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*Full Length Research Paper*

# Evaluation of mice, sheep and human IgG and IgE antibody responses against the mice Crude Hydatid Cyst Fluid (HCF) antigens

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**Hydatidosis is a dangerous parasitic disease which commonly occurs in humans and animals. It annually causes many economic and health problems throughout the world. The existing diagnostic kit uses sheep cystic fluid antigen as an available antigen to evaluate the human IgG antibody. The current study was designed to evaluate an alternative antigen which is the mice HCF antigen for such diagnosing kit. For this purpose the IgG responses in sera of human, mice and sheep against the mice crude hydatid fluid antigens was applied using ELISA and Western Blot. Thirty Balb/c mice were immunized using sheep hydatid cyst and complete freunds adjuants. The hydatid cyst fluid antigens of human and sheep were obtained from naturally infected human and sheep and for mice from experimentally infected mice with protoscolices of hydatid cysts (HC). Antigens were used in ELISA and SDS PAGE after their concentrations were measured applying Bradford procedure. Thirty positive samples sera from mice, sheep and human were employed as the case and 30 healthy sera from each as the control group. The statistic analysis tests were ANOVA and post Hoc Analysis Model. The highest mice antibody response against mice HCF antigens was IgGAM antibody (with mean OD value 0.4) while the lowest was IgE (with mean OD value 0.26). The best antibody response was seen for human total IgG against mice HCF with mean OD value of 0.71 while the lowest response against this antigen belonged to human IgG3 and IgE each with mean OD value of 0.12. ANOVA analysis indicated significant differences between the human IgG class and subclass responses to mice HCF antigens ($P < 0.05$). The sensitivity and specificity of the ELISA for antibody responses to this antigen was mostly more than 90% almost for all sera of different hosts. The mean OD value of sheep IgG against mice HCF antigen was 0.34. The studied sera (mice, human and sheep) considerably showed a positive response against mice HCF antigens by ELISA test. The human and mice sera showed a higher response over the sheep sera in that their mean OD values and their OD ratio were significantly higher than that for the sheep. Totally there are some immune responses from the serum of each animal recognizing the crude hydatid cyst fluid antigen of mice. According to the results of the current study the mice HCF is an appropriate candidate for diagnosing of hydatidosis of human and animal.**

**Key words:** Hydatid cyst, cross-reaction, IgG response, hydatid cyst fluid.

## INTRODUCTION

Hydatidosis is a parasitic disease of domestic and wild animals including human causing by infection with the larval stages of dog/fox tapeworms of genus *Echinococcus* and is also referred to as echinococcosis (Lightowlers et al., 1989). It is estimated that more than 3 million global cases are occurred annually of which human

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hydatid cyst is the most common presentation (Zhang et al. 2008). Actually these complex multicellular patho-gens are able to modulate antiparasite immune responses and as a result they can persist and flourish in their mammalian hosts. In order to understand how the immune system deals with these parasites it is important to evaluate the immune response of different hosts against the most accessible antigens of such parasites (Zhang et al. 2008). Though recent application of modern molecular and immunological approaches has elucidated some insights on the nature of immune responses generated during the course of hydatid infection, many aspects of the *Echinococcus* host interplay remain unexplored yet (Zhang et al. 2008).

Hydatidosis is a worldwide disease especially widespread in sheep raising areas that can cause so many economic and health problems annually (Thompson, 1995; Todsorov and Boeva, 1999). As parasite can habituate in different organs like lung, heart, brain, liver, spleen and spinal cord in asymptomatic form for a period of 20 years therefore, the diagnosis of disease is so difficult and usually is based on para-clinical methods like serology (Movasagh et al., 2008). As the disease is dangerous and surgery is the only way for the treatment of this disease therefore definitive and immediate diagnosis of hydatidosis is vital for the hosts (Guisantes, 1997). The use of crude hydatid (HCF) cyst fluid antigen for the diagnosis of cystic hydatid (CH) is one method which along with immediate serological investigation can be helpful and effective in rapid treatment of the disease (Vuitton and Wen, 2007). Rapid and definite diagnosis is really important not only in human but also in animals. To achieve such a goal using the HCF antigens of different hosts including human and animals can be used to evaluate the diagnosis of hydatid cyst as it was reportedly confirmed that the HCF from a given host (human) shows a relatively stronger positive reaction (Zhang et al. 2003).

