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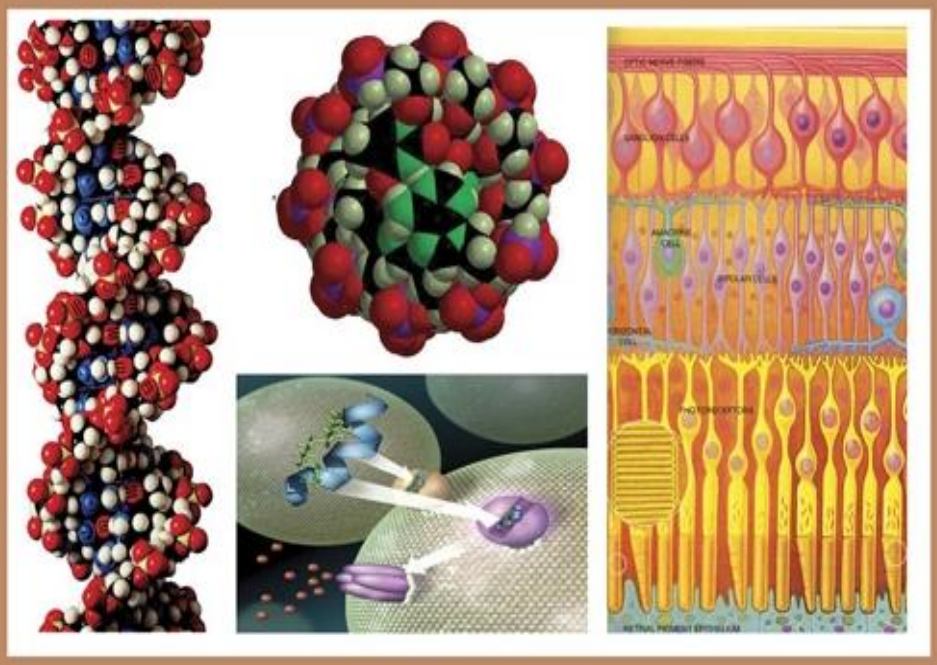

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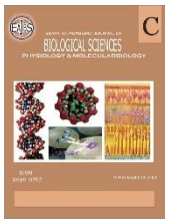

Molecular Characterization of *Echinococcus granulosus* isolates from Human Cases Using Gold Nanoparticles-Based DNA Microarray with Silver Enhancement Simple Colorimetric Technology

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ABSTRACT

The Middle East including Egypt is considered as an endemic area for hydatid disease which predominantly affects the liver. Identification of *Echinococcus* strains is essential for proper vaccines development and control programs. The current study described the development of gold nanoparticles (AuNP) based genomic microarray that specifically identifies specific COX1 mitochondrial gene and concurrently provides genetic typing of *E. granulosus* complex without amplification process, aided by colorimetric silver enhancement technology. Signals are initiated by silver precipitation on AuNP combined with streptavidin, utilized to detect the biotinylated DNA. The qualitative purpose of our technique enables a simple detection by CCD camera connected to a computer. Thus, proposing a relatively easy and cost-effective alternative to the complex DNA amplification system can be applied in low-equipped laboratories. In this work, a total of 30 samples from human hydatid cysts were investigated by oligonucleotide microarray with 10 amino labelled probes, corresponding to the 10 commonly known *E. granulosus* genotypes to be subsequently confirmed by DNA sequencing. Accordingly, camel strain, G6 was the predominant source of human hydatidosis among our cases and was found in 19 cases out of 30 (63.3%), while G1 genotype was reported among the remaining cases (36.7%**)**. There was a statistically significant association between G6 and positive serological results plus multiple organs' involvement, possibly indicating its superior antigenic effect. The predominance of the camel strain in Egyptian hydatidosis highlights the impact of ecological factors on camels' importation strategies with the recommendation of considering vaccination to the imported animals to break the parasite cycle.

