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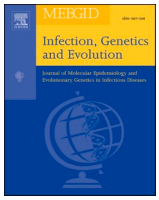

Genetic diversity of *Echinococcus multilocularis* specimens isolated from Belgian patients with alveolar echinococcosis using EmsB microsatellites analysis

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ABSTRACT

The genetic diversity of *Echinococcus multilocularis* (*E. multilocularis*) specimens isolated from patients with alveolar echinococcosis (AE), is a major field of investigation to correlate with sources of infection, clinical manifestations and prognosis of the disease. Molecular markers able to distinguish samples are commonly used worldwide, including the EmsB microsatellite. Here, we report the use of the EmsB microsatellite polymorphism data mining for the retrospective typing of Belgian specimens of *E. multilocularis* infecting humans. A total of 18 samples from 16 AE patients treated between 2006 and 2021 were analyzed through the EmsB polymorphism. Classification of specimens was performed through a dendrogram construction in order to compare the similarity among Belgian samples, some human referenced specimens on the EWET database (EmsB Website for the *Echinococcus* Typing) and previously published EmsB profiles from red foxes circulating in/near Belgium. According to a comparison with human European specimens previously genotyped in profiles, the 18 Belgian ones were classified into three EmsB profiles. Four specimens could not be assigned to an already known profile but some are near to EWET referenced samples. This study also highlights that some specimens share the same EmsB profile with profiles characterized in red foxes from north Belgium, the Netherlands, Luxembourg and French department near to the Belgian border. Furthermore, Belgian specimens present a genetic diversity and include one profile that don't share similarities with the ones referenced in the EWET database. However, at this geographical scale, there is no clear correlation between EmsB profiles and geographical location. Further studies including additional clinical samples and isolates from foxes and rodents of south Belgium are necessary to better understand the spatial and temporal circumstances of human infections but also a potential correlation between EmsB profiles and parasite virulence.

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1. Introduction

Alveolar echinococcosis (AE) is a severe zoonosis caused by the larval stage (metacestode) of *Echinococcus multilocularis (E. multilocularis)*. This serious parasitosis is a significant public health issue worldwide with mortality over 90% in untreated or inadequately treated patients within 10–15 years after the diagnosis (Wilson et al., 1992). *E. multilocularis* is widely distributed in the northern hemisphere where it is typically maintained in a wild animal cycle with canids as definitive hosts, mostly represented by the fox in Europe, and rodents as intermediate hosts. Humans are accidental hosts meaning that they can be infected in the same way as other intermediate hosts, but they are not involved in the transmission of the infection to the definitive host because human is a dead-end host. Infection in human occurs through ingestion of parasite eggs present on contaminated food, water or soil, or after direct contact with animal hosts. Human echinococcosis is often expensive and complicated to treat and may require extensive surgery and/or prolonged drug therapy (WHO, 2015). Over the past two decades, numerous studies have addressed the epidemiology and distribution of *Echinococcus* species worldwide (Deplazes et al., 2017; Kolářová et al., 2015; Dezsényi et al., 2021; Auer, 2006; Grenouillet et al., 2013; Baumann et al., 2019; Eroglu et al., 2021). Increased prevalence of *E. multilocularis* in fox populations has been described in different European countries so the richness of genetic profiles circulating in a country increases as well (Umhang et al., 2014; Knapp et al., 2021; Knapp et al., 2009; Knapp et al., 2008). As yet, no molecular typing has been carried out to describe the genetic diversity of the *E. multilocularis* circulating in Belgium in humans. Several studies demonstrated the discriminatory power of the tandemly repeated EmsB microsatellite genetic marker to classify *E. multilocularis* specimens based on its polymorphism (Knapp et al., 2010; Maillard et al., 2009; Umhang et al., 2021a; Shang et al., 2021; Bart et al., 2006; Casulli et al., 2009; Umhang et al., 2017; Umhang et al., 2021b). This method consists of a PCR followed by a fragment size analysis that permits to generate electrophoretic diagrams, reflecting the genetic signature of the specimen. In order to explore the genetic diversity within *E. multilocularis* isolates from Belgian patients, this marker was chosen due to its high discriminatory power and its potential field applications (Bart et al., 2006; Knapp et al., 2007). Retrospectively, *E. multilocularis* specimens isolated after surgery from Belgian AE patients from 2006 to 2021 were analyzed to assess the intra-species polymorphism level on a local geographical scale. EmsB analysis provided a multi-peak profile, characterized by tandemly repeated microsatellite sequences in the *E. multilocularis* genome.

2. Material and methods

2.1. Collection of samples

Sixteen AE patients, referred from 2006 and 2021 to the University Hospital of Liege (CHU of Liege, Belgium) for diagnostic confirmation or therapeutic management, were included in the study. The samples were collected during surgery or by needle aspiration conducted to confirm a suspicious diagnosis. Samples were collected from the liver ($n = 15$), lung (n = 1), pleural fluid (n = 1) and bile (n = 1). Two samples were collected twice from the liver of the same patient at different times (2011 and 2016) and two other samples were collected from different body sites for one patient (pleural fluid and liver collected during the same intervention in 2019). This study received the approbation of the institutional ethical committee of the CHU of Liege (N° 2021/31), all data were anonymized.

