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# Use of the EmsB microsatellite-based next generation sequencing for genotyping of *Echinococcus granulosus* sensu lato in hydatid cyst tissue samples from animals and humans

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**Abstract:** *Echinococcus granulosus* (Batsch, 1786), a cestode of the Teniidae family, causes human cystic echinococcosis (CE) also known as hydatid disease. *Echinococcus granulosus* sensu lato includes the G1, G3, G4, G5, G6/7 and G8/10 genotypes which are known to cause human CE. This study aimed to differentiate genotypes of *E. granulosus* s.l. complex by employing EmsB, a tandemly repeated multilocus microsatellite, using next-generation sequencing (MIC-NGS). Human and animal histopathology-confirmed hydatid cyst tissue samples and reference DNA samples of *E. granulosus* G1, G3, G4, G5, G6/7 and G10 underwent MIC-NGS assay with custom primers amplifying a 151 bp EmsB DNA fragment. NGS data were analysed using online Galaxy analysis pipeline, a phylogenetic tree was constructed by MEGA software, and haplotype networking was performed with PopArt 1.7. All sixty samples (49 from animals and 11 from humans) included were successfully identified and genotyped with a 100% success rate. The study showed improved discrimination power to distinguish all study samples including closely related *E. granulosus* s.s. genotypes G1–G3. The maximum likelihood tree reaffirmed the monophyly of *E. granulosus* s.l. The median-joining haplotype networking revealed 12 distinct haplotypes. In conclusion, MIC-NGS assay was shown to be sensitive, specific and simple to apply to clinical samples offering a powerful discriminatory tool for the genotyping of *E. granulosus* s.l.

**Key words:** *Echinococcus granulosus* sensu stricto, echinococcosis, EmsB, genotyping, microsatellite, next-generation sequencing

This article contains supporting file (Supplementary Fig. S1) online at http://folia.paru.cas.cz/suppl/2024-71-014.pdf

Echinococcosis is a zoonotic disease that is presented in three forms: cystic echinococcosis (CE), alveolar echinococcosis (AE) and neotropical echinococcosis (NE) (Casulli et al. 2022, WHO 2023). Cystic echinococcosis (CE); also known as hydatid disease or hydatidosis; is a worldwide zoonotic disease affecting animals and humans. CE is caused by the metacestodes of the *Echinococcus granulosus* (Batsch, 1786) sensu lato (s.l.) complex.

The genetic determination of *E. granulosus* involves identifying specific genotypes within the species complex, which are designated by numbers such as G1, G2 and G3. These genotypes are distinguished through molecular and genetic analyses of mitochondrial and nuclear genes, which detect variations in their DNA sequences. *Echinococcus granulosus* sensu stricto (s.s.) (G1–G3) accounts for the majority of global CE cases (Alvarez Rojas et al. 2014).

The life cycle involves the dog as a definitive host and herbivorous animals as intermediate hosts, while humans act as the accidental intermediate host (Centers for Disease Control and Prevention 2023). *Echinococcus granulosus* s.l. complex consists of five species; *Echinococcus* Rudolphi, 1801 sensu stricto (s.s.) (G1 and G3), *E. equinus* (Williams et Sweatman, 1963) (G4), *E. ortleppi* Lopez-Neyra et Planas, 1943 (G5), *E. canadensis* Cameron, 1960 (G6, 7, 8 and 10), and *E. felidis* Ortlepp, 1937 (Lymbery 2017). *E. granulosus* s.s. includes genotypes G1 (sheep strain) and G3 (buffalo strain) with genotype G2 becoming a variant of G3, no longer a separate genotype (Kinkar et al. 2018, Casulli et al. 2022).

Human CE leads to the development of hydatid cysts in the liver and lungs, and less frequently in the kidney, spleen and other organs. Hepatic CE accounts for approximately

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60–70 % of CE cases; the symptoms include hepatomegaly, abdominal pain, nausea and vomiting. Cholestatic cirrhosis and mild to severe biliary complications may develop in some cases (Botezatu et al. 2018). In the West Bank – Palestine, the six-year average surgical incidence was 2.1 per 100,000, in which two districts (Al-Khalil and Bethlehem) showed statistically significant foci of CE (Al-Jawabreh et al. 2017). On the other hand, the socioeconomic burden of CE is related to its effect on humans and livestock, with increased disability-adjusted life years (DALYs) and monetary losses resulting from decreased livestock production and treatment costs (Budke et al. 2006, Widdicombe et al. 2022).

The variants of *E. granulosus* s.l. complex showed great intraspecific variability all over the world, leading to the emergence of five species (genotypes) based on phylogenetic patterns and evolutionary processes using mitochondrial DNA gene sequences (Lymbery 2017). Knowledge about *E. granulosus* s.l. genotypes is of great value for epidemiological studies as well as for control and prevention measures, as the genetic variation has been related to the transmission dynamics of the parasite and differences in vaccine response (Alvarez Rojas et al. 2014).

In this context, several genes have been targeted for detection identification and genotyping of the *E. granulosus* s.l. complex, such as the mitochondrial cytochrome c oxidase subunit 1 (*cox*1), mitochondrial 12S rRNA gene, and NADH dehydrogenase 1 (*nad*1) genes (Bowles et al. 1992, Stefanic et al. 2004, Trachsel et al. 2007, Bohard et al. 2023), as well as the EmsB, a tandemly repeated multilocus microsatellite that showed a higher degree of genetic diversity within *Echinococcus multilocularis* Leuckart, 1863 from rodents and human samples (Bart et al. 2006).

Recently, EmsB microsatellite marker has been compared to mitochondrial genes for the genotyping of *E. multilocularis* by Bohard et al. (2023). EmsB in the genome of *E. granulosus* contains the same number of repeats (40 copies of CA and GA) as in *E. multilocularis*, with large genetic variation and a low probability of homoplasy. The presence of two identical EmsB sequences in *E. granulosus* and *E. multilocularis* is due an evolutionary event and not to common ancestry (Valot et al. 2015). This highly polymorphic EmsB microsatellite has been employed in the genotyping of *E. multilocularis* (Knapp et al. 2007).

