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DIFFERENT ANTIGENIC PROFILES AND ENZYME-LINKED IMMUNOSORBENT ASSAY RESULTS BETWEEN *ECHINOCOCCUS GRANULOSUS SENSUS STRICTO* HYDATID CYST FLUIDS CLASSIFIED AS G1 AND G3 GENOTYPES

Alex Sebastián Vargas Soto
Facultad de Ciencias de la Naturaleza, Campus Bellavista, Universidad San Sebastián, Santiago, Chile

Marco Bugueño Aguirre
Laboratorio de Filogenia Molecular, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile

Eric Diaz Guzmán
Laboratorio de Parasitología, Hospital Público Barros Lucos-Trudeau, Santiago

Mercedes Lopez Nitsche
Programa de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile

María Isabel Jercic
Sección de Parasitología, Instituto de Salud Pública, Santiago, Chile

Gittith Ariela Sanchéz Padilla
Laboratorio de Filogenia Molecular
Programa de Biología Celular y Molecular,
Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile

Juan Antonio Venegas Hermosilla
Laboratorio de Filogenia Molecular
Programa de Biología Celular y Molecular,
Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile
ORCID:0000-0002-0492-1777

Abstract: The objective of the present study was to investigate whether G1 and G3 genotypes of *Echinococcus granulosus* sensu stricto hydatid cyst fluid (HCF), are differently detected by Western blot and Enzyme-linked immunosorbent assay (ELISA) analysis, using sera from patients infected with the parasite and the detection of different IgG subclass antibodies. The main result was that antigen recognition depends on the genotype of HCF used as antigen, even though the G1 and G3 genotypes are classified within the same parasite species. The smallest antigens detected were on the 8- 10 kDa range which may correspond to antigen B subunit. Statistically significant differences between G1 and G3 antigens were also detected by ELISA, but only using IgG4 subclass antibodies. Detection of G1 and G3 HCF depends also on IgG subclass antibodies. Thus, both with Western blot as well as ELISA significant differences were observed between IgG4 and either IgG2 or IgG3 subclasses. Taking into account our results and the literature, it is reinforcing the need for Western blot confirmation of ELISA and the detection of IgG1 and IgG4 subclass specific antibodies, in order to be sure that the serological results may not be changed by using HCF not classified as G1 genotype.
Keywords: *Echinococcus granulosus*, hydatid cyst fluids, G1 and G3 genotypes, Western blot, Enzyme-linked immunosorbent assay analysis

INTRODUCTION

Cystic echinococcosis (CE) is one of the most relevant and common zoonosis worldwide and it has an enormous impact and animal and human health (MORO & SCHANTZ, 2009; SENDEKIE *et al*., 2024)

The etiological agent is a tapewarm belongs to Cestoda class, *Echinococcus granulosus* sensu lato (*E. granulosus* s. l.), specifically, the larval form of the parasite, called metacestode (SENDEKIE *et al*., 2024). The disease is main-

tained over time by a series of cultural factors, as well as by the presence of a wide variety of hosts (NOCERINO *et al.,* 2024). The disease traditionally appears in rural environments where herding is an important source of economic activity and dogs are frequently found in these areas performing tasks such as herding, hunting and guarding. Dogs and other carnivores are the definitive hosts of the parasite while sheep, cows and goats are the intermediate hosts (CHACHA *et al.,* 2023).

The phylogenetic analysis of *E. granulosus* s. l. has allowed to establish polymorphisms in mitochondrial genes, thanks to the use of molecular biology techniques. To date, at least ten genotypes called G1-G10 and *Echinococcus felidis* would constitute the species complex called *E. granulosus* s. l. (THOMPSON, 2008; NAKAO *et al*., 2010; ROMIG et al., 2015; KINKAR *et al,.* 2017; LYMBERY, 2017; CASULLI *et al.,* 2022). Within this complex, several species can be distinguished. Thus, genotypes G1, G2 and G3 constitute the species *Echinococcus granulosus* sensu stricto (*E. granulosus* s. s.) (THOMPSON, 2008; ROMIG et al., 2015; KINKAR et al 2017; KINKAR et al., 2018).Thus, the genotype G4 corresponds to the species *Echinococcus equinus*, the genotype G5 to the especies *Echinococcus ortleppi*. (G5).

