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Development and Testing of Trial Samples for Specific Serodiagnosis of *Fasciola gigantica via* External Quality Assessment

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Abstract

Objective: To build a procedure for production of samples for specific serodiagnosis of anti-*Fasciola gigantica* antibodies *via* an external quality assessment scheme, and to evaluate the homogeneity and stability of the trial samples.

Methods: In this experimental study, samples contained an anti-*Fasciola gigantica* antibody according to screening by ELISA, followed by confirmation by Western blotting were collected. All samples were tested and found negative for other helminth, especially of trematode antibodies. The samples were also negative for anti-HIV-1 and -2 antibodies, anti-HCV antibodies, and HBs antigen. The samples were prepared by freeze-drying and freezing methods, the stability and homogeneity were evaluated each 2, 4, 8, 12 and up to 24 weeks.

Results: We produced three lots of serum samples containing anti-*Fasciola gigantica* antibodies at three levels. Lot DK1 had optical density, OD=0.350 ± 0.037; Lots DK3 and DL3 had O=0.653 ± 0.046, and Lot DL1 had OD=0.850 ± 0.047 with the wavelength of 450 nm. The specific IgG antibodies against *Fasciola gigantica* antigens were found to be positive for all three proteins, 8–9 kDa protein (P 8-9), 28 kDa (P 28), and 42 kDa (P 42) by the Western blot technique. The trial samples were confirmed to be homogeneous by Fisher's test (F statistics<F distribution and sig value>0.05) as well as to be stable during 24 weeks (with t statistics<t distribution and sig [2-tailed]>0.05).

Conclusion: Trial samples for specific serodiagnosis of anti-*Fasciola gigantica* antibodies *via* external quality assessment can be produced with homogeneity and stability lasting for 24 weeks by freeze-drying and freezing methods.

Keywords: ELISA; EQA; *Fasciola gigantica*; Western blot

Introduction

External quality assessment (EQA) is one of the critical elements of a laboratory quality management system in accordance with ISO Guide 15189:2012 [1]. It is also a criterion for assessing the level of quality of a medical laboratory based on decision No. 2429/QD-BYT of the Vietnamese Ministry of Health. In addition, EQA provides objective evidence of reliable results for all customers using the services of the laboratory.

The specific serodiagnosis of anti-*Fasciola gigantica* antibodies for EQA was designed to improve the quality of screening and diagnostic tests for anti-*Fasciola gigantica* antibodies. It plays a key role in the control and evaluation of the quality of a laboratory *via* inter-laboratory comparisons. EQA participation is vital for all medical laboratories [2]. At present, there is no study on production of trial samples for specific serodiagnosis of anti-*Fasciola gigantica* antibodies for EQA. Herein, we developed a procedure for producing standard samples that contain specific anti-*Fasciola gigantica* antibodies for use in EQA.

Materials and Methods

Sample collection

All samples were collected from patients who had clinical manifestations that met *Fascioliasis*-foodborne trematode infection criteria [3] comprising fever with unknown reason, right side abdominal pain, decreasing hemoglobin, hepatosplenomegaly and diagnostic imaging showed parenchymal heterogeneity characterised by multiple subcapsular and coalesced into tubular or tortuous structures.

Fifty serum samples that were expected to contain specific IgG anti-*Fasciola gigantica* antibodies were collected at the Institute of Malaria Parasitology and Entomology, Binh Dinh province, Vietnam.

From September to December 2017, collected serum samples that met the inclusion criteria were extracted into Eppendorf tubes and stored at -20°C. All the samples were transported to the Quality

Control Center for a medical laboratory under supervision of the Ministry of Health, HCMC University of Medicine and Pharmacy, for the next steps in the research process.

Hemolytic serum samples or sera that turned dark after 48 hours of storage were excluded from this study. To ensure homogeneity of serum samples, all samples were kept at -20°C during transportation and storage. The samples were tested by both ELISA (Diagnostic Automation/Cortez Diagnostics, California, USA) and Western blotting (LDBIO Diagnostics, Lyon, France) to confirm the presence of specific IgG anti-*Fasciola gigantica* antibodies.