Furthermore, Maillard et al. (2009) used this marker to present a detailed map of the distribution of genetic variants of *E. granulosus* s.l. in isolates from different countries. They also presented a hybrid genotypic profile that suggested genetic exchanges between *E. granulosus* s.s. and *E. ortleppi* and therefore it was used for the determination and tracking the source of CE (Maillard et al. 2009). In parallel, different DNA-based techniques have been described for the detection or genotyping of *E. granulosus* s.l. complex including PCR-Restriction fragment length polymorphism (RFLP), Sanger sequencing, high resolution melting (HRM) analysis, and LAMP (loop-mediated isothermal amplification) (Salant et al. 2012, Rostami et al. 2013, Sakalar et al. 2014, Hamamci et al. 2023). However, these molecular methods have different sensitivity and discriminatory power (Bonelli et al. 2021).

In our previous study, we chose a 440 bp target within the *cox*1 for identification of *Echinococcus* species and genotypes in CE cysts surgically-removed from patients in Palestine. The study was carried out by the conventional Sanger sequencing and revealed that *E. granulosus* s.s. (G1–sheep genotype) was the causative agent in the analysed samples (82 %; 9/11) (Al-Jawabreh et al. 2017). Our results were consistent with the world trend that *E. granulosus* s.s. (G1) was responsible for the majority of human CE cases (Alvarez Rojas et al. 2014, Debeljak et al. 2016, Al-Jawabreh et al. 2017). However, the Sanger sequencing technique required a high pure DNA yield, thus requiring amplicon cleaning prior to sequencing as well as forward and reverse sequencing, making it impractical for large scale epidemiological studies (Al-Jawabreh et al. 2017).

Probe-based methods have been recently used for the identification and genotyping of species of the *E. granulosus* s.l. complex by utilising quantitative PCR (qPCR). Regions of the *E. granulosus* s.l. complex mitochondrial genome were targeted by specific primers and their corresponding probes to identify common species (Maksimov et al. 2020). The aim of this study was to develop an EmsB-microsatellite-based detection and genotyping method using next-generation sequencing with genotype-specific virtual probes for the purpose of large-scale epidemiological surveys.

## MATERIALS AND METHODS

### Sample collection

Archived deeply-frozen hydatid cyst tissue samples and formalin-fixed paraffin-embedded (FFPE) hydatid cyst tissue, both collected from humans during the period 2012–2014, were included in this study. The samples were obtained from CE patients confirmed by histopathological examination in different Palestinian hospitals as well as DNA sequencing. In addition, hydatid cyst tissue samples were collected from livestock animals during the period 2017–2018 from different abattoirs in the West Bank, Palestine, and kept at -70 °C until use.

### Reference DNA samples

Six DNA samples of *Echinococcus granulosus* s.l. identified as G1 (*E. granulosus* s.s.), G3 (*E. granulosus* s.s.), G4 (*E. equinus*), G5 (*E. ortleppi)*, G6/7 (*E. canadensis*), and G10 (*E. canadensis*) were kindly provided by the World Health Organisation Collaborating Centre for the Epidemiology, Detection, and Control of Cystic and Alveolar Echinococcosis (in humans and animals), Higher Institute of Health, Rome, Italy to be used as standards to validate the MIC-NGS (microsatellite-next-generation sequencing) assay. Moreover, two DNA controls, *E. granulosus* (G1) and *Echinococcus multilocularis*, were provided by Ibrahim Abbasi from Al-Quds University, Palestine.

### DNA extraction from tissue samples

In preparation for DNA extraction, approximately 30–40 mg of tissue was shredded into pieces on a clean glass slide using a sterile blade (No. 20, IQONIQ Medical Supplies, Nablus Palestine). Shredded tissue was placed into a 1.5 ml microtube (Eppendorf). Five hundred µl of lysis buffer (10 mM Tris-HCl (pH 7.4),

**Table 1.** Characteristics of study samples (n = 60) and control samples (7).

**Study Samples**

| Host (n) | Site of infection | (n, %) | Origin (n) |
|---|---|---|---|
| Animal: Sheep (37) Goat (10) Cow (2) | liver | (37, 76%) | Al-Khalil (21), Ariha (11), Tulkarm (11), Nablus (5), Jenin (1) |
| | omentum | (6, 12%) | |
| | lung | (2, 4%) | |
| | spleen | (2, 4%) | |
| | esophagus | (1, 2%) | |
| | gallbladder | (1, 2%) | |
| | sub-total | 49 | |
| Human | liver | (7, 64%) | Ariha (1), Nablus (1), Ramallah (1) |
| | lung | (4, 36% ) | Al-Khalil (4), Jenin (4) |
| | sub-Total | 11 | |
| | grand total | 60 | |
| Control samples | | | |
| Host | **EURLP code** | ***Eg sl* genotype** | **Origin (n)** |
| Human | 10/0634 | G1 | Afghanistan (1) |
| Sheep | 19/0454 | G3 | Italy (1) |
| Horse | 21/0636 | G4 | Ireland (1) |
| Goat | 21/0378 | G5 | Tanzania (1) |
| Pig | 19/0726 | G6/7 | Latvia (1) |
| Reindeer | Eg/Fi-47 | G10 | Finland (1) |
| Fox | Abbasi | Em | England - laboratory |
| Total | | | 7 |

Eg sl, *Echinococcus* sensu lato complex, Em, *Echinococcus multilocularis*

10 mM EDTA, 50 mM NaCl, 0.5% sodium dodecyl sulfate, and 20 mM dithiothreitol or 0.2% (v/v) 2-mercaptoethanol) were added and incubated at 85 °C for 10 min (Eppendorf ™ Thermomixer ™), followed by the addition of 0.5 mm glass beads with constant shaking for 5 minutes at 2,850 rpm using disruptor Genie (Scientific Industries, Inc, New York, United States) until the tissue was completely lysed. Proteinase K (500 µg/ml) was added to the lysed samples, vortexed and incubated overnight at 56 °C or until tissue is totally dissolved. DNA was extracted as described by the manufacturer (NuceloSpin, Machery Nagel, Dueren, Germany). The extracted DNA samples were stored at -20 °C until use. In the case of specimens from formalin-fixed paraffin-embedded (FFPE) blocks, a deparaffinisation step preceded the extraction procedure as described elsewhere (Al-Jawabreh et al. 2017).