2.2. DNA extraction conditions

DNA was extracted from specimens using automated extraction with Maxwell instrument and DNA extraction kits. For frozen tissue, the Maxwell 16 LEV tissue kit was used (Promega, Madison, USA) according to the manufacturer instructions, preceded by a pre-treatment of the tissue with proteinase K (Qiagen, Hilden, Germany) for one hour at 56 °C. DNA were extracted from paraffin embedded tissues from the Biobank of the anatomopathology department of the CHU of Liege. Briefly, slices of the embedded tissues were cut by using a microtome Leica Histocore autocut (Leica Biosystems, Nussloch GmbH, Heidelberger, Germany). The tissues were first de-paraffined and then, a pre-treatment with proteinase K (Qiagen, Hilden, Germany) was performed for two hours at 70 °C, followed by DNA extraction using the Maxwell 16 FFPE plus LEV DNA purification kit (Promega, Madison, USA).

2.3. EmsB microsatellite amplification and data analysis

The PCR was performed according to Knapp et al. (Knapp et al., 2020).The sequences of the primers used were as followed: The EmsB A: 5′-Fam-GTGTGGATGAGTGTGCCATC-3′), EmsB C: 5′-CCACCTTCCC-TACTGCAATC-3′. The EmsB A primer was 5′-labeled with 6-carboxyfluorescein 6-FAM-fluorochrome (Integrated DNA technologies, Coralville, USA), and used for PCR with the EmsB C primer. The EmsB PCR was done twice for each sample to confirm the EmsB profile reproducibility. Samples with incorrect duplicates were removed from the analysis. Each run of PCR included a calibrator that was used to check and compare electrophoresis reliability. The EmsB calibrator consists in a plasmid construction containing four EmsB microsatellite sequences (Knapp et al., 2017). Capillary electrophoresis of PCR products and fragment size analysis was performed on an ABI 3500 automatic sequencer (ThermoFisher scientific, Waltham, USA). The fluorescence signal was detected by colorimetric reading. For each sample, size and height of the amplified fragments were studied using Gene mapper software (ThermoFisher Scientific, Waltham, USA). An EmsB profile is composed of several peaks from 209 to 241 bp. Euclidean distance matrix was generated from these data and the relationships between samples were established by applying the unweighted-pair group method using average linkages. A dendrogram was built in order to assess the genetic similarity among samples. The threshold of 0.1 of Euclidean distance was used to determine the genotypic relationship among samples and define profiles (Pn), as described by Knapp et al. (Knapp et al., 2020). The profiles obtained were then compared with a panel of previously genotyped samples from France, Switzerland, Austria and Germany, included in the EmsB Website for the *Echinococcus* Typing (EWET) data collection (Knapp et al., 2020; Knapp et al., 2017) and also compared to *E. multilocularis* adult worms isolated from red foxes in Belgium ($n = 20$), in Luxembourg ($n = 13$), Netherlands (n = 1) (Umhang et al., 2021b) and in Northern France ($n = 12$) (Umhang et al., 2014). Previously genotyped profiles were included in the present study to compare EmsB profiles described among humans and animals. Specimens from Alaska and/or Japan were used as outgroups. The EWET database contains information about 1500 *E. multilocularis* specimens from different continents (mainly from 13 European countries) from adult worms (fox, cat, and raccoon dog hosts) and metacestodes (human, monkey, and rodent hosts). In order to compare each present sample with the EWET database, a search of the most similar EWET reference samples was performed with R software as a sorted list, following EmsB guidelines (Knapp et al., 2017). Individual research of similarity was performed on 1500 referenced samples, including the present human samples.

2.4. Statistical analysis

Correlation between the genetic profiles defined by the EmsB microsatellite and the different provinces of Belgium/Luxembourg has been assessed by a Fisher's exact test. The relationship between the EmsB profile and clinical features was studied by the ANOVA test and by the chi-square test depending on the type of variable. The results are considered significant at the 5% uncertainty level ($p < 0.05$). Diversity

among the EmsB profiles was evaluated using the calculation of the diversity index (D), based on the Simpson index (L), (D = 1 – L) reflecting the number and relative abundance of EmsB profiles in the studied population. The reciprocal Simpson index (1/D) has also been calculated. Statistical tests were performed using the SAS software version 9.4.

3. Results

The main clinical characteristics of the Belgian cases included in the hierarchical clustering analysis are described in Table 1. The sex ratio M/F was 9/7 (56%) and the mean age at the time of diagnosis, was 63 years (range 27–89). In total, 62.5% of the patients were asymptomatic. Serology was positive or doubtful in 12/16 cases (75%). All patients had liver involvement, and 43% of them had extra-hepatic lesions. In the majority of the patients (68.7%), Radical resection (R0) surgery was performed. No statistical relationship has been found between EmsB profile and any clinical features by ANOVA/ Chi-square test.