To prevent the cross reaction, samples were tested by ELISA technique and found negative result for other helminth antibodies including *Taenia solium*, *Echinococcus* sp. (Diagnostic Automation/ Cortez Diagnostics, California, USA) and *Toxocara canis* (Creative Diagnostics, New York USA) especially of trematode antibodies: *Schistosoma mansoni* (Diagnostic Automation/Cortez Diagnostics, California, USA), *Paragonimus westermani* and *Clonorchis sinensis* (Creative Diagnostics, New York USA). All samples were also tested and found negative for antibodies to HIV-1, -2, HCV, and HBV were selected for further analysis.

Negative trial samples for EQA were collected from researchers and tested negative for anti-HIV-1 and -2 antibodies, anti-HCV antibodies, HBs antigen, and anti-*Fasciola gigantica* antibodies. Then, the samples were frozen at −80°C at OD=0.062 ± 0.012.

Procedure

Trial samples were divided into three batch then stocked in different condition comprising frozen samples were stored at -80°C, freeze-dried samples were stored at -20°C and one batch were stocked by both conditions. Each batch contained 100 tubes, which were subdivided into two lots, 50 tubes each.

The methods for testing the homogeneity and stability were in accordance with ISO Guide 35 and ISO 13528 [4]. Fourteen tubes from each lot were selected randomly. Optical density (OD) of each tube was measured by ELISA with the wavelength of 450 nm and was used to evaluate homogeneity. Then, stability was assessed after 2, 4, 8, 12 and 24 weeks, by taking three random tubes from each lot and determining OD by the ELISA technique as well as calculating the average of OD values at the corresponding time points.

Data analysis

The F-test analysis of variance (one-way ANOVA) was carried out to evaluate homogeneity of samples (F statistics<F distribution). The t test (independent-sample t test), sig (2-tailed) >0.05, at α=0.05, allowed us to evaluate stability of the samples. SPSS 20.0 software was used for data analysis.

Results

All collected samples were contained an anti-*Fasciola gigantica* antibody by ELISA. The specific IgG antibodies against *Fasciola gigantica* antigens were found to be positive for all three proteins, 8–9 kDa protein (P 8-9), 28 kDa (P 28), and 42 kDa (P 42), by the Western blot technique as shown in Figure 1. None of them were positive for other helminth as well as HIV, Hepatitis B and Hepatitis C.

Three batches of trial samples contain specific IgG antibodies to *Fasciola gigantica* were divided into three concentrations:

- The freeze-dried samples (Lot DK1) had OD=0.350 ± 0.037.

-The frozen samples (Lot DL1) had OD=0.850 ± 0.047.

-The freeze-dried and frozen samples (Lots DK3 and DL3) had OD=0.653 ± 0.046.

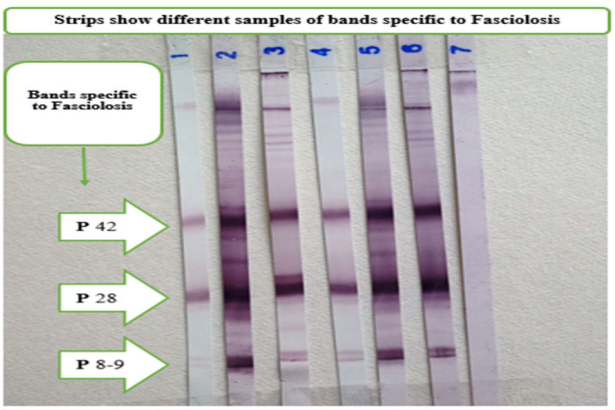

Figure 1: Protein bands detected by western blotting that correspond to *F. gigantica* antigens.

Strips number 1 and 4 contain the reaction with a positive control sample; strips number 2, 3, 5, and 6 show a reaction with positive collected samples; strip number 7 contains the reaction with a collected negative sample.