INTRODUCTION

Cystic echinococcosis (CE) or hydatidosis is a life-threatening zoonotic infection caused by the larval stage of *Echinococcus granulosus* tapeworm with a mortality rate reaching 4%, linked specifically to the affected elderly cases (Belhassen-García *et al.,* 2014). The disease is caused by *E. granulosus* is now categorized into 4 genetic groups; *sensu stricto* (sheep strain, G1 to G3), *equinus* (horse strain, G4), *ortleppi* (cattle strain, G5) and finally, *E. granulosus sensu lato* (genotpe 6 to G10) (Thompson and McManus, 2002 and Thompson, 2014). The latter group includes *E. canadensis* and *E. intermedius*, yet arguments are still going concerning these genotypes which may be related to different species (Laurimäe *et al.,* 2014).

Morphological and biological features as well as discrepancies of host susceptibility correlated to *E. granulosus sensu lato*. genotype specifically is still a matter of debate. In general, detection of genetic variation is influential to ascertain variable factors including emerging stability, transmission profiles, antigenic components and sensitivity to chemotherapeutic agents (Thompson *et al.,* 1995, Thompson & Mc- Manus 2001). Genotype identification is also vital for the successfulness of whichever control programs in a certain endemic area, including immunization trials which should be basically prepared from the existing strain in such area (Suzuki *et al.,* 2004). Unfortunately, the Middle East including Egypt is considered as an endemic area of this disease (Dakkak, 2010). Hydatidosis can involve any organs but predominantly affects the liver (70%), thus may augment the burden within a proportion of Egyptian livers that are distressed by viral hepatitis and/or schistosomiasis (El-Shazly *et al.,* 2007).

In latest years there has been cumulative awareness related to the use of nanoparticles (NP) coupled with silver staining in variable medical fields including molecular diagnostic purposes due to its relatively higher sensitivity compared to the well-known fluorescent dyes (Guidelli *et al.,* 2016). In the present study, we describe the development of a new NP-based genomic microarray that specifically identifies COX1 parasitic mitochondrial gene and at the same time provides genotyping identification of *E. granulosus* isolates without the need of amplification systems such as restriction enzyme based-PCR techniques. The method is considered simple and rapid since the specific echinococcal genomic materials are added directly to a prepared slide for hybridization with immobilized specific probes and hence can be detected using gold NP and silver enhancer technology, facilitating a highly sensitive colorimetric detection. To date, no studies have explored in depth the feasibility of such kind of microarray to access the genetic diversity of *E. granulosus senso lato* isolated from human cases.

MATERIALS AND METHODS

The study was approved by the ethical committee; Deanship of Higher Education and Scientific Researches, Faculty of Medicine, Cairo University (proposal number I-080317). The samples in the present study were collected from 30 patients with cystic echinococcosis (CE) attending the Surgery Department in the National Hepatology and Tropical Medicine Research Institute as well as patients attending the Tropical Medicine Department in the Faculty of Medicine at Cairo University, Egypt. The cases were initially diagnosed as CE based on their clinical, radiological and serological examinations.

Participants/ Sample Collection:

A total number of 30 hydatid cysts fluids (HCF) were obtained post-operatively under aseptic conditions to be examined under light microscopy to confirm the presence of protoscolices and/or germinal layers that were washed three times with phosphate-buffered saline by centrifugation at 2,000 *g* for 15 min. The obtained pellets were kept in 95% ethanol at -20^{o}C until

DNA extraction. Demographic, clinical and serological outcomes, as well as other individual patient characteristics, were also recorded pre-operatively through a comprehensive questionnaire. For serological testing, the commercially available IHA kit for echinococcosis was used to detect anti-*Echinococcus* antibodies, Fumouze [ELITech Microbio, France, Paris].

