Cystic echinococcosis in Poland: genetic variability and the first record of *Echinococcus granulosus* sensu stricto (G1 genotype) in the country

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Abstract Cystic echinococcosis is one of the most important zoonotic diseases affecting humans and livestock worldwide, and is endemic in Poland. A set of six isolates on larval stages of *Echinococcus granulosus* sensu lato tapeworms collected from three humans, two pigs and one sheep from Polish foci of CE was examined by DNA sequencing of two mitochondrial genes (*cox1*, *rrnS*). The results demonstrated the presence of *E. canadensis* and *E. granulosus* sensu stricto in the investigated hydatid cysts. The former species was found in all five isolates from pigs and humans derived from central Poland. In a sheep hydatid cyst originating from Lesser Poland Voivodeship in southern Poland, *E. granulosus* s. s. (G1 genotype) was identified. This is the first report of an unambiguously autochthonous infection with *E. granulosus* s. s. in Poland. The global distribution and host affiliations of the commonly occurring G1 microvariant with nucleotide change 56C/T in *cox1*, detected here in Polish sheep, are discussed. The finding that sheep harboured *E. granulosus* s. s. may have important consequences for developing effective hydatid control programmes in Poland due to its longer maturation rate in dogs compared with *E. canadensis* G7. This may lead to greater expenditures for purchasing anthelmintics to provide an appropriate dosing regime in sheep-raising areas of the country.

Keywords *Echinococcus granulosus* · Poland · DNA sequencing · Genotype · Sheep · Human · Pig

Introduction

The larval stages of the tapeworm *Echinococcus granulosus* sensu lato are the causative agent of cystic echinococcosis (CE), one of the most important cestode infections causing morbidity and mortality in humans and significant economic losses in livestock. Around one million or more people are currently suffering from CE globally, and the financial burden of the disease on the livestock industry is substantial, with up to $2 billion lost annually (Torgerson and Macpherson 2011).

According to the current nomenclature, *E. granulosus* s. 1. circulating in Europe has been subdivided into *E. granulosus* sensu stricto (“sheep strain” and “buffalo strain”, genotypes G1 and G3), *Echinococcus equinus* (“horse strain”, G4), *Echinococcus ortleppi* (“cattle strain”, G5) and *Echinococcus canadensis* (“camel strain”, G6; “pig strain”, G7; “two cervid strains”, G8 and G10) (Romig et al. 2015). Human cystic echinococcosis is caused predominantly (approximately 90%
of cases worldwide) by *E. granulosus* s. s., which has the most cosmopolitan distribution and is largely transmitted in areas with extensive sheep farming (Alvarez Rojas et al. 2014). That species is followed by *E. canadensis* (genotypes G6–G10), globally responsible for about 10% of human infections. Among the remaining species traditionally classified as *E. granulosus* sensu lato, only nine human infections with *E. ortleppi* (G5) and no infection with *E. equinus* (G4) have been reported to date (Alvarez Rojas et al. 2014; Grenouillet et al. 2014).

In Poland, in 2015, over 4500 cases of cystic echinococcosis in farm animals were recorded according to the report of the European Food Safety Authority (EFSA and ECDC 2016), and in 99.56% of those cases, pigs were the intermediate hosts. Only 0.43 and 0.01% of cases of cystic echinococcosis were recorded in sheep and cattle, respectively. All genotyped metacestodes originating from humans, domestic pigs and the European beaver belonged to the G7 genotype of *E. canadensis* (summarized in Cardona and Carmena 2013; Alvarez Rojas et al. 2014). In geographical terms, transmission of the G7 genotype is largely confined to a contiguous zone in central and eastern Europe including the Baltic region. No records about the circulation of highly pathogenic *E. granulosus* s. s. in Poland and/or neighboring countries are available so far.

The study was conducted to extend the knowledge about the genotype spectrum of *Echinococcus granulosus* tapeworms circulating in sheep, pigs and humans in Poland.

### Material and methods

**Sample collection** *Echinococcus* protoscoleces were collected from the livers of naturally infected pigs (two isolates from the Masovian Voivodeship in central Poland) and sheep (one isolate from Podhale district in Lesser Poland Voivodeship in southern Poland). Three human samples were derived from surgically removed hydatid cysts from patient livers at the Department of General Transplant and Liver Surgery, Medical University of Warsaw (central Poland). Sheep and pig samples were frozen at −20 °C, subsequently defrosted and stored in 70% ethanol. Human samples were stored in 70% ethanol.