**Primer design and probes**

For primer design, a representative of EmsB nucleotide sequences of *E. multilocularis* and *E. granulosus* s.l. microsatellites were retrieved from GenBank (*E. multilocularis*, Acc. No. AY680861.1 and *E. granulosus* (Acc. No. AY680860.1) and previously published sequences (Bart et al. 2006). These sequences were aligned, using the multiple sequence alignment online program (http:// multalin.toulouse.inra.fr/multalin/) which showed single nucleotide variation (SNV) in 21 sites of the known regions. A consensus DNA sequence was generated from the two sequences, *E. granulosus* s.l. and *E. multilocularis.* Primer 3 online software (https://primer3.ut.ee/) was used to design the primers, a forward primer EmsBA2NGSF (5'-GTCCATCAATACACTCAGCTCC-3') and a reverse primer NGS_EmsBCR (5'-CAGCCACCTTCCCTACTGCAATC-3). The primer sequences were selected in the conserved region (EmsB) to detect the *E. granulosus* s.l. complex. The primer pair was modified by adding the Illumina overhang adapter sequences at the 5' end of the forward (5'-CGTCGGCAGCGTCAGATGTGTATAAGAGACA-3') and reverse primers (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-3). For genotyping, control samples representing G1, G3, G4, G5, G6/7, G10 and *E. multilocularis* were sequenced using NGS. The resulting 151 bp sequences were aligned and polymorphic regions specific to each genotype were identified to design seven virtual probes, each 50 bp long, that matched the corresponding genotype.

The first ten sequence variants produced by NGS for the same genotype were included in the multiple alignments to verify the stability of the polymorphic regions, which were located within the first 90 bp of the 151 bp sequences. The virtual probes are specific DNA sequences used with the Galaxy program (Galaxy Version 1.0.4) command line called "Select lines that match an expression." These virtual probes, which achieve 100% sequence match, count the number of identical sequences retrieved from the high-throughput sequencing data, enabling the discrimination of the G1, G3, G4, G5, G6/7 and G10 genotypes of *E. granulosus* s.l. complex and *E. multilocularis* in a single tube reaction.

**DNA amplification and library preparation**

The two primers designed in this study to detect the *E. granulosus* s.l. complex (EmsBA2NGSF and NGS_EmsBCR, were used to amplify a fragment of about 270–420 bp of EmsB microsatellite under the following thermal cycling profile: 30 cycles of 98 °C for 10 s for denaturation, and 60 °C for 5 s for annealing and 72 °C for 10 s for extension. The PCR reaction was performed in a volume of 25 µL using 12.5 µl master mix (PrimeSTAR Max Premix (2X)-Takara, Wisconsin,United States), 10 pmol of each primer and 2 µl of each DNA sample. PCR reactions without DNA template were used as negative controls. Six pure DNA control samples confirmed as G1, G3, G4, G5, G6/G7, and G10 and one *E. multilocularis* were used as reference strains and genotypes. The lowest detection limit of *Echinococcal* DNA was determined using five-fold serial dilution of pure *E. granulosus* s.s. (G1) DNA. The DNA concentration was measured by Qubit machine (Thermo Fisher Scientific, Inc., Massachusetts, United States) and the PCR product (1 µl) was separated on High Sensitivity D1000 Screen Tape using Tapestation machine 4200 (Agilent Technologies, Santa Clara, California, United States).

For library preparation, 25 µl from each PCR product were cleaned by AMPure XP beads, Beckman Coulter (X1), and eluted in 25 µl elution buffer. A 7.5 µl from each sample were subjected to a second round of amplification (10 cycles) to include the index sequences (N7XX and S5XX) for barcoding using Nextera XT Index Kit (Illumina, San Diego, California, USA). The barcoded samples (5 µl) were pooled together, cleaned using X1 AMPure XP beads and deep-sequenced with the Nextseq500 machine using the 150-cycle mid output kit (Illumina, Inc., USA).

**Bioinformatic analysis**

The free online Galaxy analysis pipeline (https://usegalaxy.org/) was used for bioinformatic analysis as described previously (Nasereddin et al. 2022). Out of all the sequences in each genotype, and since too many variations within the EmsB region were expected, ten sequences (top ten) with the highest number of reads and quality score (Q) > 20 error rate above 1 : 100 were selected. The top ten collapsed sequences were selected from each genotype. The

**Table 2.** The sequences of the specific virtual probes used for the genotyping of *Echinococcus granulosus* s.l. complex.

| Probe | Primer sequences (5'-3') |
|---|---|
| G1-Probe | CAGGCCAGTGACACTCACTCTCATTGTCAAACCCACACACTCACTCACGC |
| G3-Probe | CAGGCCTGTGACACTTACTCGCACGGCCATACCCACACACTCACTCACGC |
| G4-Probe | CAGGCCAGTGACACTTACTCGCACGGCCAAACCCACACACACACTCACAC |
| G5-Probe | CAGGCCTGTGACACTTACTCGCACGGCCAAACCCACACTCACACCCACAC |
| G6/7-Probe | CAAGCCACTGACACTCACTCTCATTGTCAAACCCACACAATCATTCACGC |
| G10-Probe | CTGGCCTGTGACACTTACTCGCACGGCCAAACCTACACACTCATTCACGC |
| Em-Probe | CAGGCCAGTGACACTAACTCGCACTCACTCACGCTCACACACACACACAC |

selected sequences were aligned using a free online program (http://multalin.toulouse.inra.fr/multalin/) (Corpet 1988) Genetic</keyword><keyword>*Multigene Family</keyword></keywords><dates><year>1988</year><pub-dates><date>Nov25</date></pub-dates></dates><isbn>0305-1048 (Print. The virtual specific and conserved probes for each genotype were used to generate a new workflow to run the application (Galaxy analysis pipeline) for all the reference genotypes. The running of the application involved an overwhelming search in the raw data of the millions of sequences to find those sequences that are identical to the virtual probes and count them as the sample reads corresponding to the sample of interest.