Molecular Investigation:

DNA Extraction: *E. granulosus* genomic DNA was extracted from protoscolices and/or germinal layers using the DNeasy Blood & Tissue Kit (Qiagen Sciences, U.S.A) following the manufacturer's instructions. Isolation of high-quality genomic DNA was performed by the spin-column procedure. The DNA quality was evaluated by rate OD 260/280 using a NanoDrop (ThermoFisher Scientific).

DNA Microarray:

Probes' Immobilization: For probes design, gene bank accession numbers for variable genes were regained from the National Center for Biotechnology Information database (NCBI) (Table 1). According to Conzone and Pantano (2004) with modification, the used fluorophore-free amino-modified probes contained 10 deoxyadenosines in the 5^0 terminus were matching the amino-modified DNA labeling technology. The DNA probes corresponding to the 10 genotypes of *E. granulosus* were synthesized and purchased from Eurofins Company. The probes were dissolved in 50 mM phosphate buffer (pH 8.0, with 10 mM EDTA) at 50 mM final concentration.

Probes' Spotting*:* The probes were spotted on an epoxy silane coating glass slide with 16 sub-array chambers [ArrayIT. Company]. This was done automatically using fine pins [ArrayIT SMP3] aided by a robotic machine. To confirm the validity of the molecular technique, each sub-array chamber involved 4 replicates, and each sub-array contained 40 spots of the 10 tagged DNA probes plus 8 spots related to positive and negative control samples in quadruplicate, in which specific probe related to housekeeping 18S was used and this was followed by UV fixation for 15 min. So, to confirm the presence of a certain strain, 16 spots /sub-array chamber was prepared. For positioning, the extent of each spot was 100 µm and the distance between the individual spots was 200 µm and the space between the spots and the borders were also adjusted during the robotic machine's programming to be 100 µm. Methylene blue dye was used to optimize spots' positioning prior to the actual printing. The fabricated microarray was processed with pre-hybridization solution (1% BSA in phosphate-buffered saline (PBS) containing 10 mM EDTA) and kept at room temperature for 1 hour, to block intact amine-reactive groups on slides before hybridization.

Biotinylation, **Hybridization with Streptavidin-Conjugated with Au NPs with Silver Enhancement:**

DNA samples were labeled with biotin by using Label IT® Nucleic Acid Labelling Biotin kit (Mirus) following manufacture instructions. Biotin was reconstituted with 100 µl reconstitution solution and was incubated at 37°C for one hour. Each DNA profiling microarray was hybridized with a 1.5 ug biotinylated sample of single-stranded (ss) DNA (denaturated) in 10 µl 30 % formamide hybridization solution at 37°C overnight. The hybridized microarray was then washed with a washing buffer containing 1x saline-sodium citrate (SSC) /0.5% sodium dodecyl sulfonate (SDS) at 37 °C for 10 min and then was dried by tissue wiping. The microarray was incubated with 10 ul of 0.5 streptavidin-conjugated Nanogold solution [Sigma]. After thorough washing with distilled water, the microarray was then manipulated with a silver enhancer kit [Sigma] in which equal volumes of silver enhancer solutions A and B were mixed (0.5 ml each). Approximately 2 ml of the mixture was required per slide for individual sections. 25% sodium thio sulphate was prepared (0.39 gm in 10 ml distilled water H2O). The section was rinsed in distilled water to remove the silver enhancer mixture after achieving the needed colour intensity. Fixation was done by

immersing in 2.5% Sodium Thiosulfate Solution for 2–3 minutes followed by washing in distilled water. Probes related to the 18S housekeeping gene were used as a positive control, while double distilled water was used instead of the template as negative control (Fig. 1).