Evaluation of homogeneity

Trial samples	DK1	DL1	DK3- DL3
F $_{statistics}$	0.367	0.087	0.465
F $_{distribution}$	3.231	3.231	2.525
Sig	0.695	0.917	0.761

Table 1: Evaluation the homogeneity of the freeze-dried and frozen samples.

Table 1 shows the results of assessment of the homogeneity of samples DK1, DL1, and DK3–DL3 with F statistics<F distribution (F statistic was 0.367, 0.087, and 0.465 in comparison to F distribution at 3.231, 3.231, and 2.525, respectively), with sig value>0.05 (sig value was 0.695, 0.917, 0.761, respectively). We assumed that the H_0 hypothesis is accepted. It means that trial samples for EQA that were produced by both the freeze-drying and freezing method were homogeneous.

Evaluation of stability

The stability of the frozen samples 1

The frozen samples DL1					
Time	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
t $_{statistics}$	-2.74	1.498	1.069	1.086	1.280
t $_{distribution}$	2.10				

Sig (2-tailed)	0.787	0.152	0.299	0.292	0.217

Table 2: Results of the OD value of DL1 during the study period.

Table 2 shows that the t statistics at the time of evaluation were less than the t distribution value of 18 (n1+n2−2=16+4−2) degrees of freedom and sig (2-tailed) >0.05. It is likely that the frozen samples 1 (DL1) were stable during 24 weeks.

The stability of the freeze-dried samples 1

The freeze-dried samples DK1					
Time	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
t $_{statistics}$	-0.159	0.781	1.973	1.102	1.388
t $_{distribution}$	2.10				
Sig (2-tailed)	0.876	0.445	0.64	0.285	0.182

Table 3: Results on the OD values of freeze-dried samples 1 during the study period.

Table 3 shows that the t statistics at the time of evaluation were less than the t distribution value of (n1+n2−2=20−2) degrees of freedom and sig (2-tailed) >0.05. It is likely that the freeze-dried samples 1 (DK1) were stable during 24 weeks.

The stability of the freeze-dried samples (Lot DK3)

Freeze-dried samples DK3					
Time	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
t $_{statistics}$	0.336	0.21	1.731	1.050	1.721
t $_{distribution}$	2.10				
Sig (2-tailed)	0.741	0.984	0.101	0.150	0.102

Table 4: Results on the OD value of Lot DK3 during the study period.

Table 4 shows that the t statistics at the time of evaluation were less than the t distribution value of (n1+n2−2=16+4−2) degrees of freedom and sig (2-tailed) >0.05. It is likely that freeze-dried samples 3 (DK3) were stable during 24 weeks.

The stability of frozen samples 3

The frozen samples DL3					
Time	2 weeks	4 weeks	8 weeks	12 weeks	24 weeks
t statistics	0.034	1.754	1.993	1.369	1.856
t distribution	2.10				
Sig (2-tailed)	0.973	0.096	0.069	0.188	0.080

Table 5: Results on the OD value of DL3 during the study period.

Table 5 shows that the t statistics at the time of evaluation were less than the t distribution value of (n1+n2-2=16+4-2) degrees of freedom and sig (2-tailed) >0.05. It is likely that frozen samples 1 (DL3) were stable during 24 weeks.

Results on production of the trial serum samples containing an anti-*Fasciola gigantica* antibody for EQA: The procedure for production of samples for specific serodiagnosis of anti-*Fasciola gigantica* antibodies *via* an external quality assessment scheme have been developed as shown in Figure 2.

