



شبكة المعلومات الجامعية  
التوثيق الإلكتروني والميكرو فيلم

# بسم الله الرحمن الرحيم



**HANAA ALY**



شبكة المعلومات الجامعية  
التوثيق الإلكتروني والميكروفيلم



# شبكة المعلومات الجامعية التوثيق الإلكتروني والميكروفيلم



**HANAA ALY**



شبكة المعلومات الجامعية  
التوثيق الإلكتروني والميكروفيلم

# جامعة عين شمس

## التوثيق الإلكتروني والميكروفيلم

### قسم

نقسم بالله العظيم أن المادة التي تم توثيقها وتسجيلها  
علي هذه الأقراص المدمجة قد أعدت دون أية تغيرات



### يجب أن

تحفظ هذه الأقراص المدمجة بعيدا عن الغبار



**HANAA ALY**

# **GENOTYPING OF HUMAN AND ANIMAL ISOLATES OF *ECHINOCOCCUS GRANULOSUS* FROM EGYPT**

Thesis submitted to Faculty of Medicine, Ain Shams  
University for Partial Fulfillment of M.D. degree in Medical  
Science (Medical Parasitology)

**By**

**Doaa Ashraf Nassar**

M.B., B.Ch., M.Sc, Assistant lecturer of Medical Parasitology  
Faculty of Medicine, Ain Shams University

**Under supervision of**

**Prof. Dr. Khalifa El Sayed Khalifa**

Professor of Medical Parasitology  
Faculty of Medicine, Ain Shams University

**Prof. Dr. Hala Salah Elwakil**

Professor of Medical Parasitology  
Faculty of Medicine, Ain Shams University

**Prof. Dr. Hayam Mohammed Ezz Eldin**

Professor of Medical Parasitology  
Faculty of Medicine, Ain Shams University

**Dr. Hanan Mahmoud Mohamed Abou Seri**

Lecturer of Medical Parasitology  
Faculty of Medicine, Ain Shams University

**Medical Parasitology Department**

**Faculty of Medicine  
Ain Shams University**

**2020**



## Acknowledgement

*First of all, I would like to express my deep gratitude to **Allah** for his care and generosity throughout my life, which permitted the completion of this piece of work.*

*No words can express my sincere gratitude and deep appreciation to **Prof. Khalifa El Sayed Khalifa**, Professor of medical parasitology, Faculty of Medicine, Ain Shams University, for his precious advice and continuous guidance throughout the whole work. I am so grateful for his motivation, enthusiasm, immense knowledge, endless patience, and support.*

*I am very thankful to **Prof. Hala Salah Elwakil**, Professor of medical parasitology, Faculty of Medicine, Ain Shams University, for her keen supervision and guidance and her overwhelming support throughout this work.*

*I am thankful to **Prof. Hayam Mohammed Ezz Eldin**, professor of medical parasitology, Faculty of Medicine, Ain Shams University, for her kind help and encouragement.*

*I would also like to express my great thanks to **Dr. Hanan Mahmoud Mohamed Abou- Seri**, Lecturer of medical parasitology, Faculty of Medicine, Ain Shams University, whose encouragement and knowledge supported me over the entire course of this research, for the overwhelming continuous support and effort and for being beside me whenever I need her.*

*Special thanks to my **family** for their help and support, I could not have reached this point in my life without their prayers and continuous support.*

*Last but not least, thanks to **my friends** and **all members of the Parasitology department**, Faculty of Medicine, Ain Shams University, for their great help, support, love, and care. I find myself overwhelmed in offering them all my thanks.*