Regarding genotypes G6 to G10, some controversies still persist (THOMPSON, 2008; KNAPP *et al.,* 2011, 2015; NAKAO *et al.,* 2015). However, studies by YANAGIDA et al. (2017), based on the study of two nuclear loci, suggest that the G6/G7 and G8/G10 pairs could constitute the same species; however, other studies based on the analysis of six nuclear loci suggest that both pairs of genotypes would correspond to two different species (KINKAR *et al.* 2017). Other authors suggested that the G6/G7 genotype corresponds to a new species, *Echinococcus intermedius*, G8 would correspond to *Echinococcus borealis*, and only G10 would be *Echinococcus canadensis* (CASULLI *et al,*. 2022).

New information based on KINKAR (2017) studies shows that *E.* s. s. only is represented by G1 and G3 genotypes, and G2 would be a microvariant of G3; for this reason the authors recommend that G2 should be excluded from the list of genotypes. Still, the implications of the genetic variability of this parasite on phenotypic characteristics that affect its life cycle have not been totally elucidated, including host specificity, rate of parasite development, infectivity, pathogeny, antigenicity, tissue tropism, sensitivity to chemotherapy and epidemiology (GUARNERA *et al.,* 2004; CHANDRASEKHAR & CHANDR, 2009; BOUFANA *et al.,* 2015).

The definitive diagnosis of echinococcosis is made by radiographic means, however the diagnostic method commonly used is ELISA (KHURANA et al. 2023). Although several recombinant antigens have now been described as candidates for use as antigens in the serological diagnosis of CE in humans and animals and new immunology methods have been developed, still in endemic countries the most widely used source of antigen is crude hydatid cyst fluid (HCF) of the *E. granulosus* s.s. from sheep or cattle origin (SCHANTZ, 2006; ITO, 2013; SIRACUSANO *et al*., 2012). One of the reasons of this fact, is the expensive and restrictive accesses to this new methodology, and even to the fact that the pure recombinant antigens and new immune methodologies frequently are less sensitive than the traditional serological method using HCF (TAPPE *et al*., 2008; SIRACUSANO *et al*., 2012; ITO, 2013). For this reason, still today, the study of HCF as antigen, is very relevant.

The detection of serum antibodies for the diagnosis of cystic echinococcosis is based on the use of methods such as ELISA, immunochromatography and indirect hemoagglutination, while the western blot method is used for differential diagnosis (HOGEA *et al.,* 2024)

HCF is a liquid containing mainly salts, glycoproteins, lipoproteins and immunoglobulins from immune host response, in which it is possible to find antigen B and antigen 5, which have been extensively studied due to their high concentration and immunoreactivity. Structurally, antigen B is a 160 kDa lipoprotein, while antigen 5 corresponds to a protein with two subunits of 22 and 38 kDa. The smallest antigens detected by Western blot mostly correspond to a molecule of about 8 kDa (GONZÁLEZ *et al.,* 1996; MAMUTI *et al*., 2006).

Although there is information on different isotypes and subclasses of immunoglobulin, most of the studies have been performed using ELISA (SHAMBESH *et* al., 1997; DAEKI *et al*., 2000; MAMUTI *et al*., 2002). In these studies, the most predominant IgG subclasses are IgG1 and IgG4, which are also correlated with the progression of the parasite infection and cyst maturation. However, there are some reports showing IgG2 rather than IgG4 as the predominant subclass (RAMZY *et al*., 1999; LAWN *et al*., 2004). In these studies, possible causes to explain the differences were not proposed. In addition, studies indicate that the glycome profile of serum IgG is altered during disease progression suggesting its potential as a biomarker (FENG *et al.,* 2023)

Given that still, all over the world, the most used serological diagnosis of human CE is based on HCF as a source of antigen, and still immune characteristics of the different genotypes of *E. granulosus s.s.* is poorly known, the main objective of our study was to determine whether the genotypes G1 and G3 used as antigen (and corresponding to the same parasite species), have effects on the detection of Western-blot antigenic profiles and ELISA titers obtained with sera from human patients infected with this parasite. We think that this information could be relevant to deepen the knowledge of this host - parasite interaction.

METHODOLOGY

Sera from Chilean patients with clinically confirmed CE were used in this study. All sera were diluted (1:50) in phosphate-buffered saline containing 0.05% Tween 20 and analyzed according to a standard ELISA procedure using HCF collected from two *E. granulosus* cysts of cattle. The antigens were obtained from two fertile HCF isolated from lungs of cattle. The genotypes of parasites were previously identified by us using *cox1* and *nd1* as mitochondrial markers (ESPINOZA *et al*., 2014).

