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ORIGINAL ARTICLE

Genotyping of human *Echinococcus granulosus* cyst in Morocco

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Abstract *Echinococcus granulosus* is the etiological agent of cystic echinococcosis (CE), also commonly called hydatidosis. This is a zoonotic infection endemic worldwide, including the Mediterranean basin and Morocco. The genetic variability of *Echinococcus granulosus* is known to influence development of parasitic cysts in different intermediate hosts, and therefore the epidemiology of infection. Molecular studies have identified nine genotypes of *Echinococcus granulosus*, eight of them affect humans, grouped today in four distinct species. In Morocco, molecular studies on CE cysts from animals showed the presence, for the large majority of cases, of the G1 genotype (« sheep strain » or *Echinococcus granulosus* sensu stricto), which is also the cause of the majority of human infections worldwide, and to a lesser extent of the other genotypes (G2 and G3) within *Echinococcus granulosus* sensu stricto complex. However, so far no genotyping of echinococcal cysts in Morocco has been carried out. We collected CE cysts material from 15 patients diagnosed with abdominal CE in the Meknès-Tafilalt region, Middle Atlas of Morocco, and an endemic area and genotyped by multiplex PCR. The only five cysts from which it was possible to successfully amplify the DNA were all belonging to the G1–G3 genotype, in line with the epidemiology of CE in animals in the same area. Our results add new information, on the human side, to the epidemiological picture of CE in the region, which are important in the context of any control plan for the infection.

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Introduction

In Morocco, cystic echinococcosis (CE), caused by the larval stage of the cestode *Echinococcus granulosus*, represents a real public health problem. Dogs and wild carnivores act as definitive hosts for the adult tapeworm, while livestock (sheep, goats, cattle, camels) represent the natural intermediate hosts. CE is endemic in almost all rural regions of the country, and of affected humans, 62% reside in rural areas (Derfoufi et al. 2012). Three regions, Meknès-Tafilalt, Chaouia-Ouardigha and Doukala-Abda, record the highest surgical incidence of human cases in the country (Derfoufi et al. 2012). In fact, in 2008, over 33% of all recorded surgical cases came from these three regions (Derfoufi et al. 2012). Notwithstanding the efforts put in control programs, CE remains a neglected zoonosis. According to the increasing knowledge in the molecular epidemiology of *E. granulosus*, the genotype has a great influence on various aspects of the parasite including the pattern of life cycle, the host specificity, and the pathology. Genetic variability could have important implications for the design and development of vaccines, diagnostic reagents and drugs effective against this parasite. Moreover, molecular approaches allowing the species-specific identification of *Echinococcus* can distinguish between imported or autochthonous parasitic infection. Therefore, there is a need of more studies on molecular identification of CE to understand its species diversity and molecular epidemiology.

Molecular studies on mitochondrial DNA have identified throughout the world nine genotypes of *E. granulosus*,

eight of which are known to affect humans, grouped today in four distinct species (Alvarez Rojas et al. 2014). In Morocco, molecular studies carried out on CE cysts obtained from infected animals (sheep, cattle, camels) in different regions identified in the vast majority of cases (91.4%) the G1 genotype, and to a lesser extent the G2 (1.7%) and G3 (2.6%) genotypes, all in the *E. granulosus* sensu stricto complex (Azlaf and Dakak 2006; Alvarez Rojas et al. 2014; El Berbri et al. 2015). However, paradoxically, no studies have so far characterized the genotype of *E. granulosus* cysts in humans in Morocco. Our genotyping study, the first in Morocco on CE cysts from infected patients, aims to add new data on the epidemiology of CE in the country, allowing a comparison between the parasite circulation in humans and in animals, for which more data are available, and on the susceptibility of humans to *E. granulosus* strains endemic in the country.

Materials and methods

Ethics statement

Approval was granted by the Ethics Committees of the University of Pavia, Italy, and of the University Hospital center Hassan II of Fez, Morocco.

Cases definition and samples collection

The samples were collected in the context of the project « Clinical management of Cystic Echinococcosis in Morocco », in the aim of evaluation of prevalence of human abdominal CE in the provinces of Ifrane and El Hajeb, Meknès-Tafilalt region, Middle Atlas, by ultrasound screening of 5221 people of 10–80 years of age residing in the target provinces between October 2014 and January 2015 (Chebli et al. 2017).

CE was diagnosed and staged by ultrasound using portable machines with 3,5-5 MHz convex probes.

Serology was used to confirm CE, and performed in the Parasitology Laboratory of the Teaching Military Hospital Mohammed V of Rabat, using ELISA RIDASCREEN ® *Echinococcus* IgG (code K7621, R85 Biofarm AG, Darmstadt, Germany) and Western Blot *Echinococcus* IgG (code ECHWB24G 86 LDBIO Diagnostics, Lyon, France, distributed by Promalab, Casablanca, Morocco).