If serological diagnosis can be based on the use of some highly valid and reliable antigens of different animals, designing the diagnostic kit with such antigens can be a simple, non expensive and available method. Most of the serological tests used in diagnosing the hydatid cyst have their own problems like limited availability, different sensitivity and specificity and difficulty in their preparation. Some of these tests need several specific techniques, equipments and experienced personnel. In many the materials, reagents and equipment to perform the IgG-ELISA are readily available, and this technique is probably the best overall choice for use in immunodiagnosis for human CE. However, there is still no standard, highly sensitive, and specific serological test for antibody detection in cases of different domestic animals (Mamuti et al., 2002). Therefore, for clinical practice, it should be noted that the results of serological tests depend on multiple factors, such as antigen quality, test system, organ site and number of hydatid cysts, individual variability of immune responses, etc. Different studies have reported a sensitivity range of 60 to 90% for ELISA and a specificity range of 75 to 90% (Hashemitabar et al. 2008; Pushpinder et al., 2007). Usually the range is variable based on antigen type, methodology, the geographical region in which the test is performed and also the endemic region of the disease. Antigens have different fractions e.g. antigen B has 8, 16, 24 and 38 KD with different sensitivities in diagnosis of the disease (Pushpinder, 2007; Bradford, 1976). Also these fractions have different sensitivities in each animal as well, therefore finding an antigen with a fraction which its sensitivity and specificity is high in response to the sera of human or other animals can be a considerable progress in developing a reliable and non- expensive method for diagnosis of HC. In addition there is a good trial to test the reactivity of human, mice and sheep sera to mice HCF antigens in order to analyze such a hypothesis for animals too. The current study was designed to find antibody responses in sera of human, mice and sheep against the mice HCF antigens in order to obtain a better evaluation of mice antigens for diagnostic purposes in human and animal hydatidosis.

## MATERIALS AND METHODS

This is an analytical case-control study using mice crude HCF as the source of antigen for performing ELISA, Western blotting and immunization the mice. Sample size was calculated with using $n = \frac{\lambda}{\Delta}$ formula, that $\lambda = 15.4$ (λ, for α=%5, β=%10 and k=5 groups, is 15.4), $\Delta = \frac{\sum_{i=1}^{5}(\mu_i - \bar{\mu})^2}{\sigma^2}$, $\mu_i$ were mean OD in three groups. ANOVA was used to test the difference mean of OD values among groups. Test of homogeneity of variances was done by Levens statistics. Tukey test was used in the POST HOC analysis. P-values less than 5% were considered as statistically significant.

### HCF preparation

Hydatid cyst fluid antigen preparation was carried out according to the procedure described by Mamuti (2002) with slight modifications. Briefly, hydatid cysts were isolated from sheep by a trained and licensed technician under the codes of animal rights. Hydatid cyst fluids were aspirated aseptically from cysts and centrifuged at 3000 rpm for 5 min. The Protoscolices and salts were sedimented and the supernatant was separated and kept in sterile falcon tubes. The collected sheep HCF were centrifuged again at 1500 g for 2 to 3 min for purification of remaining protosolices in order to obtain some fluid antigen to immunize the mice. The supernatant of all samples were homogenized by 3 times freeze-thawing in liquid nitrogen. After the solution became constant it was sonicated by the sonicator machine regulated on 50 $\%$ with a maximum tone of 30 s 4 times. Dialyzing of antigens was carried out to concentrate the proteins. The dialyzed extracts were assessed by using Bradford procedure for detecting protein concentrations (Bradford, 1976). This fluid was kept at 20°C as a fluid antigen source for later use. The sedimented protoscolices were transmitted into some sterile tubes performing viability experiment.

**Dialyzing the antigens against PBS**

First, the required segments of dialyzing tube with 12 KD cut off were cut. Tubes were washed under the running water for 3 to 4 h to remove the glycerin then treating with a 0.3% (w/v) sodium sulfate solution at 80°C to remove the sulfur compounds and washing with hot water for 2 min followed by acidification with 0.2% (v/v) sulfuric acid and rinsing with hot water to remove the acid. Afterwards, it was boiled in distilled water for 20 min until the adhesion of bag disappeared and its two ends opened widely. Hydatid cyst fluid was loaded into the tubes with two ends of the bag tied. Then it was dialyzed against PBS at 4ºC for 74 h while the PBS was replaced 3 times a day.