Western blots (WB) were performed by the conventional protocols previously described (VENEGAS *et al*., 2009) in which the proteins separated in polyacrylamide gels were transferred to nitrocellulose filters for 30 min at 40 mA by the semi-dry system transblot (Sartoblot II-s, Sartorius). The filters were incubated overnight with sera of patients and revealed by a secondary antibody against human immunoglobulins conjugated with alkaline phosphatase. The human immunoglobulins detected were total IgG, IgG1, IgG2, IgG3 and IgG4 (AbCam Inc, MA USA). The detection was performed by a colorimetric system using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Promega Corporation) and nitro blue tretrazolium (NBT, Promega Corporation).

Enzyme-linked immunosorbent assay (ELISA) was performed based on conditions previously described (GRIMM *et al*., 1998) using 25 µl of hydatid fluid as antigen in each assay. The serum samples were diluted 1:20 in a TBS (25 mM Tris-HCl pH 7.2; 137 mM NaCl; 2.7 mM KCl) + 0.05 % Tween 20 solution. The antibodies were detected using anti-human secondary antibody diluted to 1:400 conjugated with alkaline phosphatase (AbCam Inc, MA USA). p-Nitrophenyl phosphate Tablets (Sigma-Aldrich MO, USA) were used as substrate for the colorimetric reaction that yields a soluble yellow product which was

detected by absorbance at 405 nm (A_{405}). In order to determine the significant differences between the results of the ELISA (Table 1) were performed the Welch´s t-test method using the GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

RESULTS AND DISCUSSION

Clear differences, between the antigenic patterns of G1 and G3 hydatid fluids used as antigens, were found with Western blot methods (Fig. 1 – 3). The main dissimilarities were observed between the molecular mass range of 8-10 kDa using IgG1 and IgG4 subclass antibodies (Figs. 1 and 3). Based on the extend literature published, about the study of hydatid fluid composition in which the most abundant and immune dominant antigen on this range of molecular mass, are subunits of antigen B (AgB) (MAMUTI *et al*., 2006; VACIRCA *et al*., 2011; SIRACUSANO *et al.,* 2012), we suggest that these low molecular mass antigens detected in our work, may correspond to AgB subunits. Few comparative studies on the antigenic properties between different *Echinococcus* spp. genotypes have been performed (MAMUTI *et al*., 2006; SIRACUSANO *et al.,* 2012). As expect, considered that the G1 – G3 genotypes belong to the same *E. granulosus s.s.* species (MOKS *et al*., 2008; THOMPSON, 2008; NAKAO *et* al., 2013; ESPINOZA *et al*., 2014; ROMIG *et al*., 2015), in previous work no differences between antigenic pattern were found among G1-G5 genotypes (ITO *et al*., 2007). These results are dissimilar with the evidence found in our work, on which clear distinct differences among the G1 and G3 antigenic patterns were observed, mainly on the 8- 10 kDa range, as was mentioned above. It is worth mentioning that significant differences between these two genotypes antigens, even was detected with ELISA, using IgG4 antibodies (Tables 1 and 2). These molecular differences are not due to dissimilar technical characteristic of Western blot method, due that both hydatid fluid antigens were analyzed in the same SDS-PAGE (Fig. 1 and 3). It is not known, at what molecular biology level are based the mechanism which caused these differences among both genotypes, but considering the known structural and polymorphic properties of the AgB gene family (MAMUTI *et al*., 2006; SIRACUSANO *et al.,* 2012), it is very probably that in these two genotypes, the length of AgB genes could be dissimilar. Obviously, other and complementary mechanisms such as differential expression of dissimilar genes, and/or differential splicing and/or editing, cannot be ruled out. Further studies should be conducted to uncover the real molecular biology mechanisms of this phenomenon.