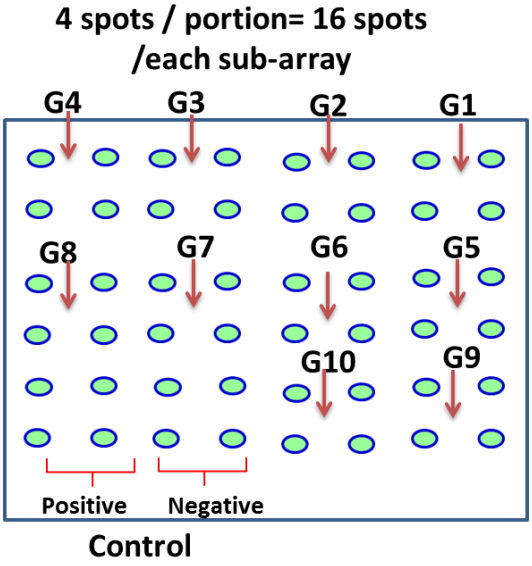

Fig. 1: A diagram represents the printing and positioning process of the amino-tagged DNA probes into the microarray sub-array chambers.

Reaction Detection: Detection was done using a commercial CCD camera connected to a computer with soft war image analysis.

DNA Sequencing:

For DNA product purification, QIAquick PCR Purification Kit was used [Qiagen, Germantown Rd, USA], following manufacturing instructions in all steps including washing and elution. Forward and reverse amplification primers were designed to amplify a product size of 750 bp that involve all genotypes and sequencing was performed using ABI 3730xl DNA analyzer and then BLAST programs and databases were used for nucleotide sequence analysis.

Statistical Analysis:

All data analyses were performed in IBM Statistical Package for the Social Sciences (SPSS) version 20 ([SPSS Inc., Chicago, IL, USA]. The properties of our data distribution were investigated using the Kolmogorov-Smirnov and Shapiro-Wilk tests. In each group, all continuous variables (demographic data, clinical and serological) were compared across genotypes (G1 and G6) using a one-way ANOVA for normally distributed variables and a Kruskal-Wallis test for non-normally distributed variables. Chi-square analyses were used for nominal variables. The results were evaluated in 95 % confidence intervals and $p \leq 0.05$ was accepted as statistically significant.

Table 1: Accession number and sequence of the designed probes.

Gene bank accession number for variable genes	**Position**
P1: AB551110.1	368 -621
P2: KX685890.1	51- 111
P3: KX685891.1	301 - 491
P4: KX685892.1	391 – 461
P5: KX685893.1	441 - 601
P6: KX685894.1	241 - 300
P7: KX685895.1	661 - 741
P8: KX685896.1	351 - 551
P9: KX685897.1	591 - 702
P10: KX685898.1	541 - 701

RESULTS

Concerning demographic data, the overall analysis of gender representation in the 30 enrolled CE patients under study revealed that females constituted a significantly higher prevalence of CE (n=20, 66.66%), in comparison to the male gender (n=10, 33.33%) with high statistical value (p= 0.002). The population under study was categorized by age into 3 groups young adults (ages 18-35 years), middle-aged adults (ages 36-55 years) and older adults (aged older than 55 years). Cystic echinococcosis was more prevalent in the young adult age group (n=17, 57%), than the other 2 age groups (middle-aged: n= 9, 30% and old age: n= 4, 13%) (P-=0.014). Concerning residency, there was a significantly higher number of cases from rural areas, 19 cases (63.33 %), in comparison to the remaining 11 cases (36.66 %) who lives in urban areas (P-value of 0.05), with a higher incidence in farmers (12 out of 30 cases, 40 %). Regarding relation to genotypes, 19 samples proved positive for G6 and 11 samples were related to G1 genotype by microarray technology which was all confirmed by BLAST nucleotide sequence analysis. Characteristics of the study groups are shown in Table (2). Age, gender, contact with dogs and clinical manifestations showed no statistically significant difference ($p>0.05$) across the G1 and G6 genotypes. The occupational risk factor was found significant with p-value (0.042) with the *Echinococcus* genotypes G1 (higher among farmers) and G6 (higher among housewives).