## List of Abbreviations

|                         |                                        |
|-------------------------|----------------------------------------|
| <b>A</b>                | Adenine                                |
| <b>AFLP</b>             | Amplified fragment length polymorphism |
| <b>AgB</b>              | Antigen B                              |
| <b><i>AluI</i></b>      | <i>Arthrobacter luteus I</i>           |
| <b><i>Atp6</i></b>      | ATP synthase subunit 6                 |
| <b>AW1</b>              | Wash buffer 1                          |
| <b>AW2</b>              | Wash buffer 2                          |
| <b>BLAST</b>            | Basic Local Alignment Search Tool      |
| <b>BMZ</b>              | Benzimidazole                          |
| <b>bp</b>               | base pair                              |
| <b>BSA</b>              | Acetylated Bovine Serum Albumin        |
| <b>buffer AE</b>        | Elusion Buffer                         |
| <b>Buffer AL</b>        | Lysis buffer                           |
| <b>Buffer ATL</b>       | Tissue lysis buffer                    |
| <b>C</b>                | Cytosine                               |
| <b>CE</b>               | Cystic echinococcosis                  |
| <b>Cm</b>               | Centimeter                             |
| <b>cDNA</b>             | Complementary DNA                      |
| <b>cox1</b>             | Cytochrome c oxidase 1                 |
| <b>CT</b>               | Computerized tomography                |
| <b>Cytb</b>             | Cytochrome b                           |
| <b>ddF</b>              | dideoxy fingerprinting                 |
| <b>ddH<sub>2</sub>O</b> | Deionized distilled water              |
| <b>ddNTPs</b>           | Dideoxynucleoside triphosphates        |
| <b>DNA</b>              | Deoxyribonucleic acid                  |

|                                  |                                              |
|----------------------------------|----------------------------------------------|
| <b>dNTPs</b>                     | Deoxynucleoside triphosphates                |
| <b><i>E. granulosus</i></b>      | <i>Echinococcus granulosus</i>               |
| <b><i>E. granulosus s.l.</i></b> | <i>Echinococcus granulosus sensu lato</i>    |
| <b><i>E. granulosus s.s.</i></b> | <i>Echinococcus granulosus sensu stricto</i> |
| <b>EDTA</b>                      | Ethylene-diamine-tetra-acetic-acid           |
| <b>ELISA</b>                     | Enzyme-linked immune sorbent assay           |
| <b>Fig.</b>                      | Figure                                       |
| <b>G</b>                         | Guanine                                      |
| <b>g</b>                         | Gram                                         |
| <b><i>HaeIII</i></b>             | <i>Haemophilus aegyptius III</i>             |
| <b>HCF</b>                       | Hydatid cyst fluid                           |
| <b><i>Hinfl</i></b>              | <i>Haemophilus influenza I</i>               |
| <b>IHAT</b>                      | Indirect haemagglutination test              |
| <b>ITS1</b>                      | Internal transcribed spacer gene I           |
| <b>kDa</b>                       | Kilo Dalton                                  |
| <b>LAMP</b>                      | Loop Mediated Isothermal Amplification       |
| <b>Mg</b>                        | Milligram                                    |
| <b>µg</b>                        | Microgram                                    |
| <b>MgCl<sub>2</sub></b>          | Magnesium chloride                           |
| <b>µl</b>                        | Microliter                                   |
| <b>ml</b>                        | Milliliter                                   |
| <b>mm</b>                        | Millimeter                                   |
| <b>µM</b>                        | Micro Molar                                  |

|                 |                                                                    |
|-----------------|--------------------------------------------------------------------|
| <b>mM</b>       | Milli Molar                                                        |
| <b>mg/kg</b>    | Milligram/kilogram                                                 |
| <b>MRI</b>      | Magnetic resonance imaging                                         |
| <b>mtDNA</b>    | Mitochondrial DNA                                                  |
| <b>ND</b>       | Not determined                                                     |
| <b>No.</b>      | Number of samples                                                  |
| <b>nad1</b>     | NADH dehydrogenase subunit 1                                       |
| <b>NADH</b>     | Nicotinamide adenine dinucleotide hydrogen                         |
| <b>NCBI</b>     | National Center for Biotechnology Information                      |
| <b>PAIR</b>     | Puncture, aspiration, injection, and reaspiration                  |
| <b>PBS</b>      | Phosphate buffered saline                                          |
| <b>PCR</b>      | Polymerase chain reaction                                          |
| <b>PCR-RFLP</b> | Polymerase chain reaction-restriction fragment length polymorphism |
| <b>PT</b>       | Percutaneous treatment                                             |
| <b>RAPD-PCR</b> | Random amplified polymorphic DNA-PCR                               |
| <b>rDNA</b>     | ribosomal DNA                                                      |
| <b>RFLP</b>     | Restriction fragment length polymorphism                           |
| <b>RNA</b>      | Ribonucleic acid                                                   |
| <b>RNase A</b>  | Ribonuclease A                                                     |
| <b>rpm</b>      | round per minute                                                   |
| <b>rRNA</b>     | ribosomal RNA                                                      |
| <b>RT-PCR</b>   | Real-time PCR                                                      |
| <b>SSCP-PCR</b> | Single-strand conformation polymorphism-PCR                        |
| <b>TAE</b>      | Tris Acetate EDTA                                                  |
| <b>U/S</b>      | Ultrasonography                                                    |
| <b>UV</b>       | Ultraviolet                                                        |
| <b>WHO</b>      | World Health Organization                                          |