**DNA extraction** Fragments of ethanol-preserved hydatid cyst samples were dried at room temperature, homogenised and subjected to DNA isolation by the silica-guanidinium procedure (Boom et al. 1999).

**DNA amplification and sequencing** A gene fragment of cytochrome c oxidase subunit 1 (*cox1*, 396 bp) was amplified with JB3/JB4.5 primers (Bowles et al. 1993) from mitochondrial DNA of all isolates. In the sheep isolate, a portion of the small subunit ribosomal RNA gene (*rrnS*, 372 bp) was amplified with P60/P375 primers (Dinkel et al. 1998). Amplified PCR products of both mitochondrial genes were then subjected to automated Sanger sequencing.

**Sequence analysis** The sequences of the *cox1* gene were compared to the reference sequences (Bowles et al. 1992) of *E. granulosus* (genotypes G1–G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5) and *E. canadensis* (genotypes G6 and G7). The sequences of the mitochondrial small subunit rRNA were compared to the reference sequences (Dinkel et al. 2004; Busi et al. 2007) of *E. granulosus* s. s. (G1 and G3) and *E. canadensis* (G6 and G7). The multiple sequence alignments were performed using the CLC Main Workbench 7 software. Generated haplotypes were identified through BLASTn analysis. To distinguish synonymous and non-synonymous mutations, EMBOSS transeq software for deriving protein sequences was used. The sequences reported in this paper were deposited in the GenBank database with the accession numbers KJ831062, KM191134, MF580386 and MF580387.

**Morphological analysis** Protoscoleces were mounted in Hoyer’s medium (Cielecka et al. 2009) and pressure was applied to the coverslip to cause the hooks to lie flat. All measurements were taken by the same person (D. C.) using a calibrated eyepiece micrometer under oil immersion. The number of rostellar hooks, the length of the blades of large and small hooks and total length of the large and the small hooks were considered. The hooks were measured according to Ponce Gordo and Cuesta Bandera (1997). Only invaginated, viable protoscoleces were analysed.

**Results**

Based on the sequences the *cox1* gene fragment five isolates (2 from pigs and 3 from humans) were classified as bearing the G7 genotype. The isolate from sheep was identified as the G1 genotype based on the sequence of the fragments of *cox1* and *rrnS* genes and herein was provisionally designated as G1A microvariant sensu Šnábel et al. (2009) (Table 1).