Serology was done for all patients in our study by IHA test. Serum samples with antigen-antibody titre above 1/320 were considered seropositive. Accordingly, the total number of seropositive cases represented 60% (18 out of 30 cases). A significantly higher number of serologically positive cases was reported among G6 genotype, indicating the possible superior antigenicity of this genetic type. Concerning the micro-array technique applied in the current study, visual detection of positive reaction was proved to be enough to identify the genotype of the *Echinococcus* cytochrome gene (Purity ratio was 2.004 for a relatively good yield of 812.0 µg/ml). As the yield of DNA was of high quality in all enrolled samples and no crucial need for quantitative estimation of genomic materials, a simple detection was achieved aided by CCD camera connected to a computer. Applying the previously explained spots' positioning within the sub-array chamber, the exact genotype was identified (Fig. 2).

Table 2: Demographic and clinical data related to the enrolled cases in relation to the genetic types

Variables	N=30	G1 (n=11)		G6 (n=19)		Chi-square	
		N	%	N	%	X^2	P-value
Age							
Young adult	17	7	63.6	10	52.6	0.426	0.808
Middle-aged adult	9	3	27.3	6	31.6		
Older adult	4	1	9.1	3	15.8		
Sex							
Males	10	3	27.3	7	36.8	0.287	0.592
Females	20	8	72.7	12	63.2		
Residence							
Rural	19	10	90.9	9	47.4	3.445	0.063
Urban	11	1	9.1	10	52.6		
Occupation							
Farmers	12	8	72.7	4	21.1	9.904	0.042*
Housewives	9	3	27.3	6	31.6		
Students	2	0	0.0	2	10.5		
Employees	3	0	0.0	3	15.8		
Unemployed	4	0	0.0	4	21.1		
History of contact with dogs							
Yes	12	4	36.4	8	42.1	0.096	0.757
No	18	7	63.6	11	57.9		
Complaint							
Complaining	22	6	54.5	16	84.2	3.135	0.077
No complaint (Accidently diagnosed during the investigation).	8	5	45.5	3	15.8		
Site of the cyst							
Single liver cyst	21	11	100	10	52.6	5.359	0.021*
Multiple cysts	9	0	0.0	9	47.4		
Serological results							
Positive results	18	2	18.2	16	84.2	12.656	<0.001**
Negative results	12	9	81.8	3	15.8		

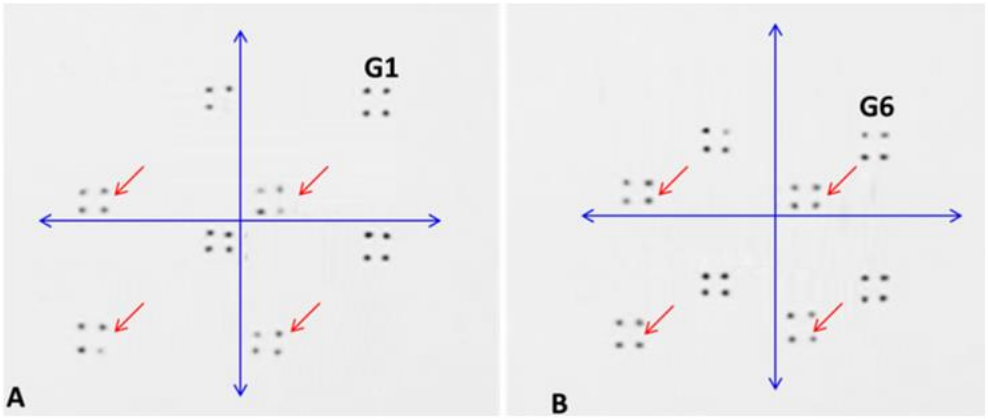

Fig. 2: The photograph shows the different positions of positive reaction of a sample in G1 (A) and G6 position (B) within the sub-array chamber. Red arrows represent positive control samples.