### Phylogenetic tree and Haplotype networking

The study DNA sequences, along with randomly GenBank-retrieved *Echinococcus granulosus* s.l. complex from different hosts and genotypes across the globe, were used to construct a phyloge-

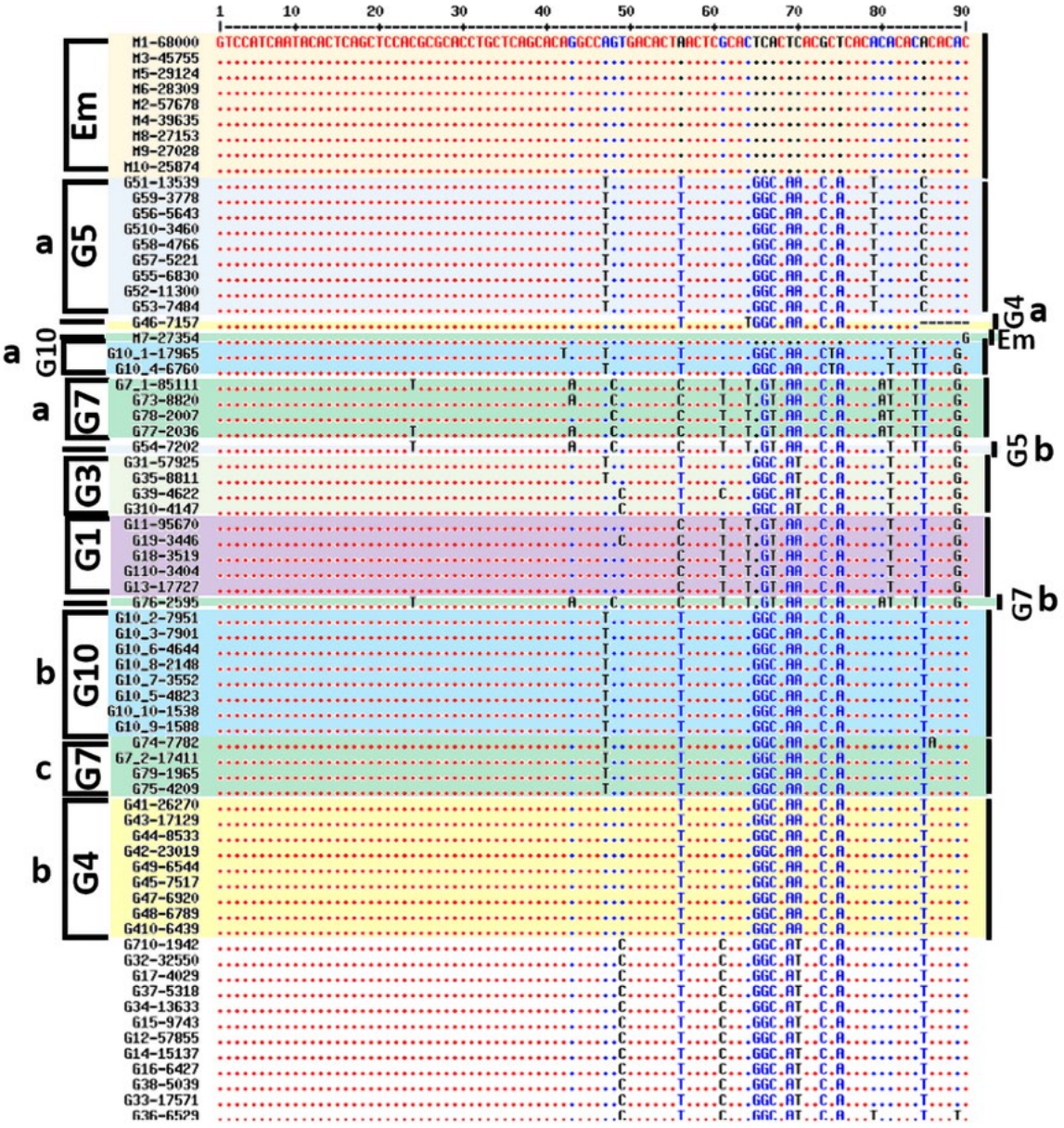


**Fig. 1.** Multiple alignments of EmsB sequences of the control samples of *Echinococcus multilocularis* Leuckart, 1863 (Em) and *E. granulosus* (Batsch, 1786) s.l. (G) genotypes using the first ten sequence variants produced by NGS. The code G51-13539, G5 is genotype 5, 1 is sequence number, and 13539 is number of reads of that sequence. The small letters (a–d) represent different variants of the aligned sequences. G7 is *E. canadensis* (G6/7). The colours were used to separate genotypes.