As it was expected according to the literature, out of total IgG, in our work the most intense absorbance with ELISA were found with IgG1 and IgG4 subclass antibodies (IOPPOLO *et al*., 1996; GRIMM *et al*., 1998; SHAMBESH *et al*., 1997; MAMUTI *et al*., 2002). According to some authors, during the progression of human CE infection, there is a switch between IgG1 to IgG4 into the humoral immune response of intermediary host (SHAMBESH *et al*., 1997; DAEKI *et al*., 2000; MAMUTI *et al*., 2002; SIRACUSANO *et al*., 2012). For this reason, the presence of IgG4 subclass antibody, not only it is associated with prolonged chronic infection, but even to evasion mechanisms of host immune response against the parasite (GARRAUD *et al*., 2003; SIRACUSANO *et al*., 2012). In our work, the ELISA absorbance obtained with IgG4 was significantly higher than those obtained with either IgG2 or IgG3, but not with IgG1 (Table 2). Due to these results, it cannot be estimated to what infection state of patients correspond these sera. A follow up with a higher CE patient group and comparing earlier with

advance infection, could be confirmed the previous finding above described in the literature (SHAMBESH *et al*., 1997; DAEKI *et al*., 2000; SIRACUSANO *et al*., 2012).

It is worth mentioning that there are some reports in the literature which suggested that IgG2 subclass is more informative than the IgG4 subclass for studying disease progression (RAMZY *et al*., 1999; LAWN *et al*., 2004). Interestingly, in these reports the hydatid cyst fluids used as antigens were from camel and horse intermediary hosts, respectively. It is known that the most probable genotypes of the cysts which come from these hosts are G6 and G4, respectively which do not correspond to the *E. granulosus* s.s. specie (THOMPSON, 2008; NAKAO *et al*., 2013; ROMIG *et al.,* 2015). For this reason, we suggest that it should be investigated whether using other than *E. granulosus* s.s. genotypes, such as G4 and G6, there is a change in the humoral immune response against the parasite. Considering these facts shown by the literature and our results, we suggest that it is probably that using different genotype of hydatid cyst fluid may be obtain dissimilar serological results. For this reason, we suggest that it is very important to identify the corresponding genotype, to be sure that the expected serological results not be altered and really correspond to the expected G1 genotype antigen and not to other, even include into the same specie *E. granulosus* s.s.

CONCLUSIONS

Our results clearly detected different antigenic profiles between the G1 and G3 hydatid fluids (particularly in the range of 8 – 10 kDa corresponding to the smallest AgB subunit). These differences were also detected with ELISA when using IgG4 as a second antibody. These results support our suggestion that to avoid misinterpretations of serological studies, it is very convenient determine the genotype of hydatid fluid, to be sure that it is effectively G1 and not another genotype.

Finally, our results suggest that the confirmation of ELISA results by Western blot, in addition to the use of total IgG, it would be highly recommended to include a second antibody, IgG1 or IgG4, to confirm the detection of specific anti-*E. granulosus* s.s. antibodies that bind to 8-10 kDa antigens.

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Conflict of interest None to declare.

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FIGURES:

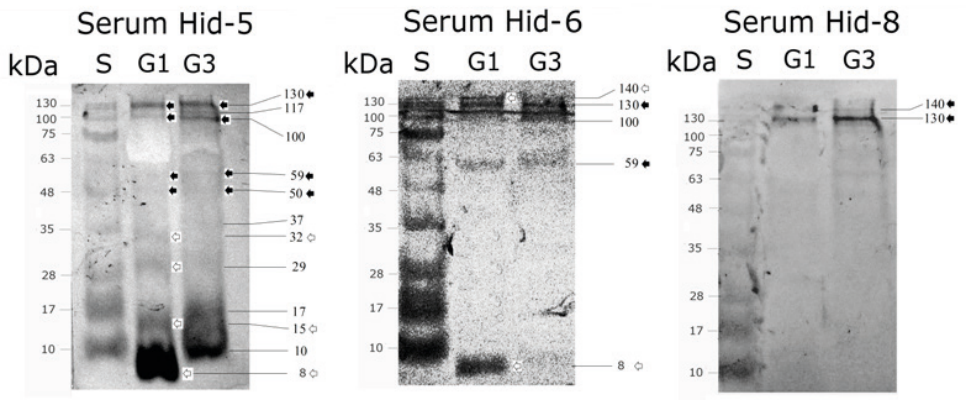

Fig. 1. Detection of hydatid antigens with IgG1 as second antibody. Western blots of two hydatid cyst fluids classified as *Echinococcus granulosus* genotypes G1 and G3 were analyzed with three sera (Hid-6, Hid-6 and Hid-8) from patients infected with this parasite. The Western blots were performed as described in Material and Methods. The detection of antigens was conducted by a chromogenic system using BCIP/NBT alkaline phosphatase substrates.

