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Two haplotype clusters of *Echinococcus granulosus* sensu stricto in northern Iraq (Kurdistan region) support the hypothesis of a parasite cradle in the Middle East

Zuber Ismael Hassan[a,1], Azad Abdullah Meerkhan[a,1], Belgees Boufana[b,c,*,1], Abdullah A. Hama[d,e], Bayram Dawod Ahmed[f], Wijdan Mohammed Salih Mero[a], Serra Orsten[g], Maria Interisano[b], Edoardo Pozio[b], Adriano Casulli[b,c]

[a] *Department of Biology, Faculty of Science, Zakho University, Kurdistan Region, Iraq*
[b] *Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità (ISS), Rome, Italy*
[c] *World Health Organization Collaborating Centre for the epidemiology, detection and control of cystic and alveolar echinococcosis (in humans and animals), ISS, Rome, Italy*
[d] *Technical College of Health, Medical Laboratory of Science, Sulaimani Polytechnic University, Kurdistan, Iraq*
[e] *College of Science, Komar University of Science and Technology, Kurdistan, Iraq*
[f] *Infection control unit, Directorate of Health, Duhok city, Kurdistan, Iraq*
[g] *Hacettepe University, Faculty of Medicine, Department of Medical Microbiology, Ankara, Turkey*

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ABSTRACT

Human cystic echinococcosis (CE) caused by *Echinococcus granulosus* s.s. is a major public health problem in Iraqi Kurdistan with a reported surgical incidence of 6.3 per 100,000 Arbil inhabitants. A total of 125 *Echinococcus* isolates retrieved from sheep, goats and cattle were used in this study. Our aim was to determine species/genotypes infecting livestock in Iraqi Kurdistan and examine intraspecific variation and population structure of *Echinococcus granulosus* s.s. in this region and relate it to that of other regions worldwide. Using nucleotide sequences of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox 1*) we identified *E. granulosus* s.s. as the cause of hydatidosis in all examined animals. The haplotype network displayed a double-clustered topology with two main *E. granulosus* s.s. haplotypes, (KU05) and (KU33). The 'founder' haplotype (KU05) confirmed the presence of a common lineage of non-genetically differentiated populations as inferred by the low non-significant fixation index values. Overall diversity and neutrality indices indicated demographic expansion. We used *E. granulosus* s.s. nucleotide sequences from GenBank to draw haplotype networks for the Middle East (Iran, Jordan and Turkey), Europe (Albania, Greece, Italy, Romania and Spain), China, Mongolia, Russia, South America (Argentina, Brazil, Chile and Mexico) and Tunisia. Networks with two haplotype clusters like that reported here for Iraqi Kurdistan were seen for the Middle East, Europe, Mongolia, Russia and Tunisia using both 827 bp and 1609 bp *cox1* nucleotide sequences, whereas a star-like network was observed for China and South America. We hypothesize that the double clustering seen at what is generally assumed to be the cradle of domestication may have emerged independently and dispersed from the Middle East to other regions and that haplotype (KU33) may be the main haplotype within a second cluster in the Middle East from where it has spread into Europe, Mongolia, Russia and North Africa. Further studies using metacestodes of human origin are required to investigate the biological importance of *E. granulosus* s.s. haplotypes/clusters and their association, if any with clinical manifestations of CE infection.

1. Introduction

Cystic echinococcosis (CE) caused by the metacestode stage of *Echinococcus granulosus* sensu lato s.l. is a parasitic zoonosis of major importance and worldwide distribution (Craig et al., 2007). Members of *E. granulosus* s.l. species complex include *Echinococcus granulosus* sensu stricto s.s. (genotypes G1-G3), *Echinococcus felidis* ("lion strain"), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) and *Echinococcus*

* Corresponding author at: Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanitá, Viale Regina Elena, 299, 00161 Rome, Italy.
E-mail address: belgees.boufana@iss.it (B. Boufana).
[1] Equal contribution.

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canadensis (G6/G7, G8, G10) (Nakao et al., 2007; Thompson, 2008; Huttner et al., 2008; Romig et al., 2015). The life cycle of *E. granulosus* s.s. is perpetuated between dogs and ungulates (mainly sheep) that serve as definitive and intermediate hosts, respectively. Infection occurs through the ingestion of the tapeworm eggs that develop into fluid-filled metacestode larvae primarily in the liver and lungs of livestock animals and humans.