**Table 3.** Polymorphic sites of consensus MIC-NGS sequences obtained from the first 90 nt of the control samples of *Echinococcus granulosus* (Batsch, 1786) s.l. complex compared to the reference isolate *E. multilocularis* Leuckart, 1863 from England.

| Probe | A47 | A56 | C64 | T65 | C66 | A67 | T69 | C70 | G73 | T75 | A79 | C80 | A81 | C84 | A85 | A89 | # polymorphisms |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G1-Probe | - | C | T | - | G | T | A | A | C | T | - | - | T | - | T | G | 11 |
| G3-Probe | - | T | - | G | G | C | A | T | C | T | - | - | T | - | T | G | 11 |
| G4-Probe | - | T | - | G | G | C | A | A | C | T | - | - | - | - | T | - | 9 |
| G5-Probe | T | T | - | G | G | C | A | A | C | T | T | - | - | - | C | - | 11 |
| G6/7-Probe | - | Y | T | G | G | T | A | A | C | T | - | R | N | K | T | M | 14 |
| G10-Probe | T | T | - | G | G | C | A | C | C | T | - | - | - | - | T | - | 10 |

Y = T/C, R = C/A, N = A/T, K = C/T, M = A/G

**Table 4.** The percentage of recovery and identity of the reference *Echinococcus multilocularis* Leuckart, 1863 and *E. garnulosus* (Batsch, 1786) s.l. complex based on BLAST search.

| GenBank/reference samples | *E. multilocularis* | XX (% coverage)–YY (% identity) *E. granulosus* s.l. | | | | | |
|---|---|---|---|---|---|---|---|
| | | G1 | G3 | G4 | G5 | G6/7 | G10 |
| *E. granulosus* Y680860.1 | 66–87 | 100–98 | 90–95 | 85–94 | 70–91 | 90–85 | 86–91 |
| *E. multilocularis* AY680861.1 | 100–92 | 63–83 | 42–91 | 62–85 | 80–84 | 41–86 | 100–71 |

netic tree and a haplotype network. MEGA-X software was used to construct a maximum likelihood (ML) tree of the EmsB microsatellite with 1,000 iterations for bootstrapping (Tamura and Nei 1993, Kumar et al. 2018). The analysis included six control DNA samples of *E. granulosus* s.l. complex genotypes as G1, G3, G4, G5, G6/7 and G10, one human case from Jericho sequenced as G1 and one sequence-confirmed *E. multilocularis.* The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option). On the other side, the same set of DNA sequences was analysed by PopART 1.7 software (Bandelt et al. 1999) to draw a median-joining haplotype network. The network was based on single nucleotide variation (SNV) with zero epsilon as a default parameter.

## RESULTS

### Study samples

A total of 60 samples were included in this study. Forty-nine of the total samples were collected from animals: sheep (n = 37), goats (n = 10) and cows (n = 2). The remaining samples (n = 11) were obtained from humans (five males and six females; the median age was 26 years). Six of the samples were from formalin-fixed paraffin-embedded (FFPE) blocks, and five were frozen tissue cysts. The host, site of infection and geographical origin of the study and control samples are shown in Table 1. Al-Khalil district in the south represented 42% of the animal study samples, and 36% of the human study samples with Yatta village in Al-Khalil as the main source. Al-Khalil district is the most CE-prevalent area in the West Bank in Palestine (9.6 per 100,000) (Al-Jawabreh et al. 2017).

### Discrimination between genotypes

To improve the discrimination capability of the closely related *Echinococcus granulosus* s.s. genotypes G1–G3, complete 151 bp sequence data were used. Using the whole range of sequence length (151 bp) of each genotype, seven 50 bp-virtual probes were selected that have enough variation capable of accurately genotyping their target sequence (Table 2, Fig. S1). Supplementary Fig. S1 shows that at positions 116 and 133–134, where a subgroup of eight isolates exhibited genetic variations, replacing nucleotide C with T at position 116 and inserting dinucleotides AC repeat at positions 133–134.

In the first 90 bp, five polymorphisms existed between G1 and G3; C–T, T–C, T–G, T–C and A–T that had the potential to differentiate between these two closely-related genotypes (Table 3). The first 90 bp were sufficient to discriminate between the other *E. granulosus* s.l. genotypes (G4–G10) with the number of polymorphic sites ranging from 9 to 14 representing different polymorphism profiles across the tested *E. granulosus* s.l. genotypes (Fig. 1, Table 3). The sequences between 90 and 151 bp were found to be highly polymorphic. The 151 nt for each species and genotype were deposited in the GenBank (accession numbers: OR570745–OR570802).

### Validity of MIC-NGS assay

To validate the MIC-NGS assay, the sensitivity and specificity were assessed using the *Echinococcus* DNA of the reference samples. The analytical sensitivity, minimum detectable concentration of DNA by the MIC-NGS assay, was investigated using a serial dilution of a G1 DNA sample starting from 15 ng/µl. The lowest detection limit of *E. granulosus* s.l. G1 DNA was 0.12 ng/µl (Fig. 2).

The assay was specific in that, upon identical sequence pattern selection with specific virtual probes, all reference isolates of *E. granulosus* s.l. (G1, G3, G4, G5, G6/7, G10, and *Echinococcus multilocularis*) were correctly identified (Fig. 3). BLAST search of the obtained *E. multilocularis* sequence showed a 92% similarity and 100% coverage with the GenBank reference strain of *E. multilocularis* (AY680861.1), while sequences of *E. granulosus* s.l. complex showed variable similarity ranging from 85–98% with the reference sequence strain of *E. granulosus* (Y680860.1) based on the obtained genotype, as shown in Table 4.

In Table 5, the diagonal line in the square matrix contains the highest numbers of reads, and these diagonal meeting points represent the high agreement between the numbers of reads for each genotype, representing the up-

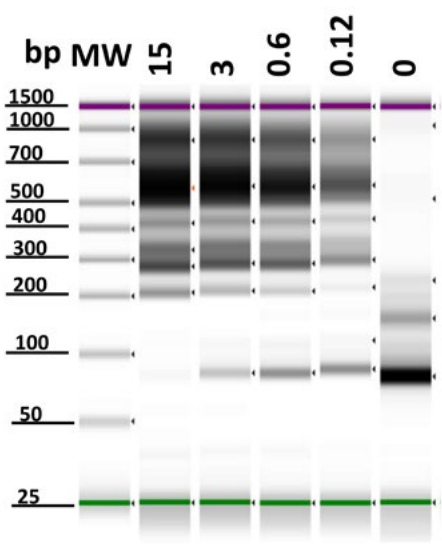


**Fig. 2.** Amplification of a partial fragment of EmsB microsatellite (270–420 bp) as revealed by TapeStation using different concentrations of *Echinococcus granulosus* (Batsch, 1786) s.l. G1 DNA (15–0.12 ng/µl). DNA was evaluated by using Qubit fluorometer (Invitrogen). Zero panel showed the primers dimers at the level of 90 bp on the DNA molecular marker.