*List of Abbreviations*

---

|                 |                                                                    |
|-----------------|--------------------------------------------------------------------|
| <b>WHO-IWGE</b> | World Health Organization-Informal Working Group on Echinococcosis |
| <b>+ve</b>      | Positive                                                           |
| <b>-ve</b>      | Negative                                                           |

## List of Tables

| Table      | Title                                                                                                                                                                                                      | Page           |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <b>2.1</b> | WHO-IWGE Classification of CE                                                                                                                                                                              | <b>13</b>      |
| <b>2.2</b> | Sensitivities of various assays for antibody detection in patients with confirmed CE                                                                                                                       | <b>15</b>      |
| <b>4.1</b> | Accession numbers of reference genotypes retrieved from GenBank for <i>in silico</i> RFLP analysis                                                                                                         | <b>84</b>      |
| <b>4.2</b> | Reagents provided with the extraction kit (50 preparations)                                                                                                                                                | <b>85</b>      |
| <b>4.3</b> | PCR Reaction Mix for each sample                                                                                                                                                                           | <b>93</b>      |
| <b>4.4</b> | <i>HaeIII</i> restriction enzyme kit content                                                                                                                                                               | <b>100</b>     |
| <b>4.5</b> | <i>HinfI</i> restriction enzyme kit content                                                                                                                                                                | <b>100</b>     |
| <b>4.6</b> | Components of the reaction mix of <i>HinfI</i> and <i>HaeIII</i> restrictions enzymes                                                                                                                      | <b>101</b>     |
| <b>5.1</b> | <i>In silico</i> RFLP patterns after digestion of nad1 gene of <i>E. granulosus</i> species with <i>HinfI</i> and <i>HaeIII</i> restriction enzymes                                                        | <b>109</b>     |
| <b>5.2</b> | Demographic and clinical data of human CE patients                                                                                                                                                         | <b>114,115</b> |
| <b>5.3</b> | Results of PCR amplification of the <i>E. granulosus</i> nad1 gene from human, camel and pig HCFs and/or germinal layer samples                                                                            | <b>118</b>     |
| <b>5.4</b> | Expected and obtained restriction fragment patterns and their corresponding genotype based on <i>in silico</i> combined <i>HinfI</i> and <i>HaeIII</i> PCR-RFLP analysis of <i>E. granulosus</i> nad1 gene | <b>122</b>     |
| <b>5.5</b> | The restriction patterns of nad1 gene of <i>E. granulosus</i> human samples and their corresponding genotype based on <i>in silico</i> PCR-RFLP analysis                                                   | <b>126</b>     |

|             |                                                                                                                                                           |            |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>5.6</b>  | The restriction patterns of nad1 gene of <i>E. granulosus</i> camel samples and their corresponding genotype based on <i>in silico</i> PCR-RFLP analysis. | <b>126</b> |
| <b>5.7</b>  | The restriction patterns of nad1 gene from <i>E. granulosus</i> pig samples and their corresponding genotype based on <i>in silico</i> PCR-RFLP analysis  | <b>127</b> |
| <b>5.8</b>  | <i>In silico</i> genotypes of human, camel and pig <i>E. granulosus</i> samples based on PCR-RFLP analysis of nad1 gene                                   | <b>131</b> |
| <b>5.9</b>  | Results of sequence analysis of 11 samples representing different <i>in silico</i> RFLP patterns of <i>E. granulosus</i> nad1 gene                        | <b>134</b> |
| <b>5.10</b> | Genotypes of the 46 samples under study as determined by <i>in silico</i> PCR- RFLP analysis and sequencing of <i>E. granulosus</i> nad1 gene             | <b>135</b> |
| <b>5.11</b> | Genotypes among the human, camel and pig <i>E. granulosus</i> samples based on <i>in silico</i> PCR-RFLP analysis and sequencing of nad1 gene             | <b>135</b> |

