. canadensis* G8/G10. To test the specificity of the assay, reference DNA of *E. multilocularis*, *Taenia multiceps*, *T. saginata*, and sample DNA of *T. hydatigena* were also included.

2.2 DNA extraction (Phase1)

Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A negative control (nuclease-free water) was included in each working session to verify absence of contamination.

DNA was stored at -20 °C until use.

2.3 PCR-RFLP (Phases 2-3)

A 444 bp fragment of the mitochondrial Cytochrome Oxidase I gene (COX1) was amplified using primers originally described by Bowles and colleagues (1992) and modified by Bart and colleagues (2006). The PCR reaction was performed in a final volume of 50 µL, and comprised 2µL of DNA template, 25µL of 2X PCR Master Mix HotStart (Qiagen GmbH, Hilden, Germany), 0.25 µM of each primer and nuclease-free water.

The cycling conditions were as follows: an initial denaturation step at 94 °C for 15 min, 38 cycles (denaturation: 94 °C for 30 sec, annealing 55°C for 30 sec, elongation: 72 °C for 30 sec) and a final extension step of 5 min at 72°C. A negative control (in which nuclease-free water replaced the DNA template) was included in each experiment.

PCR products were visualized by capillary gel electrophoresis (Qiaxcel, Qiagen GmbH, Hilden, Germany). For the PCR-RFLP assay, 10 µl of each product was digested with 10U of AluI (5′-AG/CT-3') (New England Biolabs, Ipswich, MA, USA), 2 µl of 10X Restriction Buffer (CutSmart®Buffer), as previously described (Kim et al., 2017), and water, in a final volume of 20 µl.

The mixture was incubated at 37ºC for 3 hours. Restriction fragments were analyzed by capillary gel electrophoresis.

2.4 Multiplex PCR (Phase 4)

A multiplex PCR (mPCR) was carried out on DNA that showed uncut products after the PCR-RFLP assay (Phase 3). The mPCR used five primer pairs described by Boubaker et al. (2013), namely Eg complex, Ecq Cox1, Ecnd G6/G7 ND1 (mitochondrial targets), Ecnd G8/G10, Elp1 and Eeq Cal (nuclear targets).

The mPCR was carried out in a final volume of 50 µL, and comprised 2 µL of DNA template, 25 µL 2x PCR Master Mix HotStart (Qiagen GmbH, Hilden, Germany), primer mix (molarities are shown in Table 1), and nuclease-free water.

The cycling conditions were as follows: an initial denaturation step at 95 °C for 15 min, 35 cycles (denaturation: 94 °C for 30 sec, annealing: 56°C for 30 sec, elongation: 72 °C for 60 sec) and a final extension step lasting 5 min at 72°C. A negative control (in which nuclease-free water replaced the DNA template) was included in each experiment. The

mPCR products were analysed by capillary gel electrophoresis; the species-specific patterns are shown in Table 1.

Primer name (for-rev)	Final concentration (μM)	Product size (bp)	Species (genotypes)			
Eeq Cal	2	426	*E. e.* (G4)			
Ecnd G6/G7ND1	0.3	339		*E. c.* (G6/G7)		
Ecnd G8/10 Elp1	1.5	283			*E. c.* (G8/G10)	
Eeq COX1	0.8	124	*E. e.* (G4)			
Eg complex	0.2	110	*E. e.* (G4)	*E. c.* (G6/G7)	*E. c.* (G8/G10)	*E. o.* (G5)

Table 1. The final concentration of the primer used in mPCR and the banding patterns for *Echinococcus* species identification.

E. e. (G4): *Echinococcus equinus. E. o.* (G5): *Echinococcus ortleppi* (G5). *E. c.* (G6/G7): *Echinococcus canadensis* (G6/G7). *E. c.* (G8/G10): *Echinococcus canadensis* (G8/G10).

2.5 Method performance

2.5.1 Specificity and repeatability

The method has been characterised in terms of specificity and repeatability. The specificity was evaluated at two levels: a) for PCR-RFLP and mPCR, by testing reference DNA of *E. multilocularis, T. multiceps*, *T. saginata* and sample DNA of *T. hydatigena*; b) for PCR-RFLP, by testing reference DNA of *E. equinus*, *E. ortleppi*, *E. canadensis* (G6/G7) and *E. canadensis* (G8/G10).

All reactions on reference DNA were performed in triplicate and by two different operators to reduce observer bias.