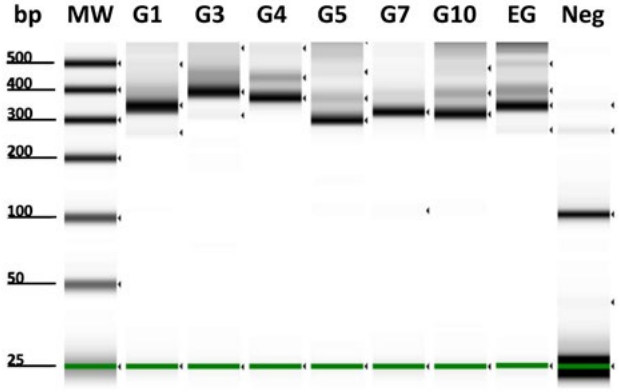


**Fig. 3.** Detection of *Echinococcus* DNA reference samples targeting the EmsB microsatellite as revealed by TapeStation with sizes ranging from 270 to 420 bp. The negative sample (Neg) showed the primer dimer at the position of 125 bp.

permost horizontal row, and the true genotype representing the first vertical column. The numbers on the off-diagonal line representing false results are very low, ranging from zero to 6 %. This indicates the high specificity of the probes to detect the true genotype with minor artifacts. Genotype G4 had the second highest number of reads, 18,991 (6 %).

### Phylogenetic and haplotype analyses

The circular ML tree showed that the study samples isolated in Palestine have grouped themselves into one major cluster. This major cluster was exclusively G1 genotype from Palestine, with *E. granulosus* clone (AY680860.1) included. All other genotypes, G3, G4, G5, G6/7, G10, and *E. multilocularis* grouped into different clusters. On the other hand, the MJ haplotype network analysis showed 12 *Echinococcus* true haplotypes. However, the size of the Palestinian node (green) was the biggest of all. Nonetheless, three minor nodes were closely connected to the major Palestinian node, with one SNV away. One of the three nodes contained seven G1 samples, with one originating from Afghanistan. One distant Palestinian node with several SNVs away, consisted of a single goat G1 sample.

The network analysis showed 20 hypothetical missing haplotypes (Fig. 4). The two methods, the ML tree and the MJ network, revealed two genetically exceptional samples. A Palestinian sample (Eg_Goat-Palestine-EI.15) was genetically diverse from the rest of the Palestinian study samples. An Afghani reference (Eg_G1-Human-Afghanistan) sample was shown to cluster with the study samples from Palestine.

## DISCUSSION

CE is considered a neglected tropical disease that has been extensively reported in the Middle East and North Africa (Sadjjadi 2006). Molecular detection and genotyping of *Echinococcus granulosus* s.l. strains is essential for planning effective control and prevention programs. This study reports the first application of NGS analysis for studying the genetic diversity and genotyping of *E. granulosus* s.l. in human and animal CE using EmsB microsatellite nuclear markers in samples collected from Palestine. Interesting, the MIC-NGS assay succeeded to genotyping all 60 samples (100 %) included in the study.

Recent studies reported that sequencing of the whole mitochondrial genome is crucial for the identification and differentiation of *E. granulosus* sensu stricto (s.s.) (G1 and G3) and *Echinococcus multilocularis* (Kinkar et al. 2018). Other studies used parts of the mitochondrial genome, such as *cox*1 gene, to distinguish *E. multilocularis* and *E. granulosus* s.l. G6 from either of G1 or G3 genotypes based on

**Table 5.** The number of sequence reads obtained by MIC-NGS analysis for each genotype of *Echinococcus* DNA reference samples using the genotype specific probes in the first column. G–F10 – genotypes of *Echinococcus granulosus* (Batsch, 1786); EM – *E. multilocularis* Leuckart, 1863.

| | No. reads of each specific genotype probe | | | | | | |
|---|---|---|---|---|---|---|---|
| Reference probes | G1 | G3 | G4 | G5 | G6/7 | G10 | EM |
| G1 | 153,005 | 111 | 66 | 1,623 | 1,135 | 602 | 4,727 |
| G3 | 948 | 50,396 | 20 | 217 | 1,267 | 108 | 1,305 |
| G4 | 1,169 | 42 | 315,385 | 340 | 1,190 | 670 | 80 |
| G5 | 1 | 2 | 4 | 359,728 | 174 | 145 | 0 |
| G6/7 | 7 | 3 | 18,991 | 15,417 | 140,309 | 2 | 0 |
| G10 | 0 | 0 | 0 | 0 | 0 | 25,663 | 0 |
| EM | 0 | 0 | 0 | 0 | 0 | 0 | 2,106,587 |

