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Patients with Cystic Echinococcosis: Evaluation of Clinical and Biological Features of Cysts

Serra Orsten¹, Turkmen Çiftci², Emre Unal², Ahmet Bulent Dogrul², Devrim Akinci², Okan Akhan²

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INTRODUCTION

Cystic echinococcosis (CE) is a neglected zoonotic disease caused by metacestode form of Echinococcus granulosus sensu lato. The life cycle of E. granulosus s.l. mainly perpetuates between canids and livestock animals especially sheep and cattle. Humans are dead-end intermediate hosts that accidentally infect with parasite eggs (1). Molecular analyses have shown that E. granulosus s.l. includes at least 4 species as Echinococcus granulosus sensu stricto (s.s.) (G1 and G3 genotypes), Echinococcus equinus (G4 genotype), Echinococcus ortleppi (G5 genotype) and Echinococcus canadensis cluster (G6/7, G8, G10 genotypes) (2-5). E. granulosus s.s. has a worldwide distribution and it...
is responsible for the majority of cases (88.44%) followed by *E. canadensis* (G6/G7) (11.07%), *E. ortleppi* (G5) (0.36%), *E. canadensis* (G8) (0.06%) and *E. canadensis* (G10) (0.06%) (6). CE is a cosmopolitan disease that is more common in rural areas. According to a population-based screening study, over 100 000 people living in pastoral areas of Turkey might be suffered from abdominal CE (7). In a recent report, the number of cases substantially increased between 2008-2019 in Turkey. Although the increase in the number of registered cases is due to the revision of the surveillance system of the Ministry of Health, there are considerable cases of CE in the region (8).

The World Health Organization (WHO) has recently announced a road map for neglected diseases including Echinococcosis targeted for prevention, control, elimination, and eradication by 2030 (9). According to the document, there is a need to define target product profile and develop optimal diagnostic for humans. As known, CE diagnosis is mainly based on imaging modalities and it is sometimes difficult even in a fully equipped health facility (10). Although CE cysts can be located in every organ, the liver is the most involved organ with a 70% rate (11). The WHO-IWGE (World Health Organization-Informal Working Group on Echinococcosis) has made widely accepted classification for CE cysts. According to this classification, CE cysts can be classified as active (CE1 and CE2), transitional (CE3a/CE3b) and inactive (CE4 and CE5) (12). Whereas imaging techniques are the primary tool for the CE diagnosis, serological tests have an auxiliary role even though these are not standardized for CE (10). Direct microscopic examination of aspirated cyst fluid or DNA detection with polymerase chain reaction (PCR) technique from hydatid material can be used as a confirmatory tools for CE diagnosis. Additionally, PCR-based methods show high specificity and sensitivity rates, as well as useful for the characterization of the species, and genotypes (13).

The main purpose of the study was to evaluate CE patients in a versatile way with the most commonly used diagnostic methods and with the contribution of well-defined and confirmed 45 CE patients.

**MATERIALS AND METHODS**

**Ethical considerations**

This study was approved by the Local Ethical Committee (Ethical Committee of the Faculty of Medicine, Hacettepe University, Turkey 2018; GO 18/366-15).

**Sample Collection**

Sample collection was carried out from May to November 2018. All the patients had an active or transitional CE cysts (CE1, CE2, CE3a and CE3b) according to the WHO-IWGE classification and they have received percutaneous treatment for CE cysts. Patients with inactive CE cysts were not included in the study. Blood samples were taken from patients at the first diagnosis, therefore any medication (e.g. albendazole) was not used by patients before sample collection. In addition, hydatid material was collected during the procedure.

**Serological Tests**

All the collected sera were stored at -80 °C until they were tested. Obtained sera were evaluated using the following commercially available serological test: Hydatidosis IgG ELISA (Vircell SL, Granada, Spain) according to manufacturer's instruction. Sample Index (SI) which is calculated using Optical Density (OD) was used to interpret the ELISA results according to manufacturer's recommendation. Accordingly, ELISA results were accepted negative for SI <0.9, positive for SI 1.1 and border line for 0.9 -SI<1.1. Borderline results were accepted negative. All results were reported as positive or negative. All tests were performed in a same session.

**Direct Examination of Hydatid Material**

Aspirated cyst fluid was transferred to a new sterile tube, and after centrifugation at 3000g for 3minutes, the precipitate was examined by light microscope for the presence of protoscoleces or hooks.

**Genetic Characterization of *E.granulosus* s.l. and Data Analysis**

The DNA extraction from hydatid material was performed using GeneMATRIX Universal DNA/RNA/Protein Purification Kit (EURx, Poland) according to manufacturer's instructions. The PCR was performed to amplify a fragment within the cytochrome c oxidase subunit 1 (cox 1) mitochondrial gene, as previously reported (14). Electrophoresis in 1.5% agarose gels was used to visualize PCR products under ultraviolet light. The amplicons were evaluated as positive if a band size of ~875 bp was obtained. All products were identified by sequencing. Obtained sequence data was analyzed via FinchTV 1.4.0 (Geospiza Inc., Seattle Washington, USA). The BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find and compare the homologous sequences in the GenBank. For data analysis, sequence alignment was performed in Mega version 7 (15) and ClustalX (16).

IBM SPSS Statistics program Ver. 23 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The chi-square test was performed for categorical data.

**RESULTS**

Forty-five patients (23 female and 22 male, mean age: 34.9) were included in this study. At the first diagnosis, 37 of 45 patients (82%) had positive ELISA results. Among the ELISA-negative patients, cyst material belonging to five of them was found negative in the direct examination, as well. In patients with a positive ELISA result (33/37, 89%), almost all of their cysts were found in the liver. Features of patients and hydatid cysts are given in Table 1.
<table>
<thead>
<tr>
<th>Patient No</th>
<th>Sex</th>
<th>Number</th>
<th>Type</th>
<th>Location</th>
<th>Diameter (cm)</th>
<th>Volume (cm$^3$)</th>
<th>Protoscolex</th>
<th>ELISA</th>
<th>Species</th>
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<td>50</td>
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<tr>
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<td>CE3 b</td>
<td>Liver</td>
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A total of 58 CE cysts were recorded and cyst type were determined based on WHO-IWGE classification. According to this, 34 of 58 hydatid cysts were classified as CE1, 14 as CE2, 2 as CE3a and 8 as CE3b. The hydatid cysts were predominantly located in the liver (51/58) and also were detected in the spleen (3/58), kidney (3/58), and bone (1/58). The average diameter of hydatid cysts was found to be 8.25cm.

Of the 58 cyst samples were examined for the presence of protoscolex, 30 of them (51%) were found to be positive and 28 (49%) were negative. There was no relationship between presence of protoscolex and patient characteristics.

All the isolates were confirmed by the BLAST algorithm as E. granulosus s.l. Almost all isolates (56/58) were identified as E. granulosus s.s. (G1/G3 cluster) and only two of isolates belonged to E. canadensis (G6/7 cluster). Due to the small number of samples belonging to E. canadensis, we were not able to investigate the relationship between genetic diversity and CE related-parameters.

The relationship between serodiagnosis results and cyst characteristics such as type, number, location and diameter were also investigated. There was no relationship between serological test results and cyst type and cyst number. On the other hand, positive serodiagnosis result was significantly associated with cyst size (p=0.018) and cyst location (p=0.027).

DISCUSSION

Although the prognosis of CE predominantly proceeds asymptomatic, management of CE depends on multiple factors (e.g., cyst number and stage, cyst location, etc.). Due to this multifactor process, diagnosis and follow-up of patients are still problematic, due to several drawbacks related to the available supporting methods used to complement the imaging findings (13). Integrating many different methods for the diagnosis of CE provides more accurate results (17). In this study, forty-five well-defined CE patients were presented with details of diagnosis.

In terms of protoscolex presence, all hydatid material was evaluated and almost half of them found negative. Although direct examination of cysts is one of the confirmatory tools for diagnosis, molecular techniques such as PCR should be performed for negative samples (18). In accordance with this view, all hydatid cysts (with or without protoscolex) were confirmed molecularly and identified as E. granulosus s.l in this study. Among the ELISA-negative patients, cyst material belonging to five of them was found negative in the direct examination. Besides, four of these patients had CE cysts lower than 5 cm diameters. Therefore, it is shown that molecular techniques should be used as a confirmatory tool for CE, especially in presence of non-specific imaging findings.

Many studies have indicated that E. granulosus s.l. show high genetic diversity (19). In addition, several study were conducted to define the relationship between genetic diversity and cyst characteristics. As an example, cerebral CE was found to be associated with the G6 genotype (E. canadensis) (20). According to a retrospective study, cyst size was significantly small in G7 genotype (E. canadensis) compared to G1 genotype (E. granulosus s.s.) (21). In a recent study, cyst volume was found to be related with genetic diversity (22). In the present study, the relationship between genetic diversity and cyst characteristics could not be investigated due to the small number of isolates that belonged to E. canadensis (2/58).