Thirty-six samples were initially submitted to the fragment size analysis, but following an incorrect replicates repeatability or a total absence of profile, 18 samples have been removed from the analysis. Eighteen specimens from 16 Belgian patients were successfully analyzed. In Fig. 1, only the Belgian samples are represented on the dendrogram of similarity. From the dendrogram and based on the established genetic distance threshold value fixed at 0.1, three different groups (G) were defined among the present collection of samples. The first group (G1) comprising 9/18 (50%) samples is predominant, a second group (G2) contains 4/18 (22.2%) isolates, and one small group (G3) of two isolates is also described on the dendrogram (11.1%). Three samples are out of these former groups (16.6%), with an Euclidean distance >0.1 with the other samples.

In the present study, the attention was first focused on the national genetic diversity, inducing the construction of dendrograms which only include samples from Belgium. To obtain information from a larger geographical context, we aimed to combine larger and spatially distant EmsB profiles by comparing our samples with those from the EWET database.

According to a comparison made with European human samples previously studied (Knapp et al., 2020), specimens were classified using a second hierarchical clustering analysis into different EmsB profiles. The different Belgian “groups” previously defined were converted into “profiles” based on the similarity within AE patient genotypes previously described (Knapp et al., 2020). Results are presented in Fig. 2. Similar EmsB profiles were shared among the present samples and European human patients. Nine Belgian specimens shared the P1 profile, while another sample was qualified as near-P1 (Euclidean distance (d), d = 0.117 with the other samples described as P1). This P1 profile has already been characterized among Swiss, German, and French *E. multilocularis* specimens from humans and in the first sample from a Belgian human included in a previous study (Knapp et al., 2020). Three Belgian isolates are included into the P8 profile. This P8 profile has essentially been hitherto found among French *E. multilocularis* samples. Some French cases were isolated near to the Belgian border. Two Belgian samples shared the P4 profile (already found near the Belgian border in France, also in Switzerland and Germany), while one last isolate was assimilated to a P9 profile. P9 profile was present in France and Switzerland. Two out of the 18 samples were out of the already described profiles. However, these two samples were close to P8 profile (d = 0.135–0.154), but genetic differences were too high to be considered as P8. Individual research of similarity allowed us to find similarity between the present samples and EWET referenced samples (with an Euclidean distance found under 0.078), including samples that are OUT of profiles except for the sample B2012080188. According to a comparison made with European samples adult worms isolated from red foxes previously studied (Umhang et al., 2021b; Umhang et al., 2014), we could observe different groups regarding EmsB profile similarities. The human Belgian profiles were close to some EmsB profiles isolated from red foxes in north Belgium, Luxembourg, the Netherlands and some departments from the north of France near to the Belgian border (Moselle (57), Marne (51), Aube (Umhang et al., 2014) and Nord (59)) and included in P1, P4, P8 previously defined profiles (Knapp et al., 2020). Samples B2012080188, B17B26730, B11P03899 and B131609130027 are not clustered into defined profiles if we consider the limit of genetic distance to 0.1 (see Fig. 3).

In Fig. 4, the distribution of the different EmsB profiles found in different Belgian/Luxembourg regions were represented in comparison to profiles found in a previous study (Knapp et al., 2020). The P1 profile is present in all the Belgian regions studied in this present work. But there is no clear relationship established between the different profiles found and the geographical region of residence of the patient ($p = 0.75$ by Fisher's exact test). The diversity of profiles found in Belgium has been evaluated by the calculation of a diversity index that shows an important diversity of genetic profiles in Belgium (D = 0,41, index of diversity = 0,59, reciprocal Simpson index =2,43), especially in the province of Liege where the index of diversity reach a value of 0.9 (D = 0,1, reciprocal Simpson index = 10). However, due to the small sampling for this city, this information should be taken carefully. On the map, with icons surrounded in red, three French human cases detected near to the Belgian border and one Belgian case from a previous study (Knapp et al., 2020), were added for information.

Interestingly the patient presenting a P9 profile is geographically isolated compared to the previous study (Knapp et al., 2020), where this profile was described in the highly endemic area of Bourgogne Franche-Comté Region (France) and bordering Swiss cantons. Some isolates belong to the same patient but sampled from different organs or raised at different times. For example, for one patient, one specimen was characterized as P8 (collected in 2016, B16P04656) and the other one was “out” of known profiles (collected in 2011, B11P03899), but near P8 (d = 0.135). Moreover, the two samples from the same patient are classified into the same Belgian group 3 (G3) when considering Belgian samples alone (Fig. 1). Two other samples concerning the same patient but originating from different organs had both a P1 profile (B131905155228, B19B10914).