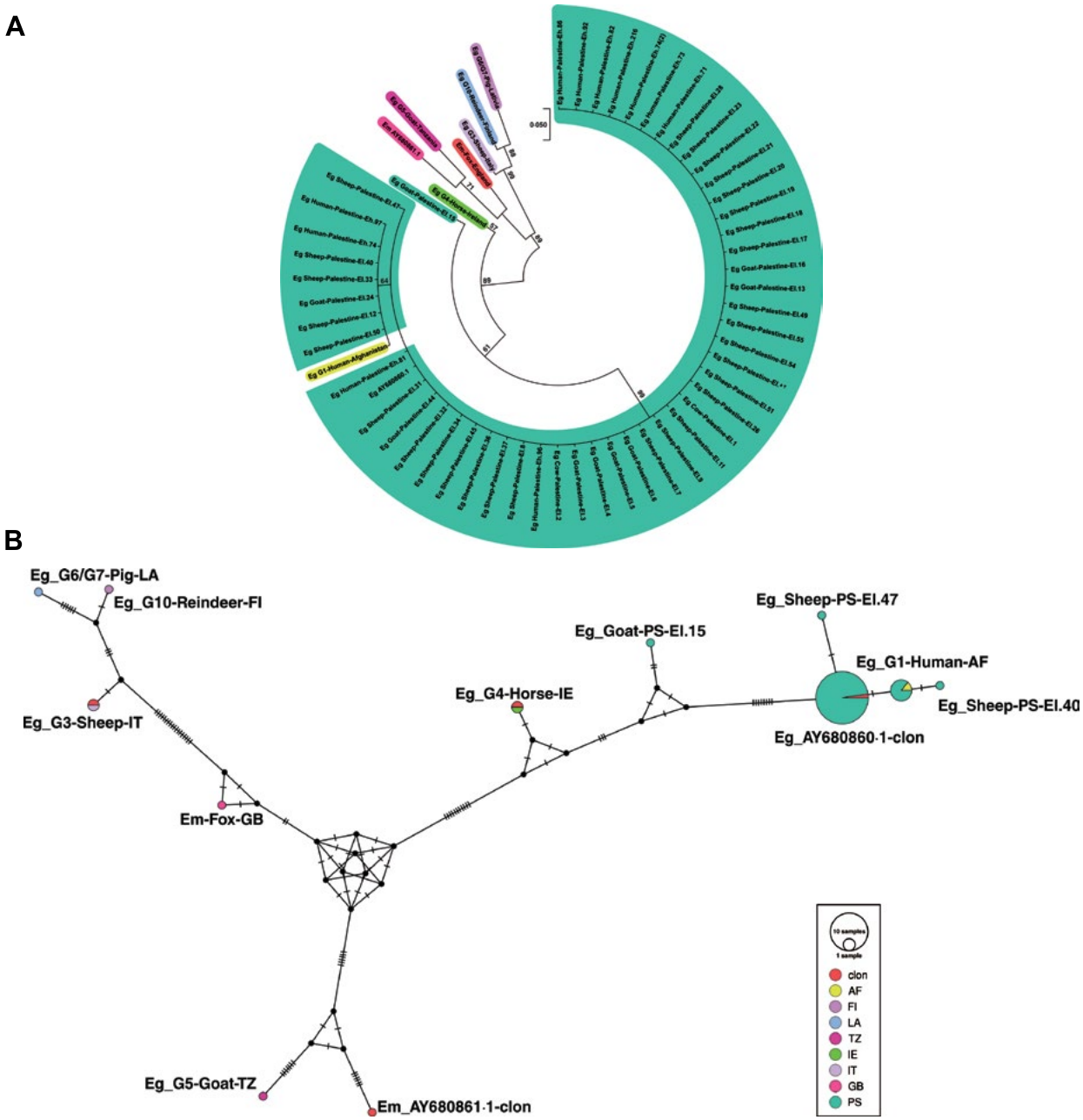


**Fig. 4. A** – Maximum likelihood circular tree of the 60 *Echinococcus granulosus* (Batsch, 1786) s.l. nucleotide sequences. The number appearing next to branches is the percentage bootstrap value out of 1,000 replicates. Phylogenetic tree was constructed by MEGA X; **B** – Median-joining haplotype network using PopART 1.7 with 1,000 iterations based on EmsB region in *E. multilocularis* Leuckart, 1863. The lines (hatch marks) on the mutated position with one in *E. multilocularis*. The lines (hatch marks) on the branches represent the mutated position with one line per mutation. Black circles represent hypothetical haplotypes. Each circle represents a unique haplotype, the colour represents the country of genome origin, and the size of the circle is proportional to the number of isolates included. Clon – Clone (obtained from the GenBank), AF – Afghanistan, FI – Finland, GB – Great Britain, IE – Ireland, IT – Italy, LA – Latvia, PS – Palestine, TZ – Tanzania.

HRM analysis or PCR-RFLP (Rostami et al. 2013, Sakalar et al. 2014, Hamamci et al. 2023). In both scenarios, limited resolving power exists to discriminate *E. granulosus* s.l. genotypes such as G1/G3 and G6/7 and there is need to sequence the whole mitogenome to achieve full discrimination (Ohiolei et al. 2020).

Conversely, our approach (MIC-NGS) was able to differentiate closely related genotypes like G1 and G3 (*E. granulosus* s.s.) by targeting the first 151 bp of the 300–420 bp tandemly repeated nuclear microsatellite EmsB. *E. multilocularis*, *E. granulosus* s.l. and E. *granulosus* genotypes (G4–G10), were all differentiated using the first 90 bp out of the 151 bp sequences (Table 5, Fig. S1). In line with this, other studies confirmed the reliability of this highly discriminant marker due to the quantitative exploitation of the amplification of about 40 copies located on chromosome 5 (Knapp et al. 2007, Valot et al. 2015).

In the present study, the MIC-NGS assay was optimised using the DNA of six reference *E. granulosus* s.l. genotypes (G1, G3, G4, G5, G6/7 and G10) and *E. mul-*

*tilocularis*. The MIC-NGS was validated in terms of sensitivity and specificity. The analytical sensitivity, or minimum detected DNA concentration, was determined to be 0.12 ng/µl, which is comparable to sensitivity in other studies performed by multiplex PCR on a mixture of three species namely *E. granulosus* s. s., *E. multilocularis* and *Echinococcus canadensis* employing the complete mitochondrial genome (mitogenome) (Shang et al. 2019). Simultaneously, the analytical specificity of MIC-NGS, detecting the intended target, was enhanced by producing a consensus DNA target sequence created out of thousands of DNA target sequences, but with minimal artifacts produced, DNA sequences with a low number of reads were ignored (0–5%) (Table 5).