As known, serology has a complementary role in the diagnosis of CE and cannot be used to guide without imaging findings for the management of CE. Serology results vary depending on the cyst characteristics such as type, number, size (13). In the present study, the majority of patients (37/45) had positive ELISA results. The relationship between serological test results and other parameters was investigated. As a result, it has been noticed that cyst diameter, as well as cyst volume, influenced serodiagnosis. (p=0.018, p=0.36). Consistent with other studies (23,24), it was noted that as the size of the cyst increased, the serological test results tend to be positive. Besides, all the patients harbored giant hydatid cysts (>10 cm) were found to be serology positive. We have shown that serological test results were influenced also by cyst location (p=0.027). In harmony with our results, most of the studies have supported that serodiagnosis is non-reliable when the cyst involved other than the liver (23). In contrast with other studies, cyst properties such as cyst type, cyst number, and presence of protoscolex were not found to be related to serological test results (23,25-28).

CONCLUSION

In conclusion, microbiological confirmation of the radiological diagnosis of CE is critical for accurate epidemiological information. In the present study, we have used different diagnostic approaches of CE and properly compared them. As a consequence, the best tool for CE diagnosis other than imaging techniques is still the subject of debate. This study suggests that even if the absence of protoscolex in direct examination and the serological test results are negative, it will definitely be useful to perform molecular techniques.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study was approved by the Local
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In Vitro Study on the Protoscolicidal Effect of Synthesized Novel Azole Derivatives on Protoscoleces of Cystic Echinococcosis

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OBJECTIVE: Echinococcosis is a zoonotic parasitic infection caused by Echinococcus granulosus sensu lato (E. granulosus s.l.) with world-wide distribution. In human, E. granulosus is found in the larval form, while the adult form is usually found in dogs. Surgery is still the treatment of choice for treatment of CE. However, chemotherapy using drugs like Albendazole, Mebendazole and Flubendazole has been used for a long time. There is still no agreement on their usage because of their high adverse effects, long-term therapy and low response. Using the new and novel drugs on protoscoleces is always interesting.

MATERIALS AND METHODS: We applied in vitro protoscolicidal effect of a group of azole derivatives on protoscoleces of CE cyst. Sheep hepatic cysts were collected from Shiraz abattoir and transferred to the parasitology department at Shiraz medical school, where protoscoleces were separated aseptically for protoscolicidal studies. The protoscoleces were incubated against 7 new synthesized azole derivatives using LC50 of the compounds.

RESULTS: Our results showed that ligand no.5 with a concentration of 50 µg/ml and 10 µg/ml, and the ligand no.3 in concentration of 50µg/ml had the most protoscolicidal effects. The lowest protoscolicidal effect was observed for ligand no.7 with 44.30±22.1 and 53.46±28.63 percent destroying effect, respectively.

CONCLUSIONS: Regarding the high protoscolicidal effect of the ligands no. 3 and 5, these compounds have in vitro protoscolicidal effects. Further ex vivo and, in vivo investigations on these compounds is suggested.

Keywords: In vitro, protoscolicidal, novel azole derivatives, Cystic echinococcosis. CE cyst
considered as the only therapeutic method for the disease despite out breaking, mortality and high recurrence rate (3). However, there are four different management modalities: percutaneous therapy, surgery, chemotherapy, and observation without intervention (watch and wait) (2). Since the discovery of Thiabendazole in 1961, different benzimidazoles have been applied for treatment of the disease. Three combinations of benzimidazoles including Albendazole, Flubendazole and Mebendazole have been applied more. The common benzimidazoles drugs stop parasite growth but do not kill the parasite. To compensate this therapeutic shortfall, new treatment alternatives are urgently needed (4,5). Mebendazole was the first combination of benzimidazoles that was applied for treatment of the disease, but its absorption from the bowel is weak. Moreover for effective results, great dose is needed for a long time duration (5). Albendazole has a better absorption with stronger effects. This drug prevents from absorbing the glucose by parasite; but it has no effect on glucose of blood concentration (5). Chemotherapy remains as an unsolved dilemma in curing the disease, as the effective drug on CE cyst, has not been found yet (5).

Many heterocyclic derivatives includingazole derivatives have been synthesized as important substrates to develop novel therapeutic agents against different diseases. Azoles are a class of five-membered nitrogen-containing heterocyclic systems that may have at least one other non-carbon atom(s) such as nitrogen, sulfur, or oxygen. Azoles such as imidazole and triazole analogs as well as their fused derivatives with benzene rings such as benzimidazoles and benzotriazoles have attracted considerable attention in drug discovery and medicinal chemistry. Up to now, there has been an increase in the number of antifungal drugs available with various structures and scaffolds. However, their clinical value has been limited by the emergence of drug resistance, high risk of toxicity, insufficiencies in their antifungal activity as well as undesirable side effects. Azole derivatives are the most extensively studied class of antifungal agents due to their high therapeutic index, a broad spectrum of action, good bioavailability, chemical stability, and favorable safety profile (6). However, the anti-parasitic effects of these compounds including protoscolicidal effect of these compounds have not been investigated.

Regarding the above mentioned items, the present study was designed to investigate in vitro effect of synthesized novel azole derivatives on protoscoleces of cystic echinococcosis. To this end, the different azole derivatives including benzotriazole, benzimidazole, imidazole, 2-phenylimidazole, and succinimide were used to synthesize ligands 1-7. These azoles were selected since they are present in the structure of many drug exhibiting different chemotherapeutic properties.

MATERIALS AND METHODS

Sheep liver cyst samples were prepared from slaughterhouse in Shiraz and transferred to the Helminthology research laboratory at Shiraz Medical School for further works. In the laboratory, surface of the cysts were sterilized by heat. Then the cysts were immediately aspirated with 10 or 20 ml syringe. The cysts were opened by sterilized blade of surgery and the contents including protoscoleces were aspirated by syringe. The contents of the cysts were transferred into 50ml falcon tubes. The tubes were putted in the laboratory for sedimentation of the protoscoleces. The liquid were poured out and the protoscoleces were washed using sterilized PBS. The viability of protoscoleces was determined as the percent of viable protoscoleces to the total protoscoleces in 1ml of the CE cyst fluid, using eosin stain 0.1% (0.1mg eosin in 100ml distilled water) (7). For staining, 10μl of protoscoleces containing solution was used with 10μl of eosin solution and checked by microscope (X100 and X400). Dead protoscoleces were stained while live protoscoleces were remained unstained (Figure 1). The viability of protoscoleces were monitored, before starting the protoscolicidal study and, only the batches with viability of more than 85% were used for the tests. For investigation the effects of ligands on protoscoleces, three concentrations of 5, 10 and 50 microgram per ml solution in DMSO (as a solvent media for chemical agents) were used. For each set of experiment cell culture microplates were used where one ml of DMSO solutions with the mentioned concentration of ligands, plus 100 to 200 protoscoleces were placed in an incubator with 37°C with 5 percent CO$_2$. RPMI$_{1640}$ containing 10 percent (FCS) and DMSO with 1:1000 concentration were used as the negative control. Albendazole drug with 50 microgram concentration per ml were used as positive control. A total of seven ligands were used as are follows:

1. $1\cdot[2\cdot(1\text{-}H\text{-}\text{Benzo[d]}[1,2,3]\text{triazole-1-yl})\cdot1\cdot\text{phenylethylidene}]\cdot2\cdot\text{phenylhydrazine}$
2. $1\cdot[2\cdot(1\text{-}H\text{-Benzo[d]}\text{imidazol-1-yl})\cdot1\cdot\text{phenylethylidene}]\cdot2\cdot\text{phenylhydrazine}$
3. $1\cdot[2\cdot(1\text{-}H\text{-Benzo[d]}\text{imidazol-1-yl})\cdot1\cdot(4\text{-}methoxyphenyl)\text{ethylidene}]\cdot2\cdot\text{phenylhydrazine}$
4. $1\cdot[2\cdot(1\text{-}H\text{-Imidazole-1-yl})\cdot1\cdot\text{phenylethylidene}]\cdot2\cdot\text{phenylhydrazine}$
5. $1\cdot[2\cdot(1\text{-}H\text{-Imidazole-1-yl})\cdot1\cdot(4\text{-}methoxyphenyl)\text{ethylidene}]\cdot2\cdot\text{phenylhydrazine}$
6. $1\cdot\text{Phenyl-2-}[1\cdot\text{phenyl-2}(2\cdot\text{phenyl-1H-imidazole-1-yl})\text{ethylidene}]\text{hydrazine}$
7. **3-[2-Phenyl-2-(phenylhydrazono)ethyl]-imidazolidine-2,4-dione.**

To investigate the effects of ligands on protoscoleces, the viability of protoscoleces were checked under invert microscope using eosin method (7).