**cox1**

The sequence of the *cox1* gene of the isolate from sheep had the highest level of similarity to reference genotypes G1–G3 of *E. granulosus* s. s., with one substitution (56C/T) compared to the reference G1 genotype, two substitutions (66T/C, 257C/T) compared to previously assigned G2 genotype, and three substitutions (56C/T, 66C/T, 257C/T) compared to the G3 genotype. The non-synonymous nucleotide change with a
| Region/countries | Host (n) | GenBank accession numbers | References |
|------------------|----------|--------------------------|------------|
| **Africa**        |          |                          |            |
| Algeria           | Human (2) | KR349028                 | Zait et al. (2016) |
| Ethiopia          | Sheep (3), cattle (1), camel (1) | AB650531 | Hailemariam et al. (2012) |
| Morocco           | Camel    | EF367279                 |            |
| Morocco           | Cattle (2) | EF367280, EF367283      |            |
| Morocco           | Goat     | EF367281                 |            |
| Morocco           | Mule     | EF367285                 |            |
| Morocco           | Sheep (2) | EF367282, EF367284      |            |
| Tunisia           | Cattle (3), human (3), sheep (1) |            | M'rad et al. (2005) |
| Tunisia           | Cattle (6), human (2), sheep (1) |            | M'rad et al. (2010) |
| Tunisia           | Donkey (7), sheep (4), cattle (1) | KM014642 | Boufana et al. (2014) |
| Tunisia           | Human    | KM014643                 | Boufana et al. (2014) |
| Tunisia           | Sheep (2), wild boar (1) | KM014641 | Boufana et al. (2014) |
| Africa (country of origin not known) |          |                          |            |
| Armenia           | Cattle (6) | KX020338, KX020339, KX020344, KX020345, KX020368, KX020372 |            |
| Armenia           | Goat     | KX020377                 |            |
| Armenia           | Human (5) | KX020337, KX020341, KX020359, KX020365, KX020367 |            |
| Armenia           | Sheep (8) | KX020336, KX020357, KX020383, KX020386, KX020388, KX020391, KX020392, KX020402 |            |
| China, Qinghai province | Sheep (3) | AB491421 | Nakao et al. (2010) |
| China, Xinjiang province | Human (2) | AB491439, AB491447 | Nakao et al. (2010) |
| China, Xinjiang province | Human (3), dog (11) | DQ356877 | Bart et al. (2006) |
| Kazakhstan        | Dog      | KT001396                 | Boufana et al. (2015) |
| Mongolia          | Human    | AB8935246                | Ito et al. (2014) |
| Mongolia          | Human (2) | AB787546, AB787548      |            |
| Mongolia          | Sheep (2) | AB787531, AB787538      |            |
| Russia (Altai Krai) | Human   | AB688139                 | Konyaev et al. (2012a) |
| Russia (Novosibirsk Oblast) | Human | AB688140 | Konyaev et al. (2012a) |
| Europe            |          |                          |            |
| Albania           | Sheep    | KU925433                 | Kinkar et al. (2016) |
| Austria           | Human    | AJ508019                 | Obwaller et al. (2004) |
| Bulgaria          | Human    | KY235681                 |            |
| Greece            | Sheep    | KM245580                 |            |
| Hungary           | Human    | JF690976                 | Šnábel et al. (2016) |
| Italy             | Sheep (3) |                          | Busi et al. (2007) |
| Moldova           | Sheep (6), cattle (2) | KJ782437 | Umhang et al. (2014) |
| Poland            | Sheep    | KJ831062                 | this study |
| Portugal          | Sheep    | HF947559                 | Beato et al. (2013) |
| Romania           | Cattle   | KU925431                 | Kinkar et al. (2016) |
| Russia (Permiskiy Krai) | Sheep | AB777906 | Konyaev et al. (2013) |
| Spain             | Sheep    | KU925419                 | Kinkar et al. (2016) |
| **Middle East**   |          |                          |            |
| Iran              | Camel    | JQ250814                 | Yanagida et al. (2012) |
| Iran              | Camel    | HM563013                 |            |
| Iran              | Dog      | KP399046                 | Gholami et al. (2016) |
| Iran              | Dog      | JN604908                 | Parsa et al. (2012) |
| Iran              | Goat     | KR337820                 |            |
| Iran              | Sheep (11), cattle (7), human (6) | KP659560 | Farhadi et al. (2015) |
| Iran              | Human    | AB677811                 | Pezeshki et al. (2013) |
| Iran              | Human (2) | JQ250810, JQ250812      | Yanagida et al. (2012) |
| Iran              | Sheep    | JQ219962                 |            |
| Iran              | Sheep    | KP751431                 |            |
| Iran              | Sheep    | HM563012                 |            |
| Iran              | Human    | KM513627                 | Sharbatkhori et al. (2016) |
| Iran              | Sheep    | KT074944                 | Sharbatkhori et al. (2016) |
| Iran              | Cattle   | KT074945                 | Sharbatkhori et al. (2016) |
| Iran              | Camel    | KT074946                 | Sharbatkhori et al. (2016) |
thymine at position 56, which induces substitution of alanine with valine, is typical of the G2 genotype, but the remaining nucleotides of sheep isolate corresponded to the sequence pattern of the G1 genotype.

The cox1 sequences obtained from human and swine isolates were identical to the reference sequence for genotype G7 of *E. canadensis*. Multiple sequence alignments are presented in Fig. 1.

**Morphological characteristics of protoscoleces**

The rostellar hook characteristics of protoscoleces of Polish sheep origin are shown in Table 2. Morphometrical data suggested that rostellar hooks in the examined sheep were apparently shorter than those previously measured from pig and humans in the same region of Europe (Poland and Ukraine), whereas a high similarity in hook sizes was found in relation to previously analysed sheep isolates from UK and Spain.