In addition, the use of highly specific virtual probes representing all genotypes increased the specificity, making MIC-NGS a more robust method for genotyping of *E. granulosus*. With EmsB having several 800 bp fragments in *E. granulosus* and being characterised by extremely high variation combinations of microsatellites, CA and GA, EmsB became an ideal tool for identification and genotyping (Maillard et al. 2009). Furthermore, the MIC-NGS is more practical for diagnostic purposes with lower costs than the mitogenome-based sequencing method. The low cost arises from the fact that the MIC-NGS amplicon size is 151 bp compared to a mean mitochondrial genome (mitogenome) size of 13,714 (13,588–13,807 bp) which is 90 fold greater (Nakao et al. 2007).

It is also less labourious as only two PCR steps are required and there is no need for a specific kit for mitochondrial DNA library preparation. Generally, low cost comes from the low sequencing depth needed, and the bulking of samples in one run. Therefore, the high sensitivity, specificity, reasonable cost, time-saving and labour-saving properties of MIC-NGS make it a good candidate for epidemiological surveys. Additionally, apart from two sequences of *E. multilocularis* (Acc. No. AY680861.1) and *E. granulosus* (Acc. No. AY680860.1), our study with the MIC-NGS method was able for the first time to deposit 57 *E. granulosus* s.l. nuclear EmsB sequences in GenBank and to make them available for future evolutionary, genetic diversity and phylogenetic studies (Bart et al. 2006).

In contrast to our method, Bart et. al. (2026) employed PCR, followed by more complicated plasmid cloning, and then Sanger sequencing the clones producing only two GenBank-deposited sequences. Genotyping *Echinococcus* spp. by mitochondrial genes such as *cox*1, *nad*2, and *cob*, has been a common theme, as mitochondrial genes are easily amplifiable due to their abundance and relatively short length. This explains the ample amount of sequences in the GenBank. Conversely, the EmsB microsatellite, with its high variation, made phylogenetic analysis more complex.

However, with the new NGS technology employed in this study and the corresponding analysis software such as Galaxy, EmsB microsatellite became easier to use, taking into account the advantages of high sensitivity, specificity and discriminatory power. A recent study recommended that both mitochondrial and EmsB microsatellite genotyping should be used simultaneously for ultimate accuracy (Bohard et al. 2023).

Recently, Bonelli et. al. (2021) employed three TaqMan real time PCR assays targeting three SNPs (SNP758, 112, and 1380) in a fragment of the mitochondrial *nad*5 gene to distinguish between G1 and G3 *E. granulosus* s.s. genotypes. In contrast to our approach, the assay was a custom-made based on using six primers and six fluorescent probes to recognise G1 or G3 genotypes, while our approach used two primers for discrimination of G1, G3, G4, G5, G6/7 and G10 genotypes and *E. multilocularis* in a single tube reaction. After optimisation, the MIC-NGS assay was applied to clinical samples obtained from humans and animals.

The present study showed the predominance of the sheep strain (G1) in sheep, cows, goats and human samples. The circulation of the sheep strain (G1) in Palestine could be attributed to the close relationships between dogs and sheep in the region, where uncontrolled slaughter of livestock is frequently carried out in open areas, where dogs can readily feed on the offal of CE-infected animals. Our findings are congruent with the global trend of genotype distribution, in which 90% is *E. granulosus* s.s. with G1 forming 67% and 33% for G3 (Alvarez Rojas et al. 2013, Mousa et al. 2020, Casulli et al. 2022, Santucciu et al. 2023). Genotype G4 showed a relatively high number of reads (18,991) along with the major high read (315,389) (Table 5), which could partly be explained by genetic exchange between genotype G4 and genotype G6/7 that gave rise to a new hybrid genotypic profile (Maillard et al. 2009).

The phylogenetic ML tree was a reflection of the prevalence of *E. granulosus* genotypes in the study area and around the world. The human and livestock isolates of the G1 genotype formed one major cluster (58 samples from the study area and one from Afghanistan), irrespective to the host or site of infection and time of collection. The other *E. granulosus* s.l. samples from five countries with different genotypes, in addition to the two *E. multilocularis* samples, were sporadically distributed without any clearly defined clustering (Fig. 4).

Our results support the monophyly of *E. granulosus* s.l. reported by previous studies that proved genetic relatedness not only at the genus level but also extended family (Taeniidae) and order (Cyclophyllidea) regardless of the DNA target, nuclear or mitochondrial (Nakao et al. 2013, Lymbery 2017). Therefore, due to its monophyletic nature, the data were displayed using MJ haplotype networking, which is capable of detecting intraspecific DNA sequence variation down to one SNV. The haplotype networking has detected 12 haplotypes with the Palestinian haplotype being the dominant one, containing most of the samples.

In comparison to previous studies and relative to the number of samples investigated, MIC-NGS of the EmsB produced more haplotypes than when using mitochondrial genes such as *cox*1 and *nad*5, indicating a higher discriminatory power than other DNA targets, especially in a confined geography like Palestine (Maillard et al. 2009, Fan et al. 2022, Santucciu et al. 2023). The Palestinian sample (Eg_Goat-Palestine-EI.15) that deviated away from the main cluster could have been imported from another

geographical location that had a genetically differentiated population. The Afghani reference sample (Eg_G1-Human-Afghanistan) that was grouped with the study samples from Palestine may indicate genetic exchange between the G1 variants.

Despite having 58 samples from the study area, the number of *E. granulosus* s.l. genotypes other than G1–3 was limited, as was the absence of the GenBank-retrieved *E. granulosus* s.l. EmsB DNA sequences. These limitations were clearly depicted in the many hypothetical missing haplotypes in the MJ haplotype networking. Although homoplasy of EmsB microsatellite in *E. granulosus* is low, control samples could have been in duplicates for each genotype from different isolates. Despite having controls from several different countries, the main bulk of samples were from Palestine, which could have limited the resolution and power of MIC-NGS as a genotyping tool.

This unique EmsB-based MIC-NGS assay is highly sensitive and specific, with extremely high discriminatory power, allowing for efficient large-scale surveillance of *Echinococcus granulosus* s.l. and can be considered a cost-effective tool alternative to other molecular techniques for *E. granulosus* s.l. complex detection and genotyping.

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