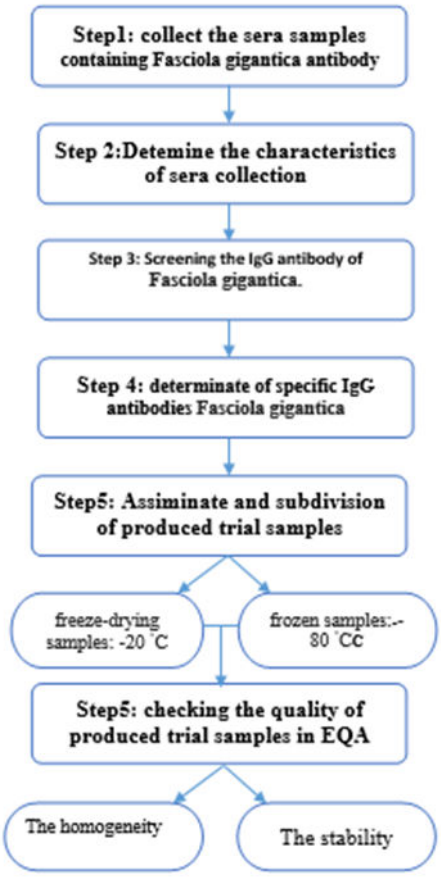

Figure 2: The workflow of preparation of trial samples containing an anti-*Fasciola gigantica* antibody used in EQ.

Discussion

Determination of quality of the trial samples containing an anti-*Fasciola* antibody used in EQA

The trial samples: The purpose of creating the trial samples with different concentrations of antibodies is to enhance the efficiency of quality assessment of participating laboratories. In comparison with other studies such as "External quality assessment scheme for parasite serology; a review of the scheme design and performance" by Collier [2], and "The United Kingdom National External Quality Assessment Service for parasitology: toxoplasma serology scheme" by Manser [5], in the EQA scheme of serological testing for *Toxoplasma* sp. in England, there is a bias at low concentrations. This observation means that our EQA sample production method yielding three different concentrations is in compliance with the trend of the EQA scheme of developed countries.

Homogeneity and stability of trial samples: In our study, trial samples produced by freeze-drying and by the freezing method had good homogeneity and stability. Nonetheless, within the limited period of our study, we could evaluate the stability of the resultant trial samples for only 24 weeks in contrast to other studies worldwide. For example, in a study by Michaut [6], serum samples stored frozen at -80°C were stable for 3.5 years, whereas in the study by Dard [7], the stability of IgG antibodies against *Toxoplasma gondii* antigens in frozen serum samples stored at -20°C lasted for 6 years. There was no change in the levels of the antibody after 6 years of storage and between the mean values before analysis (before freezing of samples) and after freezing.

The process of production of trial serum samples containing anti-*Fasciola gigantica* antibodies used in EQA

The process includes six steps as follows:

Step 1: We collected materials from the original serum sample with IgG antibodies *against F. gigantica* antigens had the clinical manifestations were mainly fever with unknown reason, right side abdominal pain, decreasing hemoglobin, hepatosplenomegaly and clearly diagnostic imaging.

Step 2: We selected the samples that were tested negative results for anti-HIV-1 & -2 antibodies, anti-HCV antibodies, HBsAg and negative screened immunoreactivity to other helminth antigens, especially of trematode antigens. This design suggests that our process of preparation of trial samples satisfies the requirements of a laboratory biosafety manual [8].

Step 3: We rechecked the IgG antibodies to *Fasciola gigantica* antigens from the original serum to eliminate false positive samples and confirm the true positive samples for production of trial samples.

Step 4: We detected protein bands by western blotting by means of the *Fasciola gigantica* antibodies including P 8-9, P 28, and P 42 kDa as specific antigens to produce the trial EQA samples.

Step 5: We generated the positive samples containing different antibody concentrations by means of the negative serum for dilution at a ratio of 1/5 and 1/10.

Step 6: We evaluated quality of the trial EQA samples in terms of homogeneity and stability: the two criteria in accordance with ISO 35: 2006 and ISO 13528: 2015.

Conclusion

1. The specific IgG antibodies against *Fasciola gigantica* antigens in collected serum samples were found to be reactive with all three protein bands (P 8-9, P 28, and P 42 kDa) in the western blot analysis.

2. The produced trial EQA samples of anti-*Fasciola gigantica* antibodies had good quality: homogeneity and stability during 24 weeks.

3. The process of preparation of the trial EQA samples of anti-*Fasciola gigantica* antibodies can be applied to produce serological EQA samples for parasitosis.

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