4. Discussion

Exploring the intraspecific genetic diversity of *E. multilocularis* favors a better understanding of epidemiological features evolution of this parasite, which is crucial for the application of surveillance and control measures. In Belgium, information about the genetic characteristics of *E. multilocularis* circulating among humans is still to be improved. This first large scale Belgian EmsB microsatellites study in humans allowed us to genetically characterize specimens collected for the first time at a large scale, from lesions or fluid of Belgian AE patients. Indeed, only one Belgian specimen has already been characterized in a French study (Knapp et al., 2020). In the present study, 18 human specimens from 16 AE patients, have been genetically characterized through an EmsB genotyping. No statistical relationship has been found between EmsB profile and any clinical features. This study highlights that some Belgian samples share the same EmsB profiles, already found in other European countries in humans and animals and that different EmsB profiles circulate in Belgium. A previous EmsB study on 66 European AE human isolates (originating from Switzerland, Germany, Belgium) highlighted nine different EmsB profiles including one Belgian sample with P1 profile (Knapp et al., 2020). The P1, P4, P8 and P9 EmsB profiles described in the present paper have been already characterized in a previous study performed on AE lesions of European patients (Knapp et al., 2020). This sharing of profiles can be done by foxes or domestic dogs circulating in neighboring countries or by humans being infected abroad. This seems to be the case in our study as human samples share some EmsB profiles with profiles characterized in red foxes from north Belgium, Luxembourg, the Netherlands and some French departments

Table 1
Main characteristics of the Belgian human alveolar echinococcosis cases included in the present study.

ID	Sex	Age at the time of diagnosis	Postal code	City	Serology	PNM classification	Extrahepatic localisation (Yes/No)	Symptoms (Yes/No)	Year of surgery	R0 Curative surgery (Yes/No)	Organ sample	Type of biological material	*EmsB* Profile
B06B05191	M	63	4100	Seraing	neg	P4N0M0	no	no	2006	yes	Liver	FFPE	P1
B11P03899[1]	F	74	4040	Herstal	pos	P1N0M0	no	no	2011	no (R1)	Liver	FFPE	OUT/ near P8
B14B13532	M	68	5376	Miecret	pos	P4N1M0	no	no	2014	yes	Liver	FFPE	P1
B131504140091	M	63	4780	St Vith	doubt	P4N1M1	yes	no	2015	no (R1)	Liver	FFPE	P4
B16P04656[1]	F	74	4040	Herstal	pos	P1N0M0	no	no	2016	yes	Liver	FFPE	P8
B131609130027	F	79	4171	Poulseur	pos	P4N1M0	yes	yes	2016	yes	Liver	FFPE	OUT/ near P1
B17B26730	F	47	4633	Melen	neg	P1N0M1	yes	no	2017	yes	Lung	FFPE	OUT/ near P9
B1905155228[2]	M	89	6852	Maissin	pos	P1N1M1	yes	yes	2019	no	Pleural fluid	Fresh	P1
B19B10914[2]	M	89	6852	Maissin	pos	P1N1M1	yes	yes	2019	no	Liver	FFPE	P1
B1906280005	M	67	9041	Ettelbruck	pos	P1N0M0	no	no	2019	yes	Liver	Fresh	P4
B131912190016	M	41	6600	Bastogne	pos	P4N1M0	yes	yes	2019	no	Liver	Fresh	P8
B1905070053	F	37	1430	Rebecq	neg	P1N0M0	no	no	2019	yes	Liver	Fresh	P1
B2003060073	F	73	5503	Dinant	neg	P3N1M0	yes	no	2020	yes	Bile	Fresh	P1
B2012080188	F	55	5660	Couvin	pos	P4N0M0	no	no	2020	yes (transplant)	Liver	Fresh	OUT
B2101050187	F	27	6640	Vaux-sur-Sûre	pos	P2N0M0	no	yes	2021	yes	Liver	Fresh	P8
B2101200041	M	61	6769	Meix-devant-Virton	pos	P1N0M0	no	yes	2021	yes	Liver	Fresh	P1
B2101260259	M	41	6880	Bertrix	pos	P1N0M1	yes	yes	2021	no	Liver	Fresh	P1
B2103080036	F	68	5170	Profondeville	pos	P2N0M0	no	no	2021	yes	Liver	Fresh	P1

Pos = positive, neg = negative, doubt = doubtful, R1 = incomplete resection, FFPE = Formalin fixed paraffin embedded tissue, Fresh = DNA extracted from frozen tissue.
[1] Liver samples from the same patient operated in 2011 and 2016.
[2] Samples from the same patient, one from the liver and one from pleural fluid, both sampled in 2019.