## List of Figures

| Figure      | Title                                                                                                                                                                | Page       |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>2.1</b>  | Life cycle of <i>E. granulosus</i>                                                                                                                                   | <b>9</b>   |
| <b>2.2</b>  | WHO-IWGE Classification of CE                                                                                                                                        | <b>13</b>  |
| <b>2.3</b>  | Algorithm for treatment of CE based on WHO-IWG-2003 international classification of US images                                                                        | <b>17</b>  |
| <b>2.4</b>  | Phylogenetic tree of <i>Echinococcus</i> spp.                                                                                                                        | <b>28</b>  |
| <b>2.5</b>  | Principle of conventional PCR                                                                                                                                        | <b>43</b>  |
| <b>2.6</b>  | Nested PCR using two set of primers                                                                                                                                  | <b>44</b>  |
| <b>2.7</b>  | Multiplex PCR                                                                                                                                                        | <b>45</b>  |
| <b>2.8</b>  | qPCR: (A) SYBR Green I assay and (B) TaqMan assay                                                                                                                    | <b>49</b>  |
| <b>2.9</b>  | <i>In silico</i> restriction banding patterns obtained after <i>HaeIII</i> and <i>HinfI</i> double digestion of the nad1 gene sequence from <i>Echinococcus</i> spp. | <b>55</b>  |
| <b>2.10</b> | A dideoxynucleotide (ddNTP)                                                                                                                                          | <b>60</b>  |
| <b>2.11</b> | First-generation DNA sequencing technologies                                                                                                                         | <b>62</b>  |
| <b>2.12</b> | Gel electrophoresis of sanger sequencing                                                                                                                             | <b>63</b>  |
| <b>2.13</b> | Capillary electrophoresis of automated sanger sequencing                                                                                                             | <b>64</b>  |
| <b>4.1</b>  | DNA extraction steps                                                                                                                                                 | <b>89</b>  |
| <b>4.2</b>  | 100 bp DNA molecular weight marker                                                                                                                                   | <b>96</b>  |
| <b>4.3</b>  | Restriction site of <i>HinfI</i>                                                                                                                                     | <b>99</b>  |
| <b>4.4</b>  | Restriction site of <i>HaeIII</i>                                                                                                                                    | <b>99</b>  |
| <b>4.5</b>  | Standard curve graph for calculating RFLP bands size                                                                                                                 | <b>102</b> |

|            |                                                                                                                                                                                                                                                                               |            |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>5.1</b> | <i>In silico</i> restriction banding patterns obtained after <i>HinfI</i> digestion of the <i>nad1</i> gene sequences from <i>E. granulosus</i> spp. (As constructed by Geneious 10.1.3 software)                                                                             | <b>110</b> |
| <b>5.2</b> | <i>In silico</i> restriction banding patterns obtained after <i>HaeIII</i> digestion of the <i>nad1</i> gene sequences from <i>E. granulosus</i> spp. (As constructed by Geneious 10.1.3 software)                                                                            | <b>110</b> |
| <b>5.3</b> | Algorithm showing expected restriction fragment patterns (bp) and their corresponding genotype based on <i>in silico</i> PCR-RFLP analysis of <i>E. granulosus</i> spp. <i>nad1</i> gene                                                                                      | <b>111</b> |
| <b>5.4</b> | <b>a:</b> Invaginated and evaginated protoscolices stained with iodine from a camel hydatid cyst (100x). <b>b:</b> Evaginated protoscolex stained with iodine from a camel hydatid cyst (400x). <b>c:</b> Unstained invaginated protoscolex from a camel hydatid cyst (400x). | <b>116</b> |
| <b>5.5</b> | An ethidium bromide-stained agarose gel electrophoresis (2%) showing the PCR product of <i>E. granulosus nad1</i> gene from human (H) samples                                                                                                                                 | <b>119</b> |
| <b>5.6</b> | An ethidium bromide-stained agarose gel electrophoresis (2%) showing the PCR product of <i>E. granulosus nad1</i> gene from camel (C) samples                                                                                                                                 | <b>120</b> |
| <b>5.7</b> | An ethidium bromide-stained agarose gel electrophoresis (2%) showing the PCR product of <i>E. granulosus nad1</i> gene from pig (P) samples                                                                                                                                   | <b>121</b> |