Human CE is highly endemic in large parts of Iraq where it has been reported from Bagdad (Senekji and Beattie, 1940; Al Jeboori, 1976; Khalili et al., 1989; AI-Naimi et al., 2012), Basrah (Al-Mukhtar, 1989; Maktoof and Tabeekh, 2015; Thamir et al., 2015), Mosul (Al-Sakkal, 1982; Salih et al., 1983; Younis et al., 2008), Babylon (Molan and Baban, 1989), the southern region (Benyan and Mahdi, 1987; Molan, 1993) and Arbil, the capital city of Iraqi Kurdistan in northern Iraq (Al-Barwari et al., 1991; Saeed et al., 2000). Using retrospective admission records of two main hospitals in Arbil, CE surgical incidence for the period between 1990 and 1998 was estimated to be 2 per 100,000 inhabitants (Saeed et al., 2000). A recent retrospective study of patients' records from public and private hospitals showed an incidence of 6.3 per 100,000 inhabitants of Arbil population (Saida and Nouraddin, 2011). However, few reports on the molecular identification of human cystic echinococcosis causative agent are known to exist (Hama et al., 2012; Ahmed et al., 2013; Baraak, 2014).

CE is also prevalent in ungulate intermediate hosts in many Iraqi provinces (Al-Abbassy et al., 1980; Molan and Saeed, 1990; Wajdi and Nassir, 1983; Saeed et al., 2000; Saida and Nouraddin, 2011; Abdullah and Mero, 2013; Mero et al., 2014; Hassan et al., 2016). Additionally, consistently high *E. granulosus* infection rates in stray dogs have been reported over time from various regions of Iraq. In a study carried out in Bagdad, 17.83% of 123 dogs were found to be infected with *E. granulosus* (Senekji and Beattie, 1940). Infection rates in dogs from 11 localities in Arbil ranged from 66.7–100% (Molan and Saida, 1989) and an *E. granulosus* prevalence of 56% in 50 stray dogs was reported from Theqar southern province (Molan, 1993). The necropsy of 120 stray dogs in Mosul between 1997 and 1999 revealed an infection rate of 16.7% (Abdullah and Jarjees, 2005). To date, molecular epidemiological studies using hydatid isolates from ungulate intermediate hosts from Iraqi Kurdistan have been limited to one report from Slemani Province (Hama et al., 2013). In addition, no molecular confirmatory reports using DNA extracted from adult tapeworms removed from infected dogs at necropsy have been published.

Considerable intraspecific variation is known to exist within *E. granulosus* s.l. (Thompson and McManus, 2002) and the genetic polymorphism of *E. granulosus* s.s. metacestodes has been extensively studied (Nakao et al., 2010; Yanagida et al., 2012; Casulli et al., 2012; Boufana et al., 2014, 2015a; Romig et al., 2015; Mahami Oskouei et al., 2016; Kinkar et al., 2016; Laurimäe et al., 2016) and to a lesser extent that of the adult tapeworms (Boufana et al., 2015b). This study was conducted to molecularly investigate *Echinococcus* species/genotypes infecting livestock animals in Iraqi Kurdistan and determine the genetic variation and population structure of *E. granulosus* s.s. in this region and relate the results to those described worldwide.