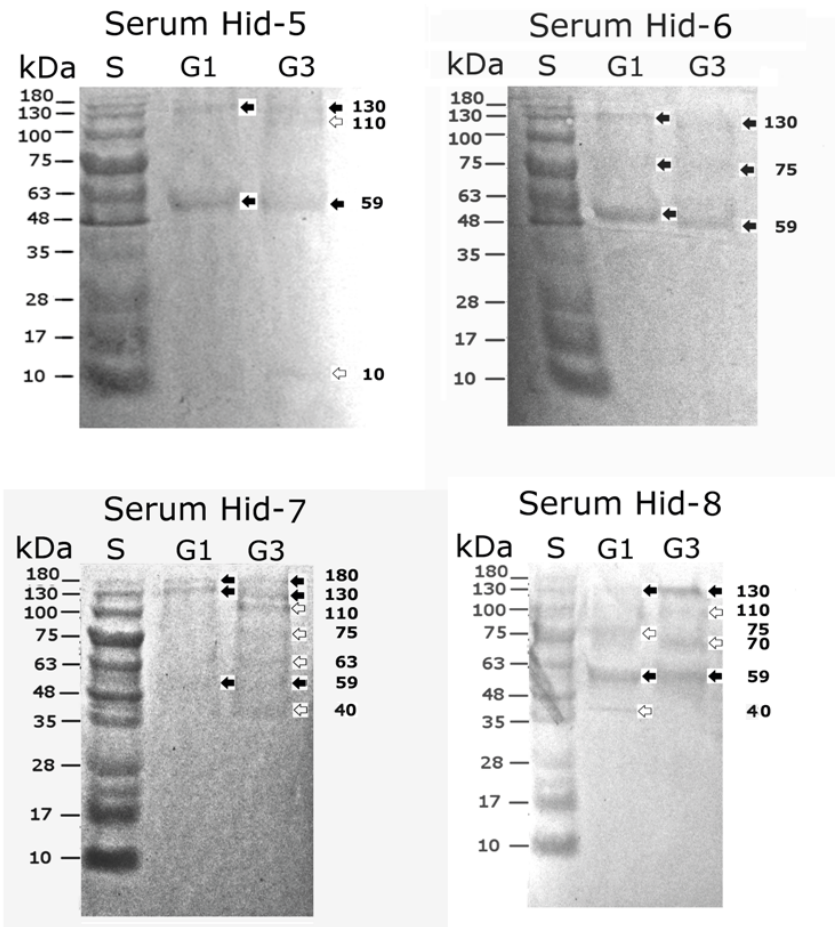

Fig. 2. Detection of hydatid antigens with IgG3 as second antibody. Western blots of two hydatid cyst fluids classified as *Echinococcus granulosus* genotypes G1 and G3, were analyzed with four sera (Hid-5 to Hid-8) from patients infected with this parasite. The Western blot was performed as described in Material and Methods. The detection of antigens was conducted by chromogenic system using BCIP/NBT alkaline phosphatase substrates.