Epidemiological data of all CE cases were collected during the screening sessions. All patients with abdominal CE requiring surgical or percutaneous (PAIR) treatment according to the WHO Informal Working Group on Echinococcosis (IWGE) Expert Consensus recommendations, were treated free of charge (Brunetti et al. 2010).

The cyst fluids obtained during these procedures were aliquoted. The first aliquot was examined under light microscope after eosin staining to determine the viability of protoscoleces. The second aliquot of each samples were centrifuged at 3500*g* for 3 min, and the pellets were washed twice for 15 min in 100 µL of PBS. Finally, the samples were centrifuged at 3500*g* for 5 min, and the supernatant was removed. The protoscoleces samples were stored at − 20 °C and used for the molecular analysis.

Molecular analysis

Genomic DNA was extracted from each sample with the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A multiplex PCR was carried out according to the protocol described in Boubaker et al. (2013) using only primers for ef1a cal, cox1, pold and elp 1 genes. The final product sizes and the targets genes are listed in Table 1. The cycling conditions were as follows: an initial denaturation step at 94 °C for 3 min, 25 cycles (94 °C–30 s, 56 °C–30 s, 72 °C–1 min) and a final extension step lasting 5 min at 72 °C. PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and subsequent UV excitation.

To confirm the obtained results, the positive samples were then amplified by PCR for cytochrome c oxidase subunit 1 gene (cox1), with the specific primers previously described in Bowles et al. (1992). Amplification was performed in a 20-µL final volume containing template DNA (1–10 ng), 0.2 mM premixed solution of dNTPs, 1 µM each primer, 1X PCR buffer, and 0.5 U of Taq DNA polymerase (GoTaq DNA Polymerase; Promega, Madison, WI, USA). The thermal profile was as follows: 2 min at 95 °C, 40 cycles of 45 s at 95 °C, 45 s at 57 °C and 1 min 30 s at 72 °C, followed by 10 min at 72 °C. After gel electrophoresis, PCR products were purified with the Wizard DNA Clean-Up System (Promega), and sequenced.

The positive controls was kindly provided by Prof Enrico Brunetti (Policlinico San Matteo Hospital Foundation, Pavia), ultrapure water was used as negative control in this experiment.

Analysis of nucleotide sequence data was performed with BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

Results

During the ultrasound screening, 102 individuals with abdominal CE were identified (Chebli et al. 2017), of whom 12 were candidate to surgery and 32 to percutaneous treatment.

Table 1 Characteristics of oligonucleotides used for *Echinococcus granulosus* complex multiplex PCR (Boubaker et al. 2013)