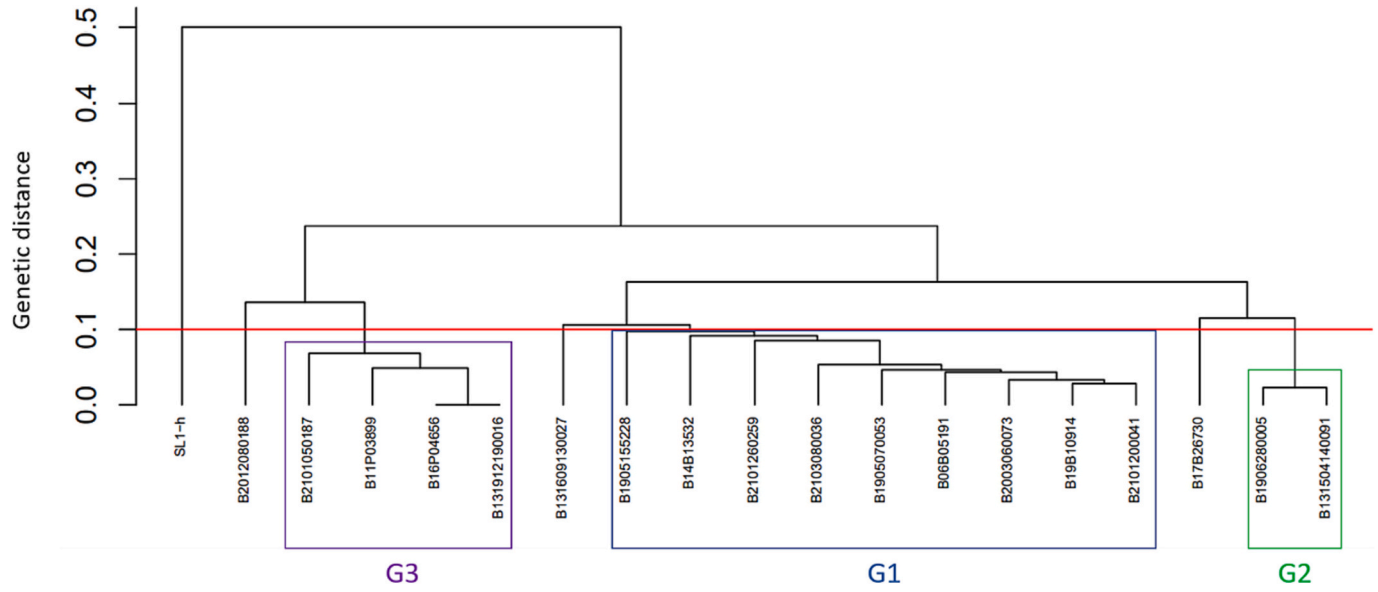

Fig. 1. *Echinococcus multilocularis* genetic classification according to the EmsB microsatellite results, representing 18 specimens from AE Belgian patients included in the present study and characterized through genetic diversity of the EmsB microsatellite polymorphism. The red line indicates the genetic distance threshold fixed at 0.1, allowing characterization of the groups G1, G2 and G3. The sample SLI-h from Saint Lawrence Island was included as outgroup control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