DISCUSSION

The present study focused on hepatic cystic hydatidosis among Egyptian patients with reference to genetic typing. Regarding the demographic data of the cases enrolled in the current work, females constituted a significantly higher number of cases, in comparison to male patients. Females were the utmost category as well in the study of Gupta *et al.* (2011) and Asghari *et al.* (2013). This observation is not surprising and may be due to the fact that

Egyptian females, being self-leadings are normally exposed to variable sources of infection in a wide range of works, including farming and raising animals in rural areas. Regarding age, the number of young adults (57%) was significantly higher than the middle-aged and older adults and this is in agreement with Mathur *et al.* (2016). About the history of contact with dogs, 12 (40 %) patients were recorded to own dogs, while the remaining 18 cases (60 %) had never owned dogs. However, Sarkar *et al.* (2017) found that 71.43% of their cases gave definite history of contact with dogs. In fact, dogs are an important origin of infection, but other sources of infection may take place, especially in rural areas via contaminated food by eggs of *E. granulosus* such as green vegetables. Our results are in accordance with that reported by Asghari *et al.* (2013) and Salama *et al.* (2014), in which a significantly higher rate of infection was recorded in patients from rural areas than those from urban areas. Living in rural areas to a certain extent increases the risk of infection. This may be due to human behaviours, such as habitual feeding dogs with the infected viscera of outdoors slaughtered livestock (Nunnari *et al.,* 2012). This is unlikely to occur in Egyptian villages which are full of stray dogs that fed on cystic stages from slaughtered animals, discharging eggs to everything around, resulting in augmentation of the life cycle in such rural communities.

Concerning the genetic type, mitochondrial genes cox1 has been used to determine the genotypes of *E. granulosus* in the present work (Bowles *et al.,* 1992). Non-fluorophore colorimetric silver detection strategy was used for visual reading of microarray's results and this in accordance with Alexandre *et al.* (2001) who found the strategy equivalent to fluorescence method, so recommended its use, being powerful with compatible scanning parameters of low-cost, suitable for researches and clinical settings. The signal is initiated by the precipitation of silver on nanogold particles combined with streptavidin which is utilized to detect the biotinylated DNA (wang *et al.,* 2002 and Cao *et al.,* 2006).

The combination of silver and gold deposition greatly improves the sensitivity, enhances light-scattering properties and additionally decreases detection limits, allowing visual detection. Using a similar approach, Zhao and co-workers developed a microarray platform using AuNP-based genomic microarray assay for specific identification of avian influenza virus H5N1 RNA (Zhao *et al.,* 2010).

In general, gold nanoparticles (AuNPs) are considered among the effective strategies used in both immunological and molecular diagnostics. Gupta *et al.* (2021) reported the lower detection limit to be 10^2 with enhancer and 10^3cells/ml without enhancer, thus confirming the relatively low quantity needed for visual detection in such an approach. AuNPs are usually utilized to overcome the drawbacks of fluorescent dyes such as photobleaching and the relatively limited potential for bioconjugation. The current accessibility of these nano-particles in different characters in addition to the option of linking them with tags results in selectivity and sensitivity's expansion of the approach (Gibriel, 2012).

This label-free methodology for detection of a point mutation within a non-amplified hybridized genomic template was applied in this study, in which accumulated silver onto the gold particles, enlarging the particle size, to eases its visual reading (Storhoff *et al.,* 2004 and Yeh *et al.,* 2009). Accordingly, proposing an exceptionally cost-effective alternative to the traditionally used, time-consuming DNA amplification procedure which is considered the cornerstone of genetic typing methodologies (D'Agata *et al*., 2017).

In general, there are several methods such as PCR, multiplex PCR, and quantitative real time PCR, which can screen for genetic types related to different infectious agents. However, these PCR-based approaches suffer from drawbacks such as low clinical sensitivity and specificity, poor precision, inadequacy for

simulations detection, besides the need for expensive devices (Hajia, 2017). Genotyping by RFLP was recorded to be a time-consuming procedure besides the constant risk of exposure to possible contamination, being muti-phases molecular technique (Dinkel *et al*., 2004; Osman *et al*., 2009; Omer *et al.,* 2011 & Ibrahim *et al*., 2011).