**Discussion**

The presented data provide the first evidence of the presence of autochthonous infection with *E. granulosus* s. s. in Poland,
Table 2  Morphometric data of protozoa of Echinococcus granulosus s.s. from pig and sheep in Europe

| Geographical origin | Total number of hooks | Blade length (μm) | Blade length (μm) | Small hooks | Large hooks | Total length (μm) | Total length (μm) |
|---------------------|----------------------|------------------|------------------|------------|------------|------------------|------------------|
| Host: sheep         |                      |                  |                  |            |            |                  |                  |
| Thompson et al. 1984 UK | 25.3 ± 0.9 (24.0–27.0) | 12.4 ± 0.5 (12.0–13.0) | 21.4 ± 1.5 (18.0–23.0) | 22.1 ± 0.8 (21.0–24.0) | 20.7 ± 2.4 (18.0–22.4) | 20.8 ± 1.13 (18.0–27) | 21.1 ± 1.33 (18.0–22.7) |
| Kumaratilake et al. 1986 UK | 24.6 ± 0.7 (24.0–27.0) | 12.8 ± 0.4 (12.0–13.0) | 21.1 ± 1.5 (18.0–23.0) | 20.7 ± 2.2 (18.0–22.4) | 20.8 ± 1.13 (18.0–27) | 21.1 ± 1.33 (18.0–22.7) | 21.1 ± 1.33 (18.0–22.7) |
| Ponce Gordo and Cuesta Bandera 1997 Spain | 21.00 ± 0.37 (20.2–21.8) | 11.1 ± 0.8 (10.8–11.8) | 19.9 ± 1.4 (19.3–20.3) | 20.8 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) |
| Eckert et al. 1993 Poland | 26.5 ± 0.5 (26.0–27.0) | 11.8 ± 0.8 (10.8–12.8) | 20.9 ± 1.4 (20.3–21.3) | 20.8 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) |
| Cielecka et al. 2005 Ukraine | 26.5 ± 0.5 (26.0–27.0) | 11.8 ± 0.8 (10.8–12.8) | 20.9 ± 1.4 (20.3–21.3) | 20.8 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) | 21.1 ± 1.4 (19.3–20.3) |

Data represent mean values ± SD. Ranges are given in parentheses.
patient (Šnábel et al. 2016). In the latter human case originating from Békes county, the microvariant G1A identical to that seen in the present study was detected. Northward from Poland, *E. granulosus* s. s. G1 was recently identified in 4 (2.2%) urban dogs in Estonia (Tartu city) in the Baltic region, although *E. granulosus* tapeworms are primarily transmitted in the country through sylvatic cycle, maintained by moose and wolves harbouring *E. canadensis* G8 and G10 genotypes (Laurimaa et al. 2015). In a part of Russia belonging to eastern Europe, three *E. granulosus* s. s. G1 cases were documented in a domestic cat from Saint Petersburg (Konyaev et al. 2012b), in a sheep from Permksiy Krai and in a human from the Republic of Bashkiria (Konyaev et al. 2013). Sheep farming strongly affects the distribution of *E. granulosus* s. s in Europe, although involvement of cattle and goats as intermediate hosts may also be considerable in some regions.

The G1A microvariant, which bears the substitution C/T at position 56 relative to the common G1 type, commonly occurs in the southern Palearctic; we have found 206 records in GenBank entries and published articles with this sequence pattern worldwide (summarized list is in Table 1). According to the available data, the highest frequencies of this cosmopolitan G1A form were to date recorded in Asia and Africa, which account for 6.34% (136/2143) and 9.03% (47/436) of the total numbers of *E. granulosus* s. s.—genetically determined isolates in these continents. Interestingly, the frequency of G1A haplotype in Europe was 1.19% (14/1172) in compiled data that is a markedly lower distribution rate than those estimated in Asia and Africa. In main intermediate hosts of *E. granulosus* s. s., sheep and cattle, the proportion of rarer haplotypes in European populations has decreased with the increased distance from the domestication centre in the Middle East (Ramnamäe et al. 2016). A similar scenario has likely occurred in their *Echinococcus* parasites, in which a part of genetic diversity was lost during their past distribution along the Mediterranean shore with livestock hosts. A low occurrence of G1A in Europe would also partially explain the lack of this haplotype in South America where only two findings (accounting for 0.20% frequency, 2/997) were to date documented in sheep from Brazil and in cattle from Argentina (Beato et al. 2013; Laurimae et al. 2016). The vast majority of cattle and sheep was imported to South America since sixteenth century from Europe (Arelovich et al. 2011), where the G1A haplotype is not abundant. Also several imports of livestock from Australia performed since the beginning of the twentieth century (Haag et al. 2004) could not contribute to G1A dispersal in South America considering its absence in the former continent according to available data.