![Image of protoscoleces](Figure 1. Dead protoscoleces stained with eosin (A). The live protoscoleces were remained unstained (B). Two dead and one alive protoscoleces. The effect of ligands on the structure of protoscoleces (D))

**RESULTS**

**The protoscolicidal effects of tested ligands, are as follows:**

Ligand number 1: protoscolicidal effects of ligand number 1, in three concentrations of 5, 10 and 50 microgram per ml, compared with positive and negative controls. Albendazole, had 100 percent protoscolicidal effect on protoscoleces at thirteenth day post exposure, while ligand number 1, had such effect at twenty eight days post exposure. Mean percentage of protoscolicidal effect in concentration of 50, 10 and 5 microgram per ml was 81.5±26.8 percent and in 5 and 10 microgram per ml was 63.2±28.4 and 62.6±26.2 percent, respectively (Figure 4). Ligand number 4: ligand number 4 used in concentrations 5, 10 and 50 microgram per ml. A 100 percentage protoscolicidal effect of this ligand was observed in concentration 50 microgram per ml in 8th day post exposure which was similar to positive control. The mean of protoscolicidal effect of this ligand in concentrations of 5, 10 and 50 microgram per ml was 73.8±32, 67.7±26 and 67.1±25.1 percent, respectively while this rate for Albendazole was 81±6±22.3 percent (Figure 5). Ligand number 5: ligand number 5 also investigated for the same three concentrations 5, 10 and 50 microgram per ml. In concentration 10, 50 microgram per ml, this ligand was compared to Albendazole as the positive control, had faster protoscolicidal effect such that in ninth day post exposure 100 percentages protoscolicidal effect was observed. While Albendazole in thirteenth day, had such effect. The mean protoscolicidal effect of this ligand in the concentration 50 microgram per ml, during 29th days post exposure was 85.6±27 percent and in the concentration 10 microgram per ml was 81.7±25 percent (Figure 6). Ligand number 6: ligand number 6 was also investigated in 3 concentrations 5, 10 and 50 microgram per ml. In concentration 50 microgram per ml, this ligand had 100 percentages protoscolicidal effect on protoscoleces in twenty thirteenth days post exposure. While in concentrations of 5 and 10 microgram per ml had 100 percentages protoscolecidal effect on 18th and 19th day. While a 100 percentage protoscolicidal effect of Albendazole as the positive control was observed on 13th day. The mean protoscolicidal effect for this ligand in the concentrations of 5, 10 and 50 microgram per ml was 68.5±27.9, 73.7±28.1 and 74.3±25.7 percent, respectively; while this rate for Albendazole was 81.7±25.9 percent (Figure 7). Ligand no. 7: The protoscolicidal effect of this ligand was also investigated in three concentrations of 5, 10 and 50 microgram per ml. In concentration of 50 microgram per ml, a 100 percent protoscolicidal effect for this ligand was observed in 23rd day post exposure while in concentration of 10 microgram per ml in 28th day and in concentration 5 microgram per ml in 26th day. A 100 percentage protoscolicidal effect was observed with Albendazole in 13th day post exposure. The mean protoscolicidal effect for this ligand in the concentrations of 5, 10 and 50 microgram per ml was 67.9±26.8, 44.3±22.0 and 53.4±28.6 percent, respectively; while this rate for Albendazole was 81.7±25.9 percent (Figure 8). The mean protoscolicidal effects of the ligands in different concentrations is shown in Table 1.

Table 1: The mean rate of protoscolicidal effects of the ligands in different concentrations

<table>
<thead>
<tr>
<th>Ligand Number</th>
<th>Drug Concentration</th>
<th>Death Rate Mean</th>
<th>Standard Deviation</th>
<th>Test Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 µg/ml</td>
<td>66.17</td>
<td>22.59</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>53.64</td>
<td>17.82</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>62.92</td>
<td>22.99</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>50 µg/ml</td>
<td>63.04</td>
<td>18.12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>60.63</td>
<td>20.42</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>60.85</td>
<td>18.67</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>50 µg/ml</td>
<td>81.57</td>
<td>26.83</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>63.24</td>
<td>28.43</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>62.62</td>
<td>26.28</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>50 µg/ml</td>
<td>73.81</td>
<td>26.06</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>67.71</td>
<td>27.15</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml</td>
<td>67.15</td>
<td>25.10</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>50 µg/ml</td>
<td>85.63</td>
<td>27.08</td>
<td>6</td>
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<tr>
<td></td>
<td>10 µg/ml</td>
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<tr>
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<td>27.97</td>
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<td></td>
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<td>73.74</td>
<td>28.19</td>
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<tr>
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<td>74.35</td>
<td>25.72</td>
<td>6</td>
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<tr>
<td>7</td>
<td>50 µg/ml</td>
<td>67.92</td>
<td>26.81</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>44.30</td>
<td>22.01</td>
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<tr>
<td></td>
<td>5 µg/ml</td>
<td>53.46</td>
<td>28.63</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 2. Protoscolicidal effect of ligand number 1 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 3. Protoscolicidal effect of ligand number 2 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 4. Protoscolicidal effect of ligand number 3 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 5. Protoscolicidal effect of ligand number 4 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole
DISCUSSION

For many years, surgery was the only therapeutic method for CE cyst treatment (3). It is still the selective method for treating CE cyst (3,7). However surgery method has over 10 percent recurrence, 0-20 percent mortality and 25-40 percent out breaking (7). Chemotherapy remains as the unsolvable dilemma about CE, because less effective drug has been found for CE cyst (5). Derivatives of Benzimidazoles like Albendazole and Mebendazole are commonly used for chemotherapy CE cyst (5). In cases of humans, high doses should be taken for a long time, therefore their side effects observing mostly (5). A study on the effects of flubendazole on protoscoleces showed similar results and showed that protoscoleces exposed to Flubendazole after 25 days, only 13.9±5.9 percent of protoscoleces were survived and after 13 days, all of protoscoleces were killed (8,9). In this research, in vitro effects of seven combination of novel azole derivatives that has been synthesized in chemistry department of Shiraz University (10,11), tested on protoscoleces Echinococcos granulosus. In comparison with effects of in vitro protoscolicidal drugs including Nitazoxanide (12), Praziquantel (13), Artemisinin and its derivatives (14), ligands had less destroying effects on protoscoleces. The effects of ligand no. 3 and 4, was similar to the results of the study by Walker et al. on Nitazoxanide (12), and Artemisinin and its derivatives (14); while in comparison with in vitro destroying effects of Flubendazole and Praziquantle (5,13), the ligand no. 3, had stronger destroying effects. Similar effects of Nitazoxanide drug scolicidal effects on protoscoleces, seems to be similar to the effect of ligand no. 5 with dose dependent effect (12). In comparison with in vitro destroying effects of Flubendazole (5) and Praziquantle (13) destroying effects of ligand no. 5 was stronger. In vitro destroying effects of ligand no. 6 was weaker in comparison with destroying effect of Nitazoxanide, Praziquantle and Artemisinin (12-14). In comparison with destroying effects of Flubendazole (5) that was 100 percent protoscolicidal effect on protoscoleces in 13th day, destroying effect of ligand no. 6 about used concentration was more stronger. Protoscolicidal effects of ligand no. 7, was similar to in vitro protoscolicidal effects of Flubendazole (5). While in comparison with in vitro effects of Praziquantle (13) Nitazoxanide (12), Artemisinin and its derivatives (14), destroying effects of ligand no. 7 was weaker. These chemicals have a higher protoscolicidal effect than natural ones (15-17). Although benzimidazoles have been the cornerstone of medical therapy since the late 1980s, many issues (e.g., duration of therapy) remain unresolved (18). Many other compounds have been tested experimentally without success. A synergy between benzimidazoles and other agents such as metformin seen in vitro, has been reported. However, still a great need for development of new chemotherapeutic agents both synthetic and natural is essential (18-21).

CONCLUSION

In conclusion, the results showed that compound no.5 with a concentration of 50 µgr/ml and 10 µgr/ml and...
compound no. 3 in concentration of 50µg/ml had the most protoscolicidal effects. The lowest protoscolicidal effect was observed for ligand no. 7 with 44.30±22.1 and 53.46±28.63 percent destroying effect, respectively. This study is the first in vitro protoscolicidal effects of these compounds, so, we suggest ex vivo and, in vivo studies on the ligands no. 3 and 5.