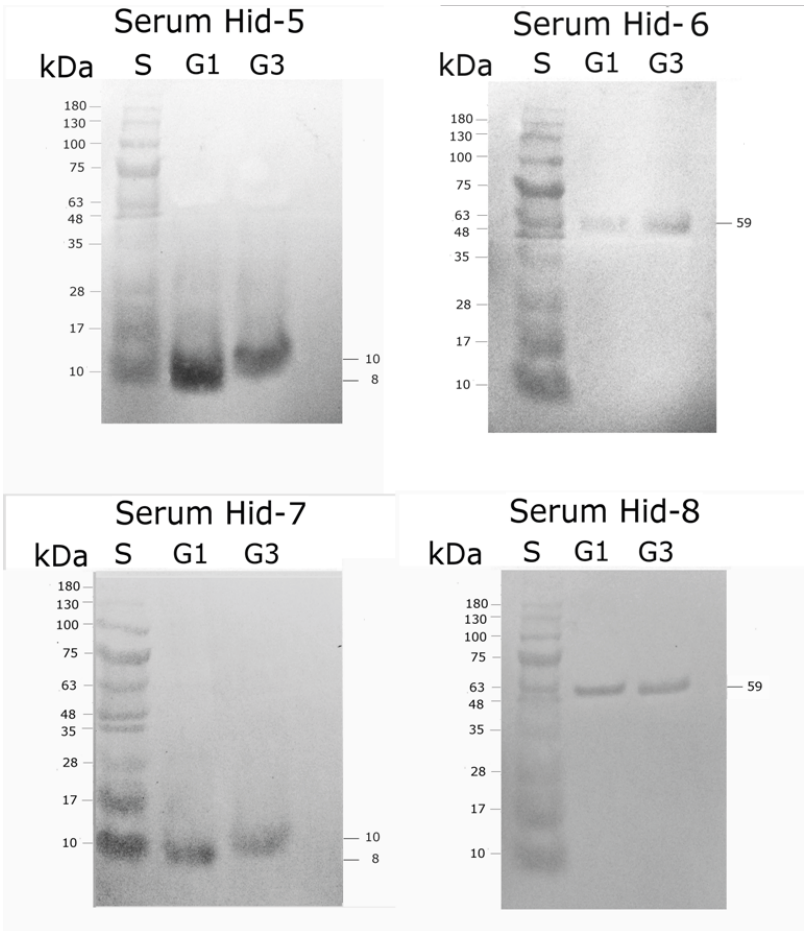

Fig. 3. Detection of hydatid antigens with IgG4 as second antibody. Western blots of two hydatid cyst fluids classified as *Echinococcus granulosus* genotypes G1 and G3 were analyzed with four sera (Hid-5 - Hid-8) from patients infected with this parasite. The Western blot was performed as described in Material and Methods. The detection of antigens was conducted by a chromogenic system using BCIP/NBT alkaline phosphatase substrates.

TABLES

Sera			A_{405} obtaining by IgG subclass antibodies and G1 or G3 HCF antigens:							
	IgG1		IgG2		IgG3		IgG4		Total IgG	
	G1[a]	G3	G1	G3	G1	G3	G1	G3	G1	G3
hid-9	0.529	0.533	0.526	0.517	0.561	0,581	0,701	0,594	1,627	1,606
hid-10	0.955	0.697	0.528	0.515	0.529	0,521	1,019	0,678	3,289	2,897
hid-11	0.548	0.523	0.522	0.526	0.583	0,566	0,761	0,575	3,451	3,451
hid-12	1.387	1.098	0.543	0.542	0.511	0,532	0,829	0,615	3,46	3,377
Mean	0.855	0.713	0.530	0.525	0.546	0.550	0.827	0.616	2.957	2.833
± SEM	0.203	0.135	0.005	0.006	0.016	0.014	0.069	0.022	0.445	0.427

Table 1. Detection by ELISA of IgG subclass antibodies against HCF antigens belong to G1 and G3 genotypes using sera from cystic echinococcosis patients.

ELISA, Enzyme-linked immunosorbent assay; HCF, hydatid cystic fluid; A_{405}, absorption at 405 nm including the cut-off value of 0.461.

Groups	Serum groups analyzed with IgG subclasses and G1 and G3 antigens:									
	A	B	C	D	E	F	G	H	I	J
	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4	IgG-total	IgG-total
	G1	G3	G1	G3	G1	G3	G1	G3	G1	G3
A	-									
B	0.5839	_								
C	0.2074	0.2668	_							
D	0.2024	0.2571	0.5605	_						
E	0.2253	0.3037	0.3940	0.2921	_					
F	0.2300	0.3133	0.2503	0.1774	0.8579	_				
G	0.9029	0.4857	**0.0226***	**0.0215***	**0.0236***	**0.0251***	_			
H	0.3238	0.5246	**0.0287***	**0.0232***	**0.0492***	0.0555	**0.0486****	_		
I	0.0114*	**0.0114***	**0.0121***	**0.0120***	**0.0123***	**0.0123***	**0.0161***	**0.0133***	_	
J	**0.0120***	**0.0118***	**0.0125***	**0.0124***	**0.0127***	**0.0127***	**0.0169***	**0.0137***	0.8473	_

Table 2. P-value of comparison by the Welch t-test among the ELISA results with different IgG subclass and HCF antigens belong to G1 and G3 genotypes.

ELISA, Enzyme-linked immunosorbent assay; HCF, hydatid cystic fluid. Asterisk, statistically significant value (P-value ≤ 0.05).