|      |                                                                                                                                                                                                                                                   |     |
|------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 5.8  | Algorithm showing obtained restriction fragment patterns (bp) and their corresponding genotype based on <i>in silico</i> PCR-RFLP analysis of <i>E. granulosus</i> nad1 gene after individual digestion by <i>HinfI</i> and <i>HaeIII</i> enzymes | 123 |
| 5.9  | An ethidium bromide-stained 2% agarose gel showing RFLP patterns of <i>E. granulosus</i> nad1 gene from human samples after digestion with <i>HinfI</i> (a) and <i>HaeIII</i> (b) restriction enzymes                                             | 127 |
| 5.10 | An ethidium bromide-stained 2% agarose gel showing RFLP patterns of <i>E. granulosus</i> nad1 gene from camel samples after digestion with <i>HinfI</i> (a) and <i>HaeIII</i> (b) restriction enzymes                                             | 128 |
| 5.11 | An ethidium bromide-stained 2% agarose gel showing RFLP patterns of <i>E. granulosus</i> nad1 gene from pig samples after digestion with <i>HinfI</i> (a) and <i>HaeIII</i> (b) restriction enzymes                                               | 129 |
| 5.12 | Sequence of human sample (H9) showing 2 <i>HaeIII</i> (GGCC) restriction sites correspond to G1 genotype                                                                                                                                          | 132 |
| 5.13 | Sequence of camel sample (C23) showing 2 <i>HinfI</i> (GATC) and 1 <i>HaeIII</i> (GGCC) restriction sites correspond to G6 genotype                                                                                                               | 132 |
| 5.14 | Sequence of pig sample (P3) showing 1 <i>HaeIII</i> (GGCC) restriction site correspond to G5 genotype                                                                                                                                             | 133 |

# Abstract

**Background:** Cystic Echinococcosis (CE), caused by the larval stage of the dog tapeworm *Echinococcus granulosus sensu lato* (*E. granulosus s. l.*), is a widespread neglected zoonotic disease that occurs in many parts of the world. Egypt is considered one of the countries where CE represents a public health concern and so far few studies were done for molecular characterization of the parasite.

**Aim of the work:** Aim of the present study was to use the PCR-RFLP technique for genotyping of *E. granulosus* isolates targeting the NADH dehydrogenase subunit 1 gene (*nad1*) and interpretation of results based on *in silico* PCR-RFLP analysis of reference strains retrieved from GenBank.

**Subjects and methods:** a pilot study was first done where reference strains retrieved from GenBank were analyzed by *in silico* RFLP analysis using two enzymes *HinfI* and *HaeIII*. Virtual graphs and algorithms for interpretation of the results were constructed. Fifty hydatid cyst fluid (HCF) and/or germinal layer samples (19 humans, 23 camels, and 8 pigs) were collected. DNA was extracted and used as a template to amplify the *nad1* gene (1069-1078 bp). The amplified PCR products were digested individually with the two restriction endonucleases to generate RFLP patterns. Samples representing the different genotypes inferred from the RFLP patterns as well as those not determined were subjected to automated DNA sequencing based on Sanger's technique.

**Results:** PCR-RFLP and sequencing showed that, except for two cases (12.5 %) which were typed as G1 among humans and one case as G5 in pigs (12.5 %), *E. canadensis* G6/7 was the predominant strain among human, camel and pig samples examined.

**Conclusion:** Camel strain (G6) is the predominant genotype in Egypt. Camels and pigs are crucial in the life cycle of the parasite in Egypt, although other animals may play a role. Control strategies should be implemented to prevent infection of dogs by consuming cysts in tissues of infected animals.

**Keywords:** *Echinococcus granulosus*, genotypes, *nad1* gene, *In silico* PCR-RFLP, Sequencing