**Competing interests:** The authors declare that they have no competing interest.

**Financial Disclosure:** This study was finically supported by Grant No. 3762 supported by the office of Vice Chancellor for Research, Shiraz University of Medical Sciences, Shiraz, financially.

**Ethical approval:** This study was approved by the research ethics committee of the Shiraz University of Medical Sciences (ethical code: IR.SUMS.REC.1386. S3762).

**REFERENCES**

**ABSTRACT**

**Objective:** Cystic echinococcosis (CE) is a disease that is endemic in our country and is caused by the contamination of eggs of the parasite *Echinococcus granulosus*. Although elective surgeries cannot be performed in certain periods during the pandemic period in our clinic, as in the whole country, from the date of the appearance of the Coronavirus Disease (COVID-19), it was aimed to examine the surgical and interventional procedures applied in the last 3 years and their results.

**Materials and Methods:** Twenty-four patients admitted to our clinic with the diagnosis of CE disease between 2019 and 2021 were retrospectively analyzed in terms of demographic, clinical, operative, and post-operative follow-ups.

**Results:** Twelve of the patients were male and 12 were female. The mean age of the patients was 43.8 years (18-99 year). There were CE lesions in the right lobe in 15 patients, in the left lobe in 2, and in both lobes in 4 patients. Lesions compatible with type 3 hydatid cysts were mostly observed in the patients. 4 patients had more than one hydatid cyst in the liver. The mean cyst diameter was 11.5 cm (5-22cm) in the examination performed considering the largest cyst diameter of patients with multiple cystic lesions. The cyst was infected in 4 patients. In 1 patient, the liver cyst ruptured and was operated under emergency conditions. In the operation, cholecystectomy was performed in 5 patients and splenectomy was performed in 1 patient simultaneously. Intraoperative primary repair was performed in a total of 4 patients with intra-cyst biliary fistula. In the postoperative period, intracystic abscess developed in 4 patients and percutaneous abscess drainage was performed. Concurrent appendiceal mucocele was present in 1 patient and appendectomy was added to the procedure. The mean hospital stay of the patients was 9.5 days (2-19 days). Mortality was not observed.

**Conclusion:** As a result of delayed elective surgery in CE disease during the pandemic, cysts were encountered in complicated and advanced stages, which increased the number of hospitalizations and additional procedures to be performed.

**Keywords:** Liver cystic echinococcosis, percutaneous treatment, surgical treatment, laparoscopic surgery, COVID-19
INTRODUCTION

Cystic echinococcosis (CE) is a disease that is endemic in our country and is caused by the contamination of eggs of the parasite *Echinococcus granulosus* (1). Although the disease is frequently seen in the Southeastern and Eastern Anatolia regions of our country, it continues to be an important health problem with its economic and public health dimensions (2). Cystic echinococcosis can affect almost all organs in the body, but it usually settles in the liver at a rate of 50-70%. The cyst is usually located in the right lobe of the liver and is single in 70-80% (2). The second most common organ is the lung with a rate of 20-30%, and it is seen less frequently in the spleen, kidney, heart, bone, central nervous system and other organs (3,4). Because CE grows very slowly, they remain asymptomatic for years. The preliminary diagnosis is usually made as a result of radiological examinations performed for other reasons. Right upper quadrant pain, jaundice, nausea, vomiting and abnormal liver function tests may be observed in symptomatic cases. Medical treatment (albendazole), percutaneous drainage and surgery are current treatment options in liver CE disease. In complicated cysts, surgery is still the best option (5-11). The aim of surgical treatment is to eliminate the scolexes, remove all viable elements of the cyst, and obliterate the remaining cavity (12). Since most of the surgeries are performed by general surgeons who are not experts in the field of liver-biliary duct surgery, conservative methods are preferred more frequently (13-16). Coronavirus Disease (COVID-19) is a disease that first developed in late December in China’s Wuhan Province, with respiratory symptoms (fever, cough, shortness of breath) (17). Although elective surgeries cannot be performed in certain periods during the pandemic period in our clinic, it was aimed to examine the surgical and interventional procedures and their results in patients diagnosed with liver CE from the beginning of the pandemic until now.

MATERIALS AND METHODS

Ethics committee approval was obtained from Antalya training and research hospital with the protocol number 17/3 dated 08/09/2022. The files of 24 hydatid cyst patients who underwent surgical or interventional procedures in the general surgery clinic of Antalya Training and Research Hospital between 2019-2021 were retrospectively analyzed. The cases were evaluated in terms of age, gender, existing symptoms, radiological findings, laboratory results, cyst locations, cyst type, types of surgery, medical treatment, post-operative follow-up of the patients (complications, recurrences) and hospital stay. All patients except emergencies were treated with albendazole (10 mg/kg/day) for 2 weeks before the operation and they were taken into the operation. Albendazole treatment was continued for 3 months in the post-operative period. Data collected were calculated with SPSS (16 for Windows, SPSS Inc., Chicago, Illinois, USA).

RESULTS

Four patients had more than one hydatid cyst in the liver. Laparoscopic partial cystectomy was performed in 3 of the patients, and in 1 case, the opening was performed due to difficulty in reaching the cyst. While PAIR (Puncture, aspiration, injection, re-aspiration) method was applied to 2 patients, partial cystectomy by laparotomy and total excision in some cases were applied to other patients. Omentopexy was added to 7 patients. Partial cystectomy and omentopexy were performed after recurrence was observed in one patient who underwent Pair. Lesions compatible with type 3 hydatid cysts were mostly observed in the patients. The cyst was infected in 4 patients. In 1 patient, the liver cyst was ruptured and operated under emergency conditions. Emergency surgery was performed in 1 patient due to the rupture of the lung cyst associated with the liver cyst. 5 patients were previously operated with the diagnosis of hydatid cyst. In the operation, cholecystectomy was performed in 5 patients and splenectomy in 1 patient simultaneously. Intraoperative primary repair was performed with 2/0 or 3/0 polypropylene or absorbable suture material in a total of 4 patients with intra-cyst biliary fistula, 3 in the right lobe and 1 in the left lobe. Three of these 4 patients required endoscopic sphincterotomy (ES) with Endoscopic Retrograde Cholangiopancreatography (ERCP) due to the persistence of bile leakage in the postoperative period. In the post-operative period, intraoperative undetected bile leakage was observed in 3 patients. These patients were also treated with ERCP and ES. Pre-op ERCP and ES were performed in 1 patient due to hydatid cyst associated with bile ducts. In the postoperative period, intracystic abscess developed in 4 patients and percutaneous abscess drainage was performed. Concurrent appendiceal mucocoele was present in 1 patient and appendectomy was added to the procedure. The mean hospital stay of the patients was 9.5 days (2-19 days). Post operative pleural effusion was observed in 2 patients. No mortality was observed.

DISCUSSION

Hydatid cyst is the larval form of the parasitic infection caused by the *Echinococcus granulosus*, which causes endemic diseases all over the world (18,19). The frequency of the disease was found to be 1.05% in our country, and approximately 2000 new cases are detected each year (20,21). Cystic echinococcosis is most frequently observed in the liver (18).

The diagnosis of CE is made by radiological methods (USG, CT, MRI). Following the USG classification developed
by Gharbi et al. (22) in 1981, the World Health Organization Informal Echinococcus Working Group (WHO-IWGE) developed the universally applicable international standardized USG classification in 1994 (23). We used this classification in our study. Half of the patients presented with Type 3 hydatid cyst.

There are basically four treatment options: surgery, percutaneous technique, antiparasitic medical treatment and follow-up (wait and see) (20). Among them, PAIR or laparoscopic surgery are effective methods that can be used safely in experienced hands in suitable patients, since they are less invasive during the pandemic period.

Before any intervention in the treatment of Cystic echinococcosis, the lesion should be evaluated radiologically, staged, and the most appropriate approach is to start patient-specific treatment planning by providing clinical and laboratory correlation (24). Albendazole is an important aid in preventing unintentional spread during surgery and percutaneous procedures (25,26). In our clinic, albendazole treatment was started for all patients 2 weeks before the surgery or percutaneous procedure, and it was observed that medical treatment was continued for at least 3 months after the procedure.

Although open surgery was the only treatment method in the past, it is still the main treatment method especially for complicated hydatid disease (9). During the pandemic period, laparotomy and partial cystectomy became the most preferred treatment method, as patients were admitted with complicated and advanced hydatid disease. While 12 patients had type 3 cysts, 4 patients had abscess due to infection of the cyst.

In the surgical treatment, 20% NaCl or betadine was used as a scolicidal agent.