2. Materials and methods

2.1. Samples, DNA extraction, PCR amplification and sequencing

A total of 125 hydatid cyst isolates collected between July 2013 and June 2014 from livestock animals (sheep n = 66; goats n = 24; cattle n = 21) slaughtered in district abattoirs of Arbil and Duhok Provinces in Iraqi Kurdistan and Mosul in the north of Iraq (sheep n = 13; cattle n = 1) were included in this study. Hydatid cysts removed from the liver, lungs and spleen were transported to the laboratory and protoscoleces and/or germinal layers were aseptically collected and stored in 70% ethanol. Total genomic DNA was extracted from hydatid cyst material using the Qiagen DNeasy Blood and Tissue DNA extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A fragment within the mitochondrial cytochrome *c* oxidase subunit 1 (*cox 1*) gene was amplified using published methodology (Nakao et al., 2000). PCR products were electrophoresed in 1.5% (w/v) ethidium bromide stained agarose gels in 1X Tris-Borate-EDTA buffer at 110 V and viewed using UV illumination (Syngene G:Box gel documentation system, Cambridge Biosciences). Amplified products were purified using QIAquick PCR Purification Kit (Qiagen) and commercially sequenced in both directions using the PCR primers (Macrogen EZ- Sequence, Amsterdam, The Netherlands). Chromatograms were viewed using FinchTV trace viewer (Geospiza, Seattle, WA, USA) to verify peak quality. The identity of the sequenced nucleotide fragments was ascertained through the use of BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Combined Echinococcus granulosus s.s. dataset

To compare *E. granulosus* s.s. isolates from Iraqi Kurdistan with those from other world regions, a broader nucleotide sequence dataset was created to include *E. granulosus* s.s. sequences from the NCBI database (http://www.ncbi.nlm.nih.gov). C*ox 1* mitochondrial nucleotide sequences of *E. granulosus* s.s. metacestodes from Albania n = 2, Greece n = 1, Italy n = 6, Romania n = 1, Spain n = 10, Turkey n = 60 (Kinkar et al., 2016); China n = 62, Iran n = 15, Jordan n = 12 (Yanagida et al., 2012; Wang et al., 2014, unpublished; Mohammadzadeh et al., 2011, unpublished); Mongolia n = 29 (Ito et al., 2014, Narankhajid et al., 2013, unpublished); Russia, Altai n = 7, Novosibirsk Oblast n = 1, Permskiy Krai = 2, Republic of Bashkiria n = 1 (Konyaev et al., 2012; Konyaev et al., 2013); Argentina n = 17, Brazil n = 6, Chile n = 4, Mexico n = 1 (Laurimäe et al., 2016) Chile n = 21 (Alvarez Rojas et al., 2017) were used (Table 1). In addition, Tunisian *E. granulosus* s.s. metacestode *cox 1* nucleotide sequences of animal and human origin (n = 123) used in a previous study (Boufana et al., 2014) were also included. The GenBank sequences were of different lengths (1609 bp or 1674 bp) to those analysed in this study and were, therefore, trimmed to equal lengths and we used 381 *E. granulosus* s.s. *cox 1* nucleotide sequences to generate regional haplo-

Table 1
Echinococcus granulosus sensu stricto nucleotide sequence data retrieved from GenBank database and used in this study.

Countries	GenBank Accession numbers	Reference
Albania	KU925432-KU925433	Kinkar et al., 2016
Argentina	KX039937-KX039953	Laurimäe et al., 2016
Brazil	KX039955-KX039960	Laurimäe et al., 2016
Chile	KX039961-KX039964	Laurimäe et al., 2016
Chile	KX227116-KX227136	Alvarez Rojas et al., 2017
China	AB688602-AB688619	Yanagida et al., 2012
	KJ628328-KJ628335, KJ628337-KJ628357, KJ628359-KJ628373	Wang et al., 2014, unpublished
Greece	KU925430	Kinkar et al., 2016
Iran	JQ250806-JQ250817	Yanagida et al., 2012
	JQ219962-JQ219964	Mohammadzadeh et al., 2011, unpublished
Italy	KU925423-KU925428	Kinkar et al., 2016
Jordan	AB688590-AB688601	Yanagida et al., 2012
Mexico	KX039965	Laurimäe et al., 2016
Mongolia	AB893242-AB893251	Ito et al., 2014
	AB787529-AB787536 AB787538-AB787548	Narankhajid et al., 2013, unpublished
Romania	KU925431	Kinkar et al., 2016
Russia	AB688136-AB688141	Konyaev et al., 2012
	AB777904-AB777908	Konyaev et al., 2013
Spain	KU925413-KU925422	Kinkar et al., 2016
Turkey	KU925351-KU925360, KU925362-KU925368, KU925370-KU925387, KU925389- KU925398, KU925400-KU925412, KU925361 KU925369	Kinkar et al., 2016