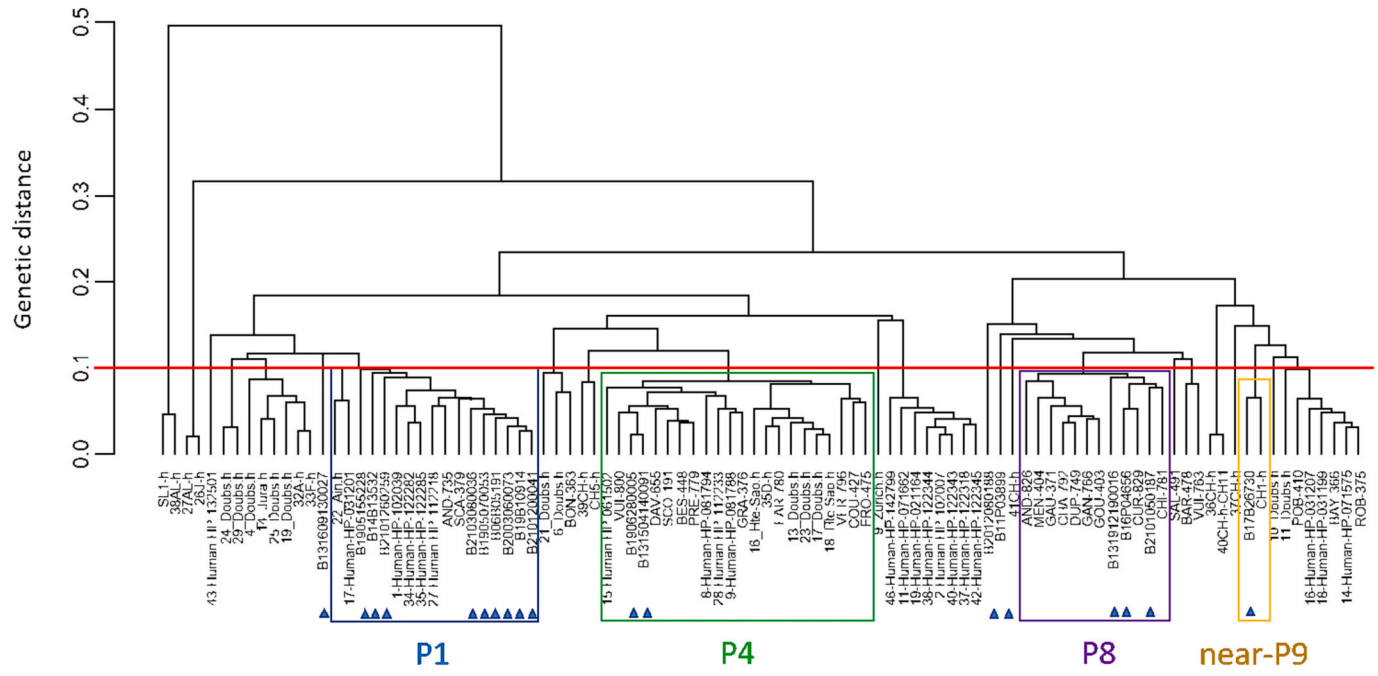

Fig. 2. *Echinococcus multilocularis* genetic classification according to the EmsB microsatellite results, representing 18 specimens from AE Belgian patients and referenced samples from the EWET database and previous study on European AE patients and characterized by the EmsB microsatellite polymorphism. Belgian specimens are highlighted with the blue arrow. The red line indicates the Euclidean distance threshold fixed at 0.1. Colored frames represent the different groups of similar genotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

near to the Belgian border. Only four of our strains do not share similarities (considering genetic distance <0.1) with previous EmsB profiles from foxes. P1 has been described as the dominant profile in Belgium. In France, P4 profile was numerically dominant among human samples, while P1 profile was characterized in a lesser extent (Knapp et al., 2020). Interestingly, P4 and P8 profiles were isolated in the three only human samples of the French study that were isolated near to the Belgian borders. These three profiles were well represented among foxes from North Belgium and surroundings. Following EWET data, the present P1 profile correspond to the G5 described in the paper of Knapp *et al*, 2009, where genetic diversity of *Echinococcus multilocularis* in red foxes at a continental scale has been investigated. This G5 profile was found to be the main profile in the studied European countries in this publication (Knapp et al., 2009). The present P4 correspond to P17 profile described by Umhang *et al* in 2014 (from worms isolated from fox intestines) and seems predominant in France following their observations. This P17 profile has been interestingly observed in Lorraine department near to the Belgian border (Umhang et al., 2014). Our results suggest that the