2.5.2 Performance on field samples

A panel of 65 metacestode samples, which were sent to the EURLP between 2019 (n=51) and 2020 (n=14) for diagnostics, were subjected to this method to evaluate the sensitivity of the assay; of these, 39 were also analyzed by PCR and sequencing. The panel of samples comprised ethanol-preserved *E. granulosus* cysts (from liver, lung or SNC) of human (n=13) and animal (n=52) origin, collected in Italy (n=32), Latvia (n=6), Hungary (n=8), Finland (n=4), Bulgaria (n=2), France (n=1) and Afghanistan (n=12).

2.5.3 Performance on frozen material

To assess the performance of the method on frozen material, three *E. granulosus* cyst samples, taken from the field samples, were stored at -20°C after being analyzed (2.5.2). Samples were thawed after four weeks and used for a second test.

3. RESULTS

3.1 Development of the assays using reference material

Amplification of the COX1 gene yielded the expected 444 bp fragment from the reference material used, which represented all the *E. granulosus s.l.* species, as well as *E. multilocularis*, *T. saginata*, *T. multiceps*, and from sample DNA of *T. hydatigena*. After digestion of the PCR products by AluI, only *E. granulosus s.s.* generated two bands of 209 bp and 235 bp, whereas the products from the remaining *E. granulosus* species, *E. multilocularis*, *T. saginata*, *T. multiceps* (Figure 2) and *T. hydatigena* remained uncut. Next, the mPCR assay was applied to reference material of *E. equinus*, *E. ortleppi*, *E. canadensis* (G6/G7), *E. canadensis* (G8/G10), *E. multilocularis*, *T. saginata*, *T. multiceps* and to sample DNA of *T. hydatigena*. As showed in Figure 3, all *E. granulosus s.l.* species were correctly identified, whereas no amplification were obtained for *E. multilocularis* and *T. saginata*. Unexpectedly, two of the four *T. multiceps* reference DNAs yielded unspecific products of 200 bp and 600 bp, and two of the seven *T. hydatigena* sample DNAs yielded unspecific products of 150 bp (data not shown).

3.2 Performance on field samples and frozen material

The PCR-RFLP was applied to 65 field samples, 50 of which (77 %) showed the two bands pattern expected for *E. granulosus s.s*. The remaining samples were consequently analysed by mPCR, and showed the pattern expected for the *E. ortleppi* (n=1), *E. canadensis* (G6/G7) (n=10) and *E. canadensis* (G8/G10) (n=4), respectively. To corroborate these results further, 39 over 65 samples were also sequenced. Sequencing confirmed their identity in all cases (i.e.: 30 *E. granulosus s.s.*, one *E. ortleppi*, four *E. canadensis* G6/G7 and four *E. canadensis* G10). The second test performed on cyst samples thawed after four weeks at -20°C lined up with the results of the first test.

4. DISCUSSION

Cystic echinococcosis is a chronic parasitic disease caused by *E. granulosus s.l.*, a species complex made up of several cryptic species and genotypes (Tamarozzi et al., 2020) characterized by specific biological features and sometimes co-existing in the same areas. Not all the genetic variants of *E. granulosus s.l.* have the same impact on public health, thus, molecular epidemiological data on circulating genotypes may support source attribution studies and *ad hoc* control measures.

Therefore, a number of molecular diagnostics protocols have been developed to distinguish *E. granulosus s.l.* species and genotypes; most are based on PCR and sequencing, but this is still a costly and time consuming step, in particular for laboratories that rely upon an external service. Therefore, protocols that skip sequencing and possibly reduce the number of tests are desirable and have been presented (Borji et al., 2018; Boubaker et al., 2013; Bowles and McManus, 1993; Chaâbane-Banaoues et al., 2016; Chen et al., 2019; Dinkel et al., 2004; Kim et al., 2017; Grech-Angelini et al., 2019; Spotin et al., 2015; Shang et al., 2019). However, only a partial resolution is offered by these protocols, and often only *E. granulosus s.s.* or *E. granulosus s.s.* and *E. canadensis* are distinguished.

In 2013, a single multiplex PCR (mPCR) able to achieve full discrimination was proposed (Boubaker et al., 2013). However, the assay is complex and difficult to optimize, being based on the use of 11 primer pairs, a high number of targets for a simultaneous amplification and the potential impact of sequence differences among *E. granulosus s.l.* genotypes.

The attempts to replicate the mPCR assay (Boubaker et al., 2013) in our laboratory, according to the original conditions, was unsuccessful in terms of both sensitivity and specificity. For this reason, we reduced the number of primer pairs included in our mPCR, however this implied the lack of additional specific bands for *E. ortleppi*.

Similarly, a recently published study observed poor efficiency using this mPCR (Tahiri et al., 2019).