The most common complication of liver hydatid cyst is cystobiliary intercourse or intrabiliary rupture, which is observed at a rate of 5-25% (27-33). In our study, intraoperative biliary involvement was detected in 4 patients and preoperative biliary involvement in 1 patient, while bile leakage was observed in 3 patients, which was not noticed during the operation and was detected in the postoperative period. The ERCP and ES procedure was applied to our patients whose post-operative biliary fistula continued.

Infection of the cyst contents with pyogenic microorganisms is another complication that may be encountered. It is treated with drainage of the abscess and appropriate antibiotics (34). Intraoperative abscess was accompanying in 4 patients. In the post-operative period, abscess developed in 4 cases and was treated with percutaneous drainage and antibiotic administration.

The most important cause of anaphylaxis, which is the most dangerous complication, is the mixing of cyst fluid into the circulation (23). No anaphylaxis or death was observed in our clinic.

CONCLUSION

Despite the advances in diagnosis and treatment in recent years, Cystic echinococcosis, which still cannot be eradicated, is endemic in our country and causes a public health problem, disrupts human and animal health and causes economic losses.

Basically, there are four treatment options, and the most appropriate treatment is chosen according to the cyst size and number, localization, cystobiliary relationship, cyst structure, and the availability of an experienced surgeon and an experienced radiologist.

As a result, due to the postponement of elective surgery in Cystic echinococcosis during the pandemic period, encountering cysts in complicated and advanced stages has increased the number of hospitalizations and additional procedures to be performed.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: The authors declare that they have received no financial support for the study.

Ethical approval: Protocol number: 17/3, Date: 08/09/2022, Institution: Antalya training and research hospital.

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**ABSTRACT**

**Objective:** Cystic echinococcosis (CE) is a parasitic zoonosis caused by *Echinococcus granulosus* sensu lato. Immunodiagnostic techniques such as Western blot (WB) or enzyme-linked immunosorbent assay (ELISA), with different antigens, can be applied to the diagnosis of sheep for epidemiological surveillance purposes in control programs. However, its use is limited by the existence of antigenic cross-reactivity between different species of taeniidae present in sheep. Therefore, the usefulness of establishing surveillance systems based on the identification of infection present in a livestock establishment, known as the (Epidemiological) Implementation Unit (IU), needs to be evaluated.

**Materials and Methods:** A new ELISA diagnostic technique has been recently developed and validated using the recombinant EgAgB8/2 antigen for the detection of antibodies against *E. granulosus*. To determine detection of infection at the IU level using information from this diagnostic technique, simulations were carried out to evaluate the sample size required to classify IUs as likely infected, using outputs from a recently developed Bayesian latent class analysis model.

**Results:** Relatively small samples sizes (between 14-29) are sufficient to achieve a high probability of detection (above 80%), across a range of prevalence, with the recently recommended Optical Density cut-off value for this novel ELISA (0.496), which optimizes diagnostic sensitivity and specificity.

**Conclusions:** This diagnostic technique could be potentially used to identify the prevalence of infection in an area under control, measured as the percentage of IUs with the presence of infected sheep (infection present), or to individually identify the IU with ongoing transmission, given the presence of infected lambs, on which control measures should be intensified.

**Keywords:** Echinococcosis, immunodiagnostics, sheep, surveillance, control
INTRODUCTION

Cystic echinococcosis (CE) is a parasitic zoonosis caused by *Echinococcus granulosus sensu lato*, a cestoda parasite in the Taeniidae family. The life cycle involves two mammalian hosts. The definitive ones are carnivores (especially dogs), and the intermediate ones are ungulates (being sheep and goats the ones of greater epidemiological importance in many parts of the world) (1).

The surveillance of CE in the framework of a control programme is directed towards the different hosts: mainly humans (who are accidental hosts), dogs, sheep and goats (2). Diagnosis in sheep and goats can be made macroscopically at slaughter (via necropsies) (3-5). However, in many endemic areas, slaughterhouses are rare: the urban supply of meat usually originates from local butchers without any type of sanitary infrastructure or veterinary inspection; in rural areas on the other hand, home slaughter for personal consumption or retail sale is the norm. Therefore, while post-mortem inspection is the technique of choice, it is challenging to support a surveillance system on this data source (6). Moreover, macroscopic diagnosis in lambs also has limitations, in particular false negatives from recent infections that may not be detected, or from newly formed small hydatid cysts that are unlikely to be observed. In older animals, false positives are also possible from the presence of degenerated or calcified cysts due to other infections or conditions (4).

Immunodiagnostic techniques such as western blot (WB) or enzyme-linked immunosorbent assay (ELISA), with different antigens, can be applied to the diagnosis of ovine CE (3,5,7,8). However, there is a limitation for its use in sheep, as infections by parasites other than *E. granulosus* (*T. hydatigena, Monezia, Tænia ovis, Tænia multiceps*) are common in sheep and goats, and there is evidence of antigenic cross-reactivity between different species of taeniidae (2,5,7).

Here, we discuss a CE surveillance strategy focusing on the implementation of sheep serology at an epidemiological unit level (IU), such as a livestock farm, rather than at individual level, that takes advantage of a novel recombinant antigen for ELISA. The immune response of the ruminant host against infection is directed towards the oncosphere, components of the immature cyst and/or fertile metacestodes and protoscolices (1,2,7). An IgG response to the fluid of the hydatid cyst of *E. granulosus*, i.e. antigens in the oncosphere, appears between four to eleven days post-infection in sheep experimentally challenged with either eggs or oncospheres, and persists for at least 4 years. However, it does not always lead to a significant increase in antibody titres and is also not maintained throughout the course of infection (9,10).

The hydatid fluid is a complex mixture of different antigens, the main ones being the antigenic lipoproteins: Ag 5 and Ag B. Ag B is the most abundant and is a thermostable lipoprotein of 120-160-kDa containing subunits: 8 or 12, 16, and 20 or 24 kDa (1,2,6). A multigenic family coding for the 8-kDa antigen (*EgAgB8/1 to EgAgB8/5*) was found to be composed of many members with high diversity, so its use can provide molecular evidence of cross-reaction, or specific reaction, for infections with metacestodes (1,2,6,11). A new ELISA diagnostic technique has been recently developed and validated using the recombinant *EgAgB8/2* antigen for the detection of antibodies against *E. granulosus* (11).

To collect the samples for the ELISA in sheep, 10ml of blood from the jugular vein can be drawn by holding the animal in a standing position with their heads fixed laterally, using 25/8 needles and disposable plastic syringes. The samples need to be labelled with one number identifying the animal and another identifying the producer. Ideally the data will be collated in a registration form that contains the numbers cited, the name of the producer, the geographical area (or otherwise be geo-referenced) and the date of sampling. It is also beneficial to record the total number of existing sheep and lambs, which can facilitate further analysis. Serum can be extracted by centrifugation and must be kept refrigerated at 5 to 8ºC until its referral to the laboratory (48 hours maximum), where it can be kept in a freezer at –20ºC until it is processed.

Sample size estimation

To determine a suitable sample size for the evaluation of infection at the IU level, outputs of a Bayesian Latent Class Analysis framework developed by Sykes et al., implementing a Markov chain Monte Carlo algorithm (12) were used. Briefly, the model infers the ‘true’ infection status of individual sheep based on multiple diagnostic techniques, without assumptions about a gold standard. In the work by Sykes et al., the model used data from necropsies, the recombinant *EgAgB8/2* antigen and western blots from 79 adult sheep.

Extending on this work, posteriors drawn from the Bayesian model were used to simulate IUs for a range of infection...
prevalence and to evaluate different sample sizes for high probabilities of CE detection. Nine farm scenarios were simulated, with prevalence in the farm of 1%, and 5% to 40% (in 5% increments). The deployment of the ELISA diagnostic technique was simulated, with different sample sizes from 1 to 100 sheep taken in each simulated farm. The posterior distribution for the sensitivity and specificity was used for the ELISA technique with an optical density cut-off value of 0.496, as defined in Sykes et al. (12). The lowest sample size needed for probabilities of detection of 80% and 90% was calculated. This can be defined as the proportion of IUs correctly identified as infected (with prevalence >0%). An IU was assumed to be infected if two or more samples came back positive (12).

<table>
<thead>
<tr>
<th>Probability of detection</th>
<th>Expected Prevalence</th>
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<tr>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>80%</td>
<td>21</td>
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<tr>
<td>90%</td>
<td>29</td>
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The sample size required at the IU level will change depending on the minimum probability of detection wanted for the programme, Table 1. Achieving a higher probability of detection requires larger sample sizes. It is important to highlight that the number of animals in the herd has a negligible effect on the sample size in these settings where IUs have hundreds (or even thousands) of animals.