There is an apparent link of the G1A haplotype with a cluster affiliated to the Turkish haplotype Tur35, which was detected as one of the two central haplotypes in a recent palaeogeographical study of G1 distribution in the Mediterranean region, conducted by screening 8274 bp of mtDNA (Kinkar et al. 2016). Sixteen of 18 haplotypes from Turkey, Albania and Romania identified in the above study as derived from Tur35 isolate, located in eastern Turkey in the vicinity of a domestication centre for the majority of livestock species, possessed the 36C/T nucleotide exchange. Dominance in a frequency of G1A findings in Africa over Europe might reflect earlier arrival and establishment of *E. granulosus* with sheep and other livestock in North Africa than in Europe, but it is more likely caused due to stochastic bottleneck events associated with founder effects.

The G1A variant was so far identified in 76 sheep, 50 cattle, 40 humans, 8 water buffaloes, 7 donkeys, 5 camels, 3 goats, 1 wild boar, 1 mule and 1 red-tailed guenon within intermediate hosts (Table 1). Humans are globally infected with G1A in similar proportions as major livestock intermediate hosts (sheep to humans ratio 1.9, cattle to humans ratio 1.25), compared to the overall figure derived from published G1 records, encompassing 1478 sheep, 1492 cattle and 929 human isolates (ratio sheep to humans 1.59, cattle to humans ratio 1.61). Although a higher number of human G1A isolates was detected especially in comparison to cattle, differences in distributions of G1 and G1A genotypes in respective hosts are not yet statistically significant at *p* < 0.05 (Fisher’s exact test; *p* = 0.27 for cattle/human comparisons, *p* = 0.43 for sheep/human comparisons). Nucleotide substitutions seen in G1A genotype thus do not seemingly confer a higher virulence for this variant towards humans and do not present any epidemiological relevance.

Results obtained from rostellar hook morphology of protoscoleces from sheep of Polish origin corroborated our genetic determination in measuring shorter hooks than those from pig cysts from Poland and Ukraine (referenced data obtained from Eckert et al. 1997; Yemets 2003). The hooks were also shorter than those from humans in Poland and Ukraine (referenced by Cielecka et al. 2005) that had been later genetically confirmed as belonging to the “pig strain” (attributable to *E. canadensis* G7). Size differences were not so striking compared to pig isolates of Spanish origin (provided by Ponce Gordo and Cuesta Bandera 1997) in some hook characteristics; however, Spanish isolates could contain a mixture of G7 and G1 genotypes considering later reports on pig findings in the country (González et al. 2002; Daniel Mwambete et al. 2004).

The hooks had similar size to those originating from Spain and UK in the material obtained from sheep (Spain, UK) and humans (Spain) (referenced by Thompson et al. 1984; Kumaratilake et al. 1986; Ponce Gordo and Cuesta Bandera 1997). The cysts isolated from Spanish patients contained “sheep–cattle strain” (sensu Ponce Gordo and Cuesta Bandera 1997) that is now presumed to be *E. granulosus* s. s. given the territory and host concerned, which applies also for morphologically examined sheep samples from the UK by the above-mentioned authors. Nevertheless, data of rostellar
hook morphology has to be interpreted with some caution given the effect of environmental factors, particularly host species (Hobbs et al. 1990).

High endemicity of human CE is being reported from areas with frequent transmission of *E. granulosus* s. s. The finding of infectious *E. granulosus* s. s. G1 in Poland, thus poses a threat to public health and may be relevant to the implementation of hydatid control in the country. As dosing regimes of dogs in control programmes are locally designed for the shorter development time of *E. canadensis* G7 (approx. 34 days p. i.), further measures should take into consideration simultaneous occurrence of more slowly developing *Echinococcus* s. s. with average maturation rate 45 days p. i. (Kumaratilake et al. 1983; Eckert et al. 1993). In the epidemiological situation in Poland characterized by intense transmission of *E. canadensis* G7 in domestic animals, a sporadic occurrence of *E. granulosus* s. s. should also be taken in account. Further metacestode samples should be analyzed from a variety of intermediate hosts (with special attention paid to sheep and humans) in concerned regions to provide a more detailed picture about the genotypic diversity of *E. granulosus* in Poland.

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