Primer name	Conc. in mPCR	Product size (bp)	Specificity	Sequence 5′–3′[a]	Primer lenght (bp)	Gene marker	Acc no. (NCBI)	Primer position
Echi Rpb2 F	1 µM	1232	All *E.* species	TTGACCAAAGAAATCAGAC	19	rpb2	FN566850.1	55–74
Echi Rpb2 R	1 µM	1232	All *E.* species	TGGTCGTCTTAATCATTTG	16	rpb2	FN566850.1	1287–1271
E.g complex F	0.15 µM	110	*E. granulosus* complex	TGGTCGTCTTAATCATTTG	19	cox2	AF297617.1	10,686–10,705
E.g complex R	0.15 µM	110	E. *granulosus* complex	CCACAACAATAGGCATAA	19	cox2	AF297617.1	10,796–10,777
E.g ss cal F	2 µM	1001	*E. granulosus* s.s. (G1/G/G3)	CAATTTACGGTAAAGCAT	18	cal	U834931.1	151–169
E.g ss cal R	2 µM	1001	*E. granulosus* s.s. (G1/G/G3)	CCTCATCTCCACTCTCT	17	cal	U834931.1	1152–1135
E.g ss Ef1a F	1 µM	706	*E. granulosus* s.s. (G1/G/G3)	TCCTAACATGCCTTGGTAT	19	ef1a	FN568380.1	594–613
E.g ss Ef1a R	1 µM	706	*E. granulosus* s.s. (G1/G/G3)	GTTACAGCCTTGATCACG	18	ef1a	FN568380.1	1300–1282
E.eq cal F	2 µM	426	*E. equinus* (G4)	GCTTATTTAGGATCCCA	17	cal	EU834936.1	566–583
E.eq cal R	2 µM	426	*E. equinus* (G4)	TCGTTTTTGCCAGTG	15	cal	EU834936.1	992–977
E.eq coxI F	0.2 µM	124	*E. equinus* (G4)	GTTGGgTTgGATGTT	15	cox1	M84664.1	143–158
E.eq coxI R	0.2 µM	124	*E. equinus* (G4)	CAAAACaGGATCACTCTT	18	cox1	M84664.1	277–259
E.ortp ATP6 R	0.05 µM	1041	*E. ortleppi* (G5)	GTGTCGTgTgTTTAgTGAG	19	atp-6	AF235846.1	6057–6076
E.ortp ATP6 F	0.05 µM	1041	*E. ortleppi* (G5)	GCACtGATAcaGGtGTtAtT	20	atp-6	AF235846.1	7098–7078
E.ortp CoxI F	0.2 µM	250	*E. ortleppi* (G5)	GGTTtTATGGGTTGTTA	17	cox1	AF235846.1	9978–9995
E.ortp CoxI R	0.2 µM	250	*E. ortleppi* (G5)	ACACCaCCAAACGTG	15	cox1	AF235846.1	10,228–10,213
E.cnd G6/G7 pold F	1 µM	617	*E. canadensis* (G6/G7)	GGCCTTCATCTCCATAATA	20	pold	FN568364.1	325–345
E.cnd G6/G7 pold R	1 µM	617	*E. canadensis* (G6/G7)	ATGAAGAGTTTGAAACTAAAG	21	pold	FN568364.1	942–921
E.cnd G6/G7 NDI F	0.3 µM	339	*E. canadensis* (G6/G7)	cTGCAGAGGTTTGCC	15	nad1	AB208063.1	7635–7650
E.cnd G6/G7 NDI R	0.3 µM	339	*E. canadensis* (G6/G7)	cACAACaGCAtAAAGCG	17	nad1	AB208063.1	7974–7957
E.cnd G8/G10 Elp F	1.5 µM	283	*E. canadensis* (G8/G10)	CCTAGTCTTCCCATGATA	18	elp1	U834894.1	450–468
E.cnd G8/G10 Elp R	1.5 µM	283	*E. canadensis* (G8/G10)	ACAGAAGGCATATCCA	16	elp1	U834894.1	733–717

Tiny characters mark additional polymorphic sites (but not strict)

[a]Strict specific bases in each primer are written in bold

A total of 157 samples were available for genotyping after microscopical assessment of cyst viability. Of these, only six samples resulted positive and the amplification size correspond to ef1a gene (G1–G3 complex; Fig. 1). The six samples were the tested for cox1 gene but only five samples resulted positive (Fig. 2). The nucleotide sequence were then obtained and analyzed and all the samples showed maximum homology (> 99%) with the G1 and G3 genotype sequences registered in GenBank, and were classified as belonging to the G1–G3 complex (*E. granulosus* sensu stricto).

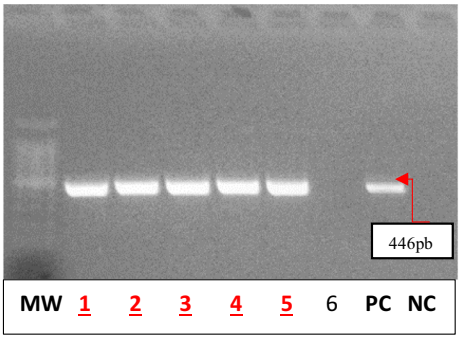

Fig. 2 Agarose gel electrophoresis of the 6 samples for Multiplex PCR using cox1 gene. *MW* Low molecular weight marker (100 bp), *lines* 1–6 the samples, *NC* Negative control, *PC* positive control, marker (100 bp Promega). In red: the positive samples (color figure online)

Discussion

Genotyping of *E. granulosus* in both human and animal hosts is important in all control programs (McManus and Smyth 1986). Different genotypes can develop differently in different intermediate hosts and it has been suggested that different genotypes may induce different clinical manifestations in humans (Schneider et al. 2010; Guarnera et al. 2004), although these data are scant and this aspect requires further investigation.