**DISCUSSION**

Traditionally, CE surveillance has been based on estimating the percentage of parasitized dogs (13). An alternative approach would be to establish surveillance systems based on the identification of infection present in a livestock establishment (i.e. a farm), here our IUs, based on an assessment of the sheep population. This has the advantage that ongoing transmission in IUs could be assessed based on the presence of lambs infected with *E. granulosus*, while presence of CE, which might not imply current ongoing transmission, could be evaluated from infections in older animals. Moreover, CE could be identified in herds that are scheduled to enter areas free from infections in older animals. Moreover, while an epidemiological implementation unit would generally consist of a single livestock establishment with enough animals to cover the sample, in the case of small producers with few animals, the IU sample may be drawn among several producers, particularly in the case

**RESULTS**

The specificity and analytical sensitivity of the ELISA diagnostic technique were evaluated with a panel of control sera of experimentally infected sheep (n=40), free of the disease (n=79), and animals naturally infected with other parasites (n=20), observing a satisfactory capacity of discrimination between positive sera of different reactivity, negative sera, and laboratory controls without antigen (11). The performance of this ELISA diagnostic at both the individual level and at the herd level was determined by ROC curves, estimating an optical density of 0.496 as an appropriate cut-off value that optimizes sensitivity and specificity at the IU level (12).

It is important to highlight that CE control programmes generally have no interest in the individual diagnosis of *E. granulosus* infection, since there is a lack of a validated specific treatment. The main objective of the programme remains the determination of infection in the herd. To use immunodiagnosis in sheep for surveillance purposes, two requirements need to be met: 1) A validated serological technique needs to be available; and 2) it should be feasible to collect a suitable, representative, sample size. The sample size needs to be appropriate to the objective, minimizing the bias given by cross-reaction with other cestodes.

As mentioned above, the recombinant EgAgB8/2 antigen recently validated could be a suitable serological technique, while, as shown in Table 1, the sample sizes required for IU are potentially feasible in many settings. Prevalence in the IU is generally not known, but the sample size needed will depend on it, therefore an “expected” prevalence needs to be assumed. This value can be inferred from knowledge of the local epidemiology and bibliographic background or reports from comparable studies (14). While a detection of 90% (or higher) would be recommended, in many resource-constrained settings 80% would be sufficient. Moreover, while an epidemiological implementation unit would generally consist of a single livestock establishment with enough animals to cover the sample, in the case of small producers with few animals, the IU sample may be drawn among several producers, particularly in the case

**DISCUSSION**

Traditionally, CE surveillance has been based on estimating the percentage of parasitized dogs (13). An alternative approach would be to establish surveillance systems based on the identification of infection present in a livestock establishment (i.e. a farm), here our IUs, based on an assessment of the sheep population. This has the advantage that ongoing transmission in IUs could be assessed based on the presence of lambs infected with *E. granulosus*, while presence of CE, which might not imply current ongoing transmission, could be evaluated from infections in older animals. Moreover, CE could be identified in herds that are scheduled to enter areas free from infections. Furthermore, the effectiveness of remedial actions could more readily be inferred, where transmission might be maintained despite control measures in place, thus requiring the intensification of activities. Baselines and trends of CE in the area under a programme could be equally evaluated from the percentage of IUs with infected animals, supporting the evidence base for future activities.

*Table 1: Reference for estimation of sample sizes according to the expected prevalence to identify transmission present with a cut-off value of 0.496, depending on the desired probability of detection. For an expected prevalence greater than 40%, it is recommended to use the sample size estimated at 40%*
of indigenous reserves and communal grounds that are shared as grazing pastures. The appropriate sample will then be selected by randomly choosing animals, which can prove more cost-effective.

While there are no field studies published yet using this approach, there are a few ongoing. A study in the northern region of San Luis province in Argentina is evaluating control in sheep and goats using the commercial vaccine Providean Hidatec EG95®, and will evaluate the recombinant EgAgB8/2 antigen in these two species, as well as another diagnostic in dogs (copro-antigen)15. Another study in the province of Misiones, Argentina is directly evaluating the recombinant EgAgB8/2 antigen in the field, in both sheep and goats (16).

CONCLUSION

Modern CE surveillance systems generally have one of two aims: either 1) characterize the prevalence of infection in an area under control measured by the proportion of IUs with infected sheep, which can be used to monitor progress of different interventions; or 2) identify IUs with ongoing transmission by investigating infection in young lambs, which again may lead to intensification of control measures such as deworming or vaccination.

To use immunodiagnosis in sheep for surveillance purposes, a validated serological technique needs to be available with an appropriate sampling design. Therefore, the ELISA in sheep described above can be used, alone or associated with the traditional CoproELISA in dogs (2,9), or other diagnostic techniques in the definitive host, as a new tool for monitoring CE in a standardized way for control and surveillance programmes. Logistically, whether it is sheep or dog samples, both are obtained by the same personnel, therefore both approaches could be used synergistically, as they are simple, economical and accessible to countries with limited resources and laboratory capacity. These would lead to enhanced surveillance of E. granulosus transmission and better evidence to adjust control measures.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: The authors declare that they have received no financial support for the study.

Ethical approval: All data used was from experiments that complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines; EU Directive 2010/63/ EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

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ABSTRACT

Objective: The aim of the study is to determine the prevalence of *Echinococcus granulosus* sensu lato by molecular analysis with Real Time PCR (RT-PCR) method by collecting stool samples from stray animals that can be reached in 9 of the 10 determined districts of Izmir province (Aliaga, Menemen, Bornova, Urla, Selcuk, Bayindir, Odemis, Tire, Kiraz).

Materials and Methods: Thirty samples from Aliaga, 41 samples from Menemen, 35 samples from Bornova, 10 samples from Urla, 40 samples from Selcuk, 40 samples from Bayındır, 33 samples from Odemis, 45 samples from Tire, 26 samples from Kiraz, totally 300 stool samples were collected. The samples were left at −80°C for 5 days for inactivate. Afterwards, all inactivated stool samples were kept at -20°C until they were taken into the study. DNA isolation from stool samples was carried out with DNA extraction kit. The purity of all isolated samples was measured by spectrophotometer. The analysis of the RT-PCR results was performed with the Rotor-Gene Q series software 2.3.1 program and the samples were evaluated for *E. granulosus* s.l. positive/negativeness.

Results: According to the results of RT-PCR analysis, *E. granulosus* s.l. positive sample was not found in stool samples collected from stray dogs in the study area.

Conclusion: Since no molecular studies have been carried out in the districts included in our study, the results obtained could not be compared. It also can be thought that the number of samples is insufficient. Therefore, a larger sample pool should be examined to find out the prevalence of *E. granulosus* s.l. Since there is limited data about the prevalence of *E. granulosus* s.l. in stray dogs, it also is important to investigate the prevalence of *E. granulosus* s.l. in different regions of Turkey.

Keywords: Cystic echinococcosis, *Echinococcus granulosus*, DNA, Real Time PCR
INTRODUCTION

Cystic echinococcosis (CE) is a chronic parasitic zoonosis caused by the larval stage of the cestode *Echinococcus granulosus* sensu lato (s. l.) (1,2). CE, which is very common in the world, affecting humans, domestic animals and wild mammals, causes significant economic losses in both the medical and livestock sectors (1,3,4). This disease, which threatens health by causing significant morbidity and mortality in humans, is on the list of 17 neglected tropical diseases, as well as on the list of priority neglected zoonotic diseases that are aimed to control or eradicate (World Health Organization (WHO) 2017) (4,5).

In the life cycle of *E. granulosus* s.l., the adult form of the parasite requires dogs and other canines as definitive hosts, while the larval form requires herbivores or omnivores as intermediate hosts. Adult Echinococcus spp. live in the small intestine of the canids. Humans are accidental intermediate hosts in this cycle, infected by accidental ingestion of eggs released in canid stool (3,5,6).

Common on all continents, including circumpolar, temperate, subtropical, and tropical regions, CE is responsible for 95% of human cases of echinococcosis and has a worldwide prevalence of approximately six million (2,3).

CE is commonly seen in livestock areas in South America, North Africa, Australia, western, central and eastern Europe and central Asia, particularly western China. Globally, at least 50 million people are infected with *E. granulosus*, with approximately more than 170,000 new cases each year. The estimated minimum global human burden is a public health problem that reaches 285,500 disability-adjusted life years (DALYs), resulting in economic losses of approximately US$3 billion annually (5,7). According to the data of the Ministry of Health between 1990 and 2005 in Turkey, 52,124 cases were operated for CE. Recently published an article by Ministry of Health reported that the number of cases was 408 in 2008 and reached 1.867 at the end of 2019. According to other research data, the estimated case incidence was published as 0.8-2 per 100,000 or 0.3-0.087%. According to limited local study data from different geographical regions, the prevalence of *E. granulosus* infection in Turkey is between 0.32% and 40% in dogs. When the current status of echinococcosis in the dogs is evaluated with the PCR technique, which is widely used in recent years, it varies between 4.0-14.0% (8,9,10).