In this work, a total of 30 samples from human hydatid cysts were investigated by oligonucleotide microarray with amino labeled 10 probes, corresponding to the 10 genotypes, in order to identify their genotypes and source of human infection. According to the results of this study, camel strain (G6) was the predominant source of human hydatidosis within our cases (19 cases out of 30, 63.3%).

The predominance of G6 in Egypt in our study may be due to the importation of camels from Sudan. (Napp *et al*., 2018 and Casulli *et al*. 2010) stressed the importance of G6 strain in highly contaminated environments which might be of greater public health significance than previously believed. In Egypt, G6 genotype involvement has been shown by a number of studies (Azab *et al.,* 2004; Magambo *et al.,* 2006; Abdel Aaty *et al.,* 2012 and Khalifa *et al.*, 2014).

G1 type produces fertile hydatid lesions chiefly in sheep and is recurrently isolated from humans (Eckert *et al*., 2001 & Thompson and McManus, 2001; 2002 and Dinkel *et al*., 2004). In the present study, we found G1 genotype in 36.7% of our cases. This is perhaps due to the nature of residential areas in which these cases are lived. Three cases mentioned that they spent part of their childhood period in Iraqi during Golf war I, from which all human cysts were referred to as sheep strain. Another 3 cases were from Northern Sinai and one was from the bedwin area in Marsa Matrouh governorate, in which shepherding is common. In Iraqi, many reports concluded that G1 sheep strain is the predominant genotype in the Golf area such as Hammad *et al.* (2018), who recorded the majority of G1 type in their study, performing molecular sequencing of (cox1) gene related to hydatid cysts collected from patients as well as different domestic intermediate hosts.

In this work, there was statistical significance between the genotype and location of hydatid cyst. It is found that the camel strain was associated with all cases with multiple cystic involvements. In addition, there was a significant correlation between G6 and positive serological results (16 out of 19, 84.2% of G6 samples showed positive serological results, while only 2 out of the 11 G1 samples, 18.2% obtained positive results). This in accordance with other studies such as Guarnera *et al.* (2004) who explained this by the growth rate of G6 genotype in the liver of human cases which was found to be faster than other genotypes of *Echinococcus*, including G1 genotype. In addition, the rate of lung infection by genotype G6 in humans is more than that of other genotypes, possibly indicating a stronger antigenic component of such genetic type. Moreover, in a study conducted by Sadjjadi and colleagues on human cerebral hydatid cysts in Iran, all investigated brain cysts (8/8) were identified as the G6 genotype of *E. Canadensis* (Sadjjadi *et al*., 2013). Generally, the taxonomy of cystic echinococcosis continues to be under discussion and is far from being completed. For example, the taxonomic status of genotypes G6, G7, G8, and G10 of *E. granulosus* has not been solved yet (lymbery *et al.,* 2015).

Conclusions

The Gold NP-based microarray assay combined with sliver enhancement strategy was able to simply detect and distinguish mitochondrial COX1 sequences directly from the genomic DNA, extracted from human hydatid cysts. The method described in this study may be useful for simultaneous detection and genotyping of the entire complex *Echinococcus granulosis senso lato*. To minimize the risk among Egyptians, procedures to prevent slaughtering at homes must be ascertained. The predominance of the camel strain (G6) in the adult Egyptian subjects highlights the

impact of ecological factors on camels' importation strategies with the recommendation of considering vaccination to the imported camels by the *Echinococcus* crude antigen to break the parasite cycle.

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Conflict of Interest Statement: On behalf of all authors, the corresponding author states that there is no conflict of interest in this study.

Consent to Participate and Publication: On behalf of all authors, all research team acknowledged their participation in this scientific work and revised the article before submitting it for publication.

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