Taking all of this into account, we have developed a simple and affordable PCR-based assay to discriminate *Echinococcus granulosus s.s.* (genotypes G1/G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6/G7), and *E. canadensis* (G8/G10). The method identifies the *E. granulosus s.s.* (genotypes G1/G3) by PCR-RFLP and, as the vast majority of human CE cases, worldwide, are caused by this species, a consistent proportion of cases will receive a definitive diagnosis at the end of the first step. Only the remaining species in human host (estimated to be around 11%; Alvarez Rojas et al., 2014) would require the second assay, mPCR.

The method has been validated at the EURLP using reference material, both parasites and DNA, provided by the WHO Collaborating Centre for the Epidemiology, Detection and Control of Cystic and Alveolar Echinococcosis. This material consists of samples with well-established properties used for the assessment of analytical methods "for which the test results are firmly established and agreed" (ISO Guide 30:2015 and ISO/IEC 17025:2017). Moreover, the method performed well on a collection of field samples (hydatid cyst material) sent to the EURLP for routine analyses, indicating its applicability to samples of various sources (geographic origin, host species and cyst location).

Furthermore, the fact that the method was validated according to the ISO standard 17025 ensured that the particular requirements for the intended use are met. This means that the method and therefore the results are confirmed by the procedures validated in terms of performance characteristics such as traceability, precision, and specificity. It therefore shows that the detection capability, and applicability-ruggedness-stability, were determined by the validation itself. Therefore, it can be stated that the method is reproducible, sensitive and specific for the chosen target.

The genotyping method here described is meant for application on *E. granulosus* cyst material. The first assay, PCR-RFLP, did not show any ambiguous result, whereas two out of

four *T. multiceps* samples, and two out of seven *T. hydatigena* samples, tested yielded unspecific products in mPCR. As the larval stage of *T. multiceps* (*Coenurus cerebralis*) forms fluid-filled cysts in the central nervous system, sometimes mimicking CE cyst (Mahadevan et al., 2011), this may be of concern. However, the different size of the mPCR products should allow the correct interpretation of the results.

Finally, the method does not allow the differentiation of closely related genotypes (G1 from G3, G6 from G7, and G8 from G10), although their distinctness has been characterized (Kinkar et al., 2018a; Lavikainen et al., 2003; Laurimäe et al., 2016a and 2018b). Indeed, they were here treated as clusters. While a deeper distinction is certainly of interest, especially for the G8 and G10 genotypes, this does not necessarily match with the necessity of a fast assay.

5. CONCLUSIONS

The method here described allows identifying the different *Echinococcus granulosus s.l.* species by two PCR steps, and does not require sequencing. The most common *E. granulosus s.s.* (genotypes G1/G3) is identified by the first step. The method was validated according to the ISO/IEC 17025.

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Declarations of interest: none.

Authors' contribution

F.S. and A.S. equally contributed to the realization of the experiments, collection and analysis of the data, and drafting and editing of the manuscript. A.C. designed the study, revised the manuscript and participated in final conceptualization. S.M. edited the final version of manuscript and participated in final conceptualization. A.R. revised and discussed the manuscript. All authors read and approved the final manuscript.

Declarations of interest: none.

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Figure 1. Flow chart summarizing the four phases of the method. Phase1: DNA extraction. Phase 2: PCR amplification of a 444 bp fragment of the mitochondrial gene COX1. Phase 3: Restriction Fragment Length Polymorphism (RFLP) assay, showing the AluI specific digestion pattern of *E. granulosus s.s.* (G1/G3). Phase 4: Multiplex PCR assay, showing the specific banding patterns for the G4-G10 genotypes.

Figure 2. Capillary electrophoresis of PCR-RFLP products. Lanes A1-A2: EgG1/G3; A3: Ladder 50-800 bp; A4: EgG4; A5: EgG5; A6-7: EgG6/G7; A8: EgG8/G10; A9: *E. multilocularis*; A10: *T. saginata*; A11: *T. multiceps*; A12: negative control. Alignment marker: 15-1k bp.

Fig. 3. Capillary electrophoresis of Multiplex PCR. Lane A1: EgG4; A2: EgG5; A3-A4: EgG6/G7; A5-A6: EgG8/G10; A7: negative control; A8: Ladder 50-800 bp; A9: *E. multilocularis*; A10: *T. saginata*; A11: *T. multiceps*. Alignment marker: 15-1k bp.

HIGHLIGHTS

- A protocol for the detection of *E. granulosus sensu lato* is described
- This method represents a practical tool for diagnosis of human and animal CE
- This method represents an alternative approach to sequencing
- This protocol was developed and validated according to the ISO/IEC 17025