Molecular analyzes were performed in Echinococcus spp. using methods such as Restriction Fragment Length Polymorphism (RFLP), Multiplex-PCR, High Resolution Melting analysis (HRM), Loop-Mediated Isothermal Amplification (LAMP), and PCR gene amplification followed by sequencing. Mitochondrial gene loci such as cytochrome c oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (ND1) are frequently used in analyzes (1).

With the molecular-based studies carried out to date, *E. granulosus* s.l. species cluster has been tried to be clarified. As a result, with phenotypic differences in morphology and biology, eight different genotypes based on genetic differences in mitochondrial genes has been clarified; *E. granulosus* sensu stricto (s.s.) (genotypes G1 and G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5), *E. canadensis* cluster (genotypes G6-8 and G10) and *E. felidis* (6,11).

In Turkey, there is limited data on the prevalence of *E.granulosus* s.l. on the definitive hosts. Therefore, the main goal of the present study is to determine the prevalence of CE with Real Time PCR (RT-PCR) method with using mitochondrial ND1 gene for characterize *E. granulosus* isolates, by collecting stool samples from stray animals that can be reached in 9 of the 10 determined districts of Izmir province.

MATERIALS AND METHODS

Material Collection

Turkish Association of Hydatidology, with “Creating Awareness of Cystic Echinococcosis in Izmir Province” project the “Local NGOs Grant Program” carried out under the Civil Society Support Program were entitled to receive grant support by the European Union and the Republic of Turkey under the Instrument for Pre Accession Assistance (IPA II) 2014 program. This project was carried out between 2019-2020 years. Stool samples collection, from stray animals, for molecular studies was carried out in 9 of the 10 districts of Izmir selected for the project, which have dog shelters (Aliaga, Menemen, Bornova, Urla, Selcuk, Bayindir, Odemis, Tire, Kiraz). Dog stool collection and storage procedure, stool collection containers (Isolab) were given to the veterinarians working in the dog shelters. Veterinarians took stool samples, according to stool collection procedure, before parasite treatment when dogs came to the dog shelter. From the dogs brought to the shelter from the districts determined within the scope of the project, 5-10 g stool samples were taken into the stool storage containers by the shelter veterinarian. Samples were kept at -20°C until the project team received them. Each sample was labeled with the ear tag number of the animal it belongs to, the date and the district it was taken from. Stool samples were delivered to the laboratory in a cold chain box as soon as possible.

Stool samples, after reaching the laboratory, labeling according to the district that collected, was performed at Ege University, Echinococcosis Research Laboratory. Stool samples were kept at -80 °C for 5 days for inactivation. The
samples were then stored at -20°C until use. Totally 300 samples were collected from selected 9 districts during the Project. The sample numbers collected by districts are shown on the map (Figure 1).

Figure 1. Map of samples which was collected from districts

Molecular Analysis

DNA isolation from stool samples was done with commercial kit (Qiagen, Qiamp Fast DNA Stool Mini Kit) according to the manufacturer's instructions. An average of 200 mg of stool samples was used for isolation. DNA isolations were performed according to the manufacturer’s protocol; Lysis of and separation of impurities from stool samples in InhibitEX Buffer, Purification of DNA on QIAamp Mini spin columns. DNA isolation steps also was performed at Ege University, Faculty of Medicine, Department of Parasitology, Echinococcosis Research Laboratory. The purity measurement of the isolated DNA was performed with Nanodrop spectrophotometer (Thermo Scientific). The purity of extracted DNA was measured by A260/A280 nm ratio. Samples of suitable purity (≥~1.8) were included in the study. Purified DNA’s from each sample was diluted approximately to 5 ng/μl, aliquoted and stored at −20°C until RT-PCR application. Mitochondrial ND1 gene used to characterize animal *E. granulosus* isolates with molecular techniques, was used also in our study.

In RT-PCR method, we used ND1 gene *E. granulosus* specific primer/probe mix (*E. granulosus*, NADH dehydrogenase subunit 1 gene, genesig Advanced Kit, Primer DesignTM Ltd.) ready-to-use, optimized by the manufacturer. We used also 2X qPCR Master Mix (oasigTM lyophilised 2X qPCR Master Mix, Primer DesignTM Ltd.), ready-to-use, optimized by the manufacturer. RT-PCR protocol also was prepared according to the protocol; Enzyme activation 95°C 2min, Cycling X 50: Denaturation 95°C 60 sec, Data Collection 60°C 10 sec. All samples were run on a Rotor Gene Corbett RT-PCR (Qiagen) instrument for amplification. RT-PCR steps was performed at Manisa Celal Bayar University, Faculty of Medicine, Department of Medical Biology, Molecular Biology Laboratory.

RESULTS

The threshold value (basing 0.3) of the obtained RT-PCR results was checked for positivity or negativity using the Rotor-Gene Q series software 2.3.1 program. Values above the threshold value were considered as positive, while the value below was considered as negative. The ND1 gene amplification curves of stool samples were observed in RT-PCR (Figure 2). Molecular analysis results of stool samples taken from dog shelters showed that *E. granulosus* s.l. agent was not found because all samples were below the threshold value, and the dogs in our study group were not infected with *E. granulosus* s.l.

DISCUSSION

Aim of this study was investigation of the distribution of *E. granulosus* s.l. in stray dogs based on stool samples collected from 9 of the 10 selected districts of İzmir, Turkey. It presents for the first data from this districts on *E. granulosus* s.l. prevalence from stray dogs.

Although dogs infected with *E. granulosus* s.l., which has a worldwide zoonotic and epidemiological distribution, are the main source of CE, little is known about the regional molecular epidemiology of adult *Echinococcus* spp. in stray dogs. With research to determine the presence and genotype frequencies of CE in definitive host populations, control programs can be established and progressed, while also assessing its distribution, host specificity, transmission dynamics, and risk of infection for humans in a given area (12). In the region where we collected samples for our study, no molecular study has been done before in stray dogs.

The epidemiology of echinococcosis has made significant progress based on molecular methods in the last ten years (13). In recent studies, serological and molecular analyzes of dog stool have been carried out in addition to traditional methods. PCR, which is one of the molecular methods; since it is an easily applicable, highly sensitive, specific method using closed tube systems, it offers the advantages...
of being able to scan a large number of samples in a short time, reducing the risk of contamination. Because of these advantages, it is frequently used in studies to determine the prevalence of *E. granulosus* from dog stool (14). Determining the prevalence of *E. granulosus* from dogs by molecular methods has been used in different parts of the world. PCR, PCR-RFLP, copro PCR, multiplex PCR and sequence analysis was done from necropsy or stool material of stray, domestic and bred dogs (12,13,15-19). In our study, RT-PCR method was performed to determine the prevalence of *E. granulosus* s.l. in stray dogs from the collected stool materials in 9 districts of Izmir province.

In studies of the molecular identification of *E. granulosus* spp., two mitochondrial genes, ND1 and cox1 were most frequently used (13,15-19). In our study, also the ND1 gene was used for molecular identification.

In the world, between 2009 and 2019, studies were conducted on the prevalence of *E. granulosus* in dogs using PCR, Copro-Antigen ELISA and necropsy methods. As a result of these studies, the prevalence of *E. granulosus* varies between 1.1-10.6% in Europe, 12.2-51.2% in the Middle East and Africa, 3.15-50.7% in Asia, Far East and Oceania, and 0.4-36% in the USA (10). In different regions of Turkey, *E. granulosus* in dogs was mostly screened by necropsy method and the prevalence was found between 0.94-54.5% (14). And also the prevalence of *E. granulosus* in dogs was reported between 0.8-10.8% by PCR and stool examination methods (10). In our study, we couldn’t find any positivity from collected samples by RT-PCR method. This result which is found in this study is different than the other studies conducted in different parts of Turkey. According to the veterinarians who were collected the samples from these stray dogs, the reason for the absence of the agent in stool samples collected from animal shelters may be that dogs are fed with dog food in dog shelters.

**CONCLUSION**

Since no molecular studies have been carried out in the districts included in our study, the results obtained could not be compared. It also can be thought that the number of samples should be insufficient. Therefore, a larger sample pool should be needed to find out the prevalence of *E. granulosus* s.l.. Except for the countries and regions where control studies have been carried out successfully, CE remains an important parasitic disease and socioeconomic problem for humans and animals in many parts of the world as well as in Turkey. In countries where economy based on livestock like Turkey, it’s so important to prevent the sheep-dog cycle. Unfortunately there is no accurate data and the map of distribution of *E. granulosus* in Turkey. As a result; CE urgently needs attention both for protecting public health and animal welfare. Control of CE can be taken under control if it is given the priority and if the necessary measures are taken by the authorities. So, serious control studies should be carried out against CE as soon as possible.