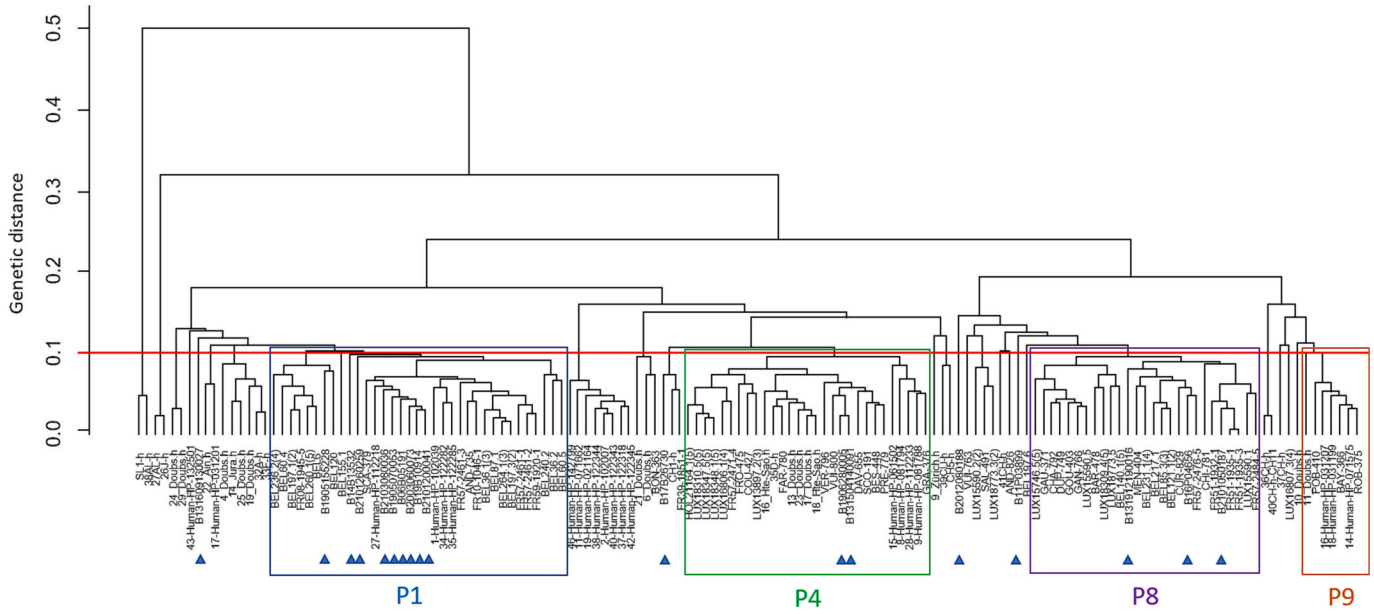

Fig. 3. *Echinococcus multilocularis* genetic classification according to the fragment size analysis on the EmsB microsatellite, representing 18 specimens from Belgian patients with alveolar echinococcosis (with blue arrows), human samples from a previous study (Knapp et al., 2020) and samples of adult worms isolated from red foxes in previous European studies (Umhang et al., 2021b; Umhang et al., 2014). The red line indicates the Euclidean distance threshold fixed at 0.1. Colored frames represent the different groups of similar genotypes (according to Knapp et al., 2020 (Knapp et al., 2020)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genetic diversity inside *E. multilocularis* is quite high in Belgium, especially given the low number of samples considered in this series and the calculation of the diversity index. So, regarding the size of Belgium compared to France, we can consider that reporting three different profiles (four if we consider the sample near P9), could foretell a more important genetic diversity in Belgium. However, additional Belgian AE specimens is necessary to confirm the present observations and characterize a potential founder event, where rare genotypes in a primary focus could become dominant in a newly colonized area (Templeton, 2008). Moreover surveillance of the parasite in foxes may bring more easily and above all more samples to estimate genetic diversity and identify this kind of potential founder event.

Important genetic diversity was also found in other small European countries or regions like Switzerland and Jura Swabe that are well known to belong to the core endemic area, the reciprocal Simpson index (1/D) for the historical focus in France is higher than in the present study (considering the entire Wallonia region) as it is estimated to be 8.25 in Jura Swabe and 9.62 in Switzerland (Knapp et al., 2009). In Poland, the reciprocal Simpson index is between 1.8 and 5 following the considered provinces (Umhang et al., 2017). In another Swiss study, among 23 samples isolated from humans, nine different EmsB profiles were described, considering isolates from Swiss pigs, humans, monkeys, rodents and foxes, the Simpson diversity (1-D) has been evaluated to be between 0.43 and 0.89 following the regions considered (Knapp et al., 2021).

The cases considered in our study were collected from 2006 to 2021. In 2006 the P1 profile has already been characterized, suggesting that EmsB profiles appeared to remain stable over the time and that some profiles are well established in the country for a long time. This profile was also previously isolated in 2010 in a European study including only one Belgian isolate from Brussels that was described as belonging to the P1 profile (Knapp et al., 2020). P4 and P8 profiles were characterized respectively in 2015 and 2016 in Belgium. These two profiles were already isolated in France among patients in 1986 and 2007 respectively (Knapp et al., 2020), reinforcing the idea of interconnections from the core-endemic areas to Northern neighboring countries (Umhang et al., 2014).

Since now, no EmsB analysis have been done on foxes circulating in south Belgium or AE lesions isolated from rodents and are limited to the north part of the country (Umhang et al., 2021b). Next studies would be focused on comparison between EmsB profiles found in humans with those found in animals in Wallonia. Exploring animal samples can either increase our knowledge on the genetic diversity of *E. multilocularis* in Belgium as it was the case in different studies (Umhang et al., 2014; Knapp et al., 2021; Knapp et al., 2021; Knapp et al., 2008; Knapp et al., 2009), could permit to identify specific profiles for humans and increase the understanding of the contamination ways. In our study, no clear correlation can be established between the geographical location of the patient and the belonging to a given profile. It is evident that the different EmsB profiles found in that study were not restricted to a specific area in Belgium as no clear link could be done between one particular profile and one specific region in Belgium, P1 profile being spread into four different provinces for example. Comparable observations have been done in a previous French study that examined EmsB profiles in a 900 km^2 area in the southeastern part of the French Regions of the Ardennes and Meuse. Indeed, six different genetic profiles were found in this restricted area (Knapp et al., 2008). However, in France, a study on EmsB profiles in humans showed that groups of patients from the same geographical area, shared identical or similar profiles (Knapp et al., 2020). This is quite surprising to find four different profiles in Belgium that are not restricted to a defined geographical area. This could be explained by the fact that Belgium is a small country interconnected with a lot of near endemic neighboring countries which can explain the spread of different EmsB profiles as foxes can run through the country easily and through neighboring countries like France, Germany or Switzerland and spread different profiles all around Belgium. Previous studies on red foxes from Belgium, France, The Netherland and Luxembourg, showed diverse EmsB profiles among these animals what can explain the different EmsB profiles found in humans (Umhang et al., 2021b; Umhang et al., 2014). The absence of high chains of mountains in Belgium and the low elevation gain between different regions can also favor the spread of different EmsB profiles. In our study, EmsB analysis especially concerns the South part of Belgium as few cases have been reported from the North part of Belgium (Jansen et al., 2020; Hanosset