Echinococcus granulosus sensu stricto, also known as the « sheep strain » is the one reported in the vast majority of human cases (Alvarez Rojas et al. 2014) As mentioned above, G1 (*E. granulosus* sensu stricto) is the most frequent genotype found in animals in Morocco (El Berbri et al. 2015). Sheep and cattle are the main intermediate hosts, with high prevalence, which is in line with the high prevalence and surgical incidence of CE in humans in endemic areas of the country. This study, for the first time, identifies and confirms that the same species *E. granulosus* sensu stricto in Morocco affects also humans, who enter in the transmission dynamics of the domestic animals cycle, and support the hypothesis that this particular species is the one probably most infective to humans (Alvarez Rojas et al. 2014). Our results are in line with epidemiological data from other countries of the same region. In Algeria, the genotype of all samples (8 in total) analyzed of human origin was the G1–G3 complex (Sadjjadi et al. 2013; Bart et al. 2004). Same results were reported from Tunisia (Boubaker et al. 2013; Maillard et al. 2007; M'rad et al. 2005; Lahmar et al. 2009; Chahed et al. 2010). We need more studies to confirm or exclude the presence of the G6, genotype in Morocco, which is globally the second most frequent genotype, affecting humans, considering that the intermediate hosts where this genotype is most often found (camels, cattle, and goats) are bred in Morocco (Alvarez Rojas et al. 2014). The G6 genotype has been described as having a faster development in the dog compared to *E. granulosus* sensu stricto (Eckert et al. 1989, 1993).

The vaccine EG95 for sheep that can help in the control of echinococcosis is a recombinant protein from parasitic material of the G1 genotype from New Zealand (Lightowlers et al. 1996).

Recent studies have shown that this protein is immunologically different from the one deriving from the G6 genotype (Alvarez Rojas et al. 2013). Additional studies will be needed to determine the efficacy of the EG95 vaccine for other genotypes, and the need for the development of different genotype-specific vaccines. Knowing that the G1 genotype is the largely dominant, in Morocco, encourages the possible introduction of this

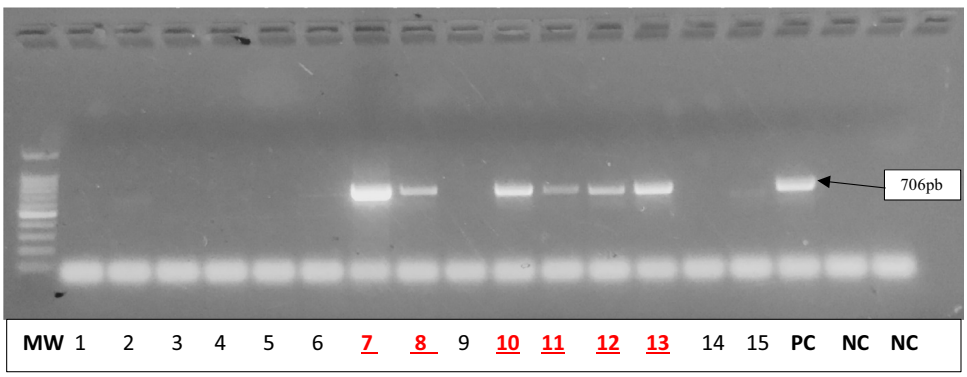

Fig. 1 The multiplex PCR results of 706 pb of Echinococcus granulosus human isolates. *Line MW* Low molecular weight marker (100 bp), *Promega; lines* 1–15: the samples, *line PC* positive control, *line NC* negative control. In red: the positive samples (color figure online)

vaccine in our country, and further highlights the reason why the genotyping of parasites circulating in the natural hosts and humans is important, as this information may influence dramatically the use of different tools in control programs. The multiple PCR developed by Boubaker et al. (2013) is a feasible method for the genotyping of hydatid cysts, even if does not allow to distinguish the different genotypes G1, G2 and G3 comprised in the *E. granulosus* sensu stricto complex.

This distinction can be done, for example, by the methods of Azlaf and Dakak (2006) and El Berbi et al. (2015) using mitochondrial cox1 and nad1 genes of *E. granulosus*. In this study, such distinction was not carried out as some authors have suggested that G1, G2 and G3 genotypes should be just indicated as the complex *E. granulosus* sensu stricto due to their global distribution and apparent lack of difference in host specificity (Thompson and McManus 2002; Jenkins et al. 2005). However, the identification of the precise genotype in human samples can give a more precise picture of the relations between genotypes affecting humans and animals. Unfortunately, the available material for this study was not enough to carry this additional characterization.

The poor efficiency demonstrated by the genotyping method applied in this study contrasts with the results shown by Boubaker et al. (2013). This may be explained by a suboptimal collection/storage of our samples. Whatever the reason, this should prompt a better and more extended analysis of the *E. granulosus* genotypes circulating in Morocco, and the Meknes-Tafilalt region, the zone of highest CE endemicity in Morocco, should constitute a privileged study site.

Conclusion

Our results show that the *E. granulosus* sensu stricto complex causes CE in humans in Morocco, which is in line with the regional and global epidemiological picture. They support the hypothesis that humans are particularly susceptible to this particular species, and highlight the role of its animal reservoirs in the transmission to humans. Larger and more discriminative studies are required in Morocco to better define the genotype distribution and confirm our results, with the aim of elaborating the better strategy for infection control.

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