**Competing interests:** The authors declare that they have no competing interest.

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**Ethical approval:** This study did not involve any experimentation with live animals.

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ABSTRACT

Cystic echinococcosis (CE) is a disease that is endemic in our country and is caused by the contamination of eggs of the parasite Echinococcus granulosus. Appendiceal mucocele is an obstructive dilatation of the appendix as a result of intraluminal accumulation of mucoid material. We aimed to present a patient with simultaneous appendiceal mucocele and liver CE. A 55-year-old male patient presented to the emergency department with abdominal pain. In the radiological examinations of the patient, CE 2 lesion with a diameter of approximately 7x6cm in liver segment 7 and a lesion compatible with an appendiceal mucocele of approximately 4x4.5cm in the right lower quadrant were observed. The patient underwent laparoscopic partial cystectomy and laparoscopic appendectomy. The patient was discharged.

In appropriate cases, laparoscopy can be safely performed simultaneously for appendiceal mucocele and liver CE.

Keywords: Liver cystic echinococcosis, appendiceal mucocele, laparoscopic surgery, appendectomy, partial cystectomy

INTRODUCTION

Cystic echinococcosis (CE) is a disease that is endemic in our country and is caused by the contamination of eggs of the parasite Echinococcus granulosus (1). The disease is caused by a cestode worm Echinococcus granulosus that lives in the small intestine of dogs and other canines as the definitive hosts. Eggs are discharged in the final host feces and when ingested by intermediate hosts such as sheep, liberate their larvae in the duodenum which penetrate...
the intestinal mucosa to enter portal circulation and then migrate and develop in different tissues (2). Although the disease is frequently seen in the Southeastern and Eastern Anatolia regions of our country, it continues to be an important health problem with its economic and public health dimensions (3). Cystic echinococcosis can affect almost all organs in the body, but it usually settles in the liver at a rate of 50-70%. The cyst is usually located in the right lobe of the liver and is single in 70-80% (3). Because CE grows very slowly, they remain asymptomatic for years. The preliminary diagnosis is usually made as a result of radiological examinations performed for other reasons. Right upper quadrant pain, jaundice, nausea, vomiting and abnormal liver function tests may be observed in symptomatic cases. Medical treatment (albendazole), percutaneous drainage and surgery are current treatment options in liver CE disease. In complicated cysts, surgery is still the best option (4).

It is an obstructive dilatation of the appendix caused by intraluminal accumulation of mucoid material. The incidence of appendiceal mucocele is 0.2-0.3%, and it is more common in women and people over 50 years of age (5).

CASE

A 55-year-old male patient presented to the emergency department with abdominal pain. The patient, who did not have any additional disease other than hypertension, had abdominal pain, mostly in the right upper and right lower quadrants. There was no previous history of surgery. On ultrasound, a lesion compatible with CE 2 (according to WHO classification-IWGE) CE was observed in Segment 7, approximately 7x6cm (A-P x transverse) in size, containing daughter vesicles and detache membrane. The patient did not know that he had cystic lesion in his liver. In the right lower quadrant, a thick-walled, oval-shaped cystic lesion with a dense content of approximately 4x4.5cm (A-P x transverse) was observed in the region corresponding to the appendix lodge. On computerized tomography (CT), a hypodense cystic appearance with a detache membrane was observed in segment 7 of the right lobe. In the right lower quadrant, a cystic appearance of approximately 4.7x4.4cm (A-P xtransverse) in size was observed, with a smooth circumscribed circumferential contrast, at the level of the appendix, lateral to the psoas muscle. The patient underwent colonoscopy. No pathology was detected in colonoscopy. Indirect hemagglutination test for CE was positive (1/1280). The patient underwent laparoscopic partial cystectomy and laparoscopic appendectomy.

In the operation, the abdomen was entered with a 10mm trocar from the epigastric subxiphoid region, the abdomen was entered with a 5mm trocar from the right lateral and a 5mm trocar from the left lateral.

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During the exploration, a cystic lesion adhering to the diaphragm was observed at the junction of the liver segment 7-8. It was observed that the diameter of the appendix increased considerably (mucocele) in the lower right side, but an area of approximately 1cm close to the root of the appendix was intact. First, appendectomy was performed. A hemoclips was placed in the root. The mass, which was thought to be a mucocele with a diameter of approximately 5x5cm, was excised, and the sub-umbilical port site was partially enlarged with the aid of an endobag and taken out of the abdomen. Hypertonic saline-impregnated gauze pads were placed around the cystic lesion in the liver. The cyst wall was opened minimally and aspirated and the pressure was reduced. Afterwards, a patient with hypertonic saline was given and waited for 10 minutes. Content aspirated. Partial cystectomy was performed. Bile fistula was not observed. With the help of the peritoneal trocar, the endobag was entered and the cystic lesion was aspirated. The cyst wall was opened minimally with a 10mm trocar.

Figure 1. Appendiceal mucocele image on CT

Figure 2. Cystic echinococcosis lesion in liver on CT
of endobag, gauze pads and daughter vesicle were taken out from the xiphoid located trocar. A rubber drain was placed in the cyst lodge and the operation was terminated. Shortness of breath was observed in the patient on the 3rd post-operative day. Minimal pleural fluid and atelectasis in the right hemithorax were observed in thorax CT. Medical treatment was applied for this. The patient was discharged on the 8th postoperative day. Medical treatment was given to the patient as albendazole 10mg/kg/day for at least 3 months.

**DISCUSSION**

Cystic echinococcosis is a parasitic infection caused by the parasite *Echinococcus granulosus*, which causes endemic diseases all over the world (6,7). The frequency of the disease was found to be 1.05% in our country, and approximately 2000 new cases are detected each year (8). CE is most frequently observed in the liver (6).

The diagnosis of CE is made by radiological methods (USG, CT, MRI). Following the USG classification developed by Gharbi et al. (9) in 1981, the World Health Organization Informal Echinococcus Working Group (WHO-IWGE) developed the universally applicable international standardized USG classification in 1994 (10). We used WHO-IWGE classification in our study. There are basically four treatment options: surgery, percutaneous technique, antiparasitic medical treatment and follow-up (wait and see) (8).

Among them, Puncture, Aspiration, Injection, and Reaspiration (PAIR) or laparoscopic surgery are effective methods that can be used safely in experienced hands in suitable patients, since they are less invasive during the pandemic period. In parallel to medical treatment with albendazole (ABZ), surgery and follow-up (wait and see) treatment options in selected cases are historically used in these diseases, various imaging-guided interventional procedures have recently emerged [drainage, stenting, or Puncture, aspiration, injection, and reaspiration (PAIR)]. These options open up a new range of therapeutic options. Consequently, diagnostic imaging and interventional expertise have brought interventional radiologists to the fore as important members of these multidisciplinary team. The interventional radiologist will need to evaluate parasite activity in both forms of the disease, to guide the choice of the appropriate therapy from among medical treatment, interventional radiology procedures and/or surgical treatment. Knowledge of the specific complications of the echinococcosis will also help interventional radiologists to discuss the appropriate treatment and management (11).

In this case we performed laparoscopic surgery since the patient has liver CE and appendiceal mucocele simultaneously. Laparoscopic liver cyst deroofing produces better outcomes with minimal invasion, less spillage, and even better postoperative outcomes and less hospital stay (12).

Mucocele of the appendix was first described by Rokitansky and is an obstructive dilatation of the appendix caused by intraluminal accumulation of mucoid material (13). The worst complication of mucocele is rupture resulting in pseudomyxoma peritonei (14). It is stated that laparoscopic approach and fine needle aspiration cytology should be avoided due to the risk of rupture. (15). Although USG, CT and colonoscopy are used for the diagnosis of appendiceal...
mucocele, the lesion is usually found incidentally during surgery (16). Surgical resection (appendectomy) is the preferred method in the treatment of benign mucocele [17]. Right hemicolectomy is recommended when there is an enlarged mesenteric lymph node, positive cytology, suspected malignant mucocele, or perforated (18). It has been stated by some authors that laparoscopic method can be performed safely and endobag should be used in appendiceal mucocele (19,20).

CONCLUSION

Despite the advances in diagnosis and treatment in recent years, hydatid disease, which still cannot be eradicated, is endemic in our country and causes a public health problem, disrupts human and animal health and causes economic losses.

Although appendiceal mucocele, which develops as a result of intralumenal accumulation of mucoid material, is detected incidentally, it causes pseudomyxoma peritonei when it ruptures. Laparoscopic surgery can be safely performed in experienced hands in the treatment of both diseases.

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