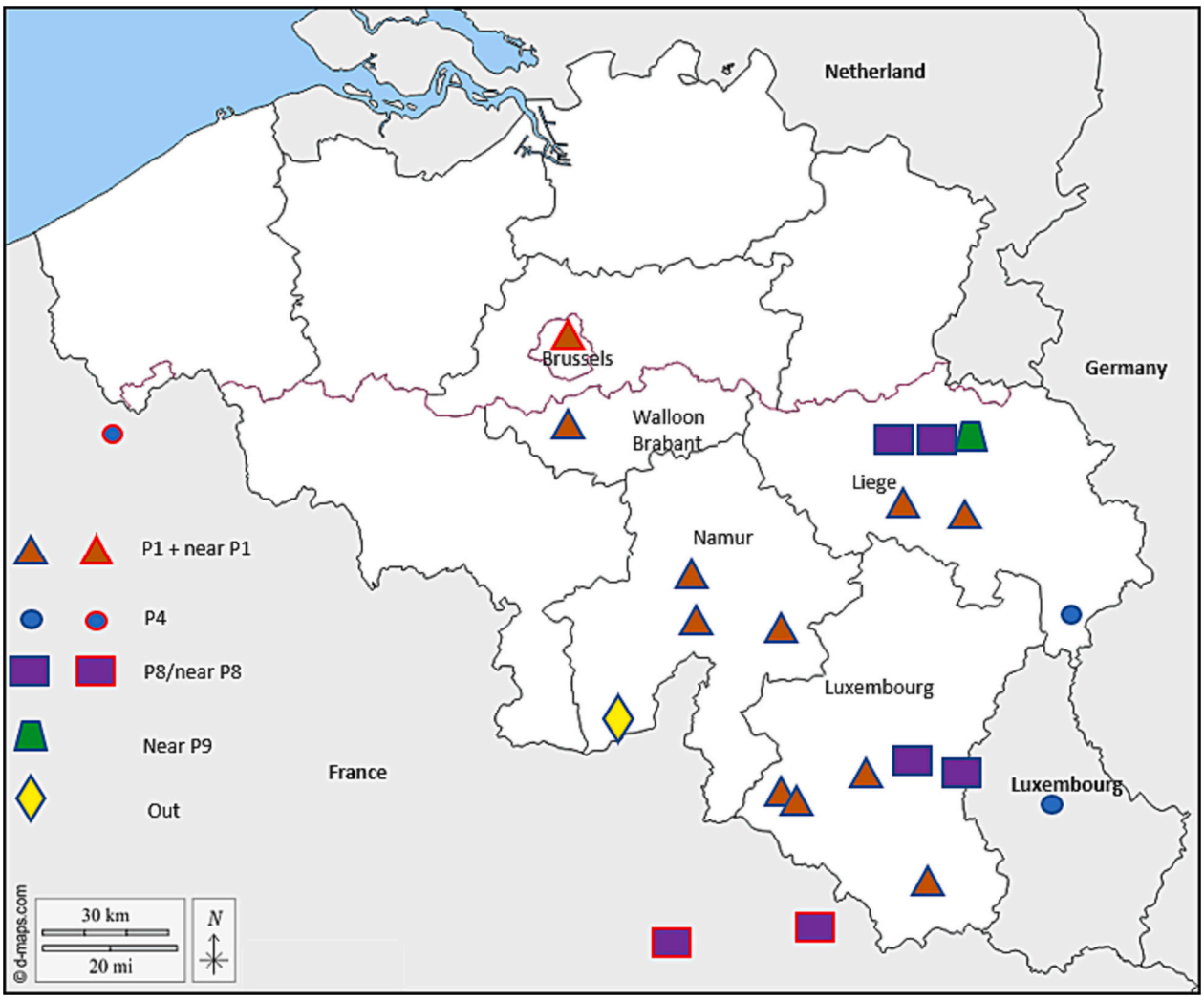

Fig. 4. Geographical distribution of *Echinococcus multilocularis* EmsB profiles obtained from Belgian AE patients included in this study. Different profiles are represented using different icons and colours. The four icons that are surrounded in red are part of the study of Knapp et al., (Knapp et al., 2020). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2008). Information about trips and risk factors experimented by patients included in this study have to be obtained to go further in the comparison between epidemiology and parasite genetic diversity. We didn't identify in our analysis different profiles in different AE lesions from a same patient. Indeed, one case detected the same P1 profile in a sample from the liver and from the pleural fluid collected at the same time, which suggests that the pulmonary localization is secondary to the liver one. The second case detected a P8 and a near-P8 profile. For this latter case, samples were collected respectively in 2016 and 2011 from the liver. So, we think that the sample of 2011 that is "near to P8" can be explained by DNA damages during sample conservation which can be responsible for the small variations observed by the EmsB method. To our knowledge multiple contaminations by different genotypes have been so far never described in humans' alveolar echinococcosis (Knapp et al., 2020).

In conclusion, this is the first study addressing the genetic diversity of *E. multilocularis* from humans in Belgium by an EmsB microsatellite genotyping. Most of the identified profiles in Belgium were already known from previous studies performed on the EmsB microsatellite. One profile did not share any similarities with other known referenced profiles and further investigations are needed to know if this genotype is the result of artefactual variations from known profiles due to DNA damages and conservation issues or if this could be a profile not yet reported in the EWET database that can also spread into other parts of Europe. Our data expand the European collection and complete the knowledge of genetic features of *E. multilocularis* in Europe, especially in parasites infecting humans. Our results also confirm that a genetic diversity of *E. multilocularis* can be described even considering a restricted geographical area and a restricted population.

Credit authors statement

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Ethical statement

All data of the study have been anonymized; this study received the approbation of the institutional ethical committee of the CHU of Liege (N° 2021/31).

Declaration of Competing Interest

None.

Data availability

No data was used for the research described in the article.

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