**Infecting the mice with protoscolices**

Laboratory model of hydatid cyst in mouse has been designed by different investigators. Previous studies have indicated that balb/c mice show a very high sensitivity to hydatid infection and are more suitable for immunologic studies of this parasite. Two groups each including 30 balb/c mice 3 weeks-old, used in this study had been bought from Iran institute of Pasteur. Immunization of mice was carried out based on the method described by Mamuti (2002) with slight modification as follows:

The mice were transferred to laboratory for vaccination and experimentally transformation of Protoscolices. The case group contains 30 balb/c mice were immunized with sheep HCF; while the control group also contained 30 balb/c mice received distilled water. Each group was placed in a specialized rack. Totally 200 µl HCF solution including; 100 µl HCF containing 10 µl penicillin and streptomycin together with 100 µl of Feround's complete adjuvant, was injected into mice intra-peritoneally. Four weeks later, the same injection was repeated but with Feround's incomplete adjuvant. Two weeks after the last injection the mice were killed and their blood for sera extraction was taken. Sera were prepared and transformed into a sterile tube and kept at -20°C for further steps.

**Antigen concentration**

Concentration of mice HCF antigens was estimated according to Bradford procedure using BSA as standard protein (Ito et al., 1999; Khosravi et al., 2011). For this assay, the dye reagent (Bio-Rad protein assay dye) was diluted with deionised water in a ratio of 1:3, respectively. The diluted solution was then filtered through fast filter paper under gravity. The calibration of spectrophotometer (GeneQuant *Pro* RNA/DNA calculator, Amersham Biosciences) was conducted using a series of blank that is, with no BSA and with, 5, 10, 20 and 40 µg of a 1 mg/ml BSA stock as a protein standard. A different dilution of crude HCF samples was prepared. In each well of a clean ELISA plate, 10 µl of each dilution of both BSA and HCF samples were placed, to which 300 µl of dye solution was added. Plate incubated at room temperature for 5 min and the OD (optical density) values were measured at 570 nm using the zero BSA sample as a blank. Using the BSA standard curve the protein concentration of samples was determined using an equation resulted from calibration as follows: Y= 0.9956X-0.0097 (Y is the absorbance value and X is the concentration of unknown protein in mg/ml).

**SDS PAGE (sodium dudecil poly achrylamide gel electrophoresis)**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Khosravi et al. (2011) with using 10% polyacrylamide gradient gels under reducing conditions. Two prestained low and high-range marker (Bio-Rad) were used for monitoring electrophoresis. Approximately 15 µl of HCFs prepared from humans was loaded into sample wells. Electrophoresis was carried out at a constant 20 mA (200v) for approximately 90 min at room temperature.

**Preparation of samples sera**

Samples were collected from hydatidosis patients that their hydatid cyst had been diagnosed definitely by their physicians. Positive sheep samples sera were collected from the naturally infected sheep in a local slaughter house that their hydatid cyst was definitely diagnosed by inspectors. Positive mice samples sera were collected from experimentally immunized mice. Negative serum of control group was collected from healthy (uninfected) individuals, and healthy sheep at slaughter house. Healthy mice (control groups) were already kept in lab and their sera were used for this purpose.

**ELISA**

ELISA was carried out as described by Khosravi et al. (2011) and Voller et al. (1974). ELISA plates were coated with 100 µl per well of the HCF antigen; typically 7.5 µlg per ml (according to the findings of checkerboard study) in carbonate coating buffer pH 9.6 and incubated at 4°C overnight. Unbound antigen was removed by three times washing with TST (Tris, sodium chloride and Tween 20) washing buffer, after which plates were pre-blocked with 5% ( w/v) skimmed milk in TST and stored at 4°C overnight. Plates were washed 3 times with TST. Sample sera from human or animals (mice, sheep) were diluted at a ratio of 1:100 with blocking buffer, of which 200 µl was added per well comparing to one positive control (100 µl of human or animal serum) and one negative control (100 µl of human or animal normal serum) at the same dilution in order to determine the reaction of each sample serum to HCF antigen. After washing five times with TST, a horseradish peroxidase (hrp)-conjugate anti human/animal IgG/IgG subclasses or was added at a dilution of 1: 1,000, incubating at room temperature for two hours. The plates were washed five times with TST and fresh ABTS (2, 2'-azino-di-3-ethylbenzthiaoline sulfonic acid) substrate was added to develop the reaction. Once the background colour had changed so that the difference between the positive and negative control was at the highest level and before the background of negative control was changed, the reaction was stopped by adding 100 µl of $H_2SO_4$ 1M to each well to terminate the enzyme reaction and to stabilize the developed colour. The OD values were read at 405 nm using a Dynatech Microtiter Plate reader after 60 min. Positive samples were defined as those giving a specific OD above the normal range for the control sera. The normal range was taken as the mean ± 2 standard deviation (SD) of 30 normal sera.

## RESULTS

### Human IgE, IgG class and subclass antibody against mice HCF antigens

Results of human IgG class and subclasses (IgG2, IgG3 and IgG4) together with IgE against HCFs antigens of mice are summarized in Table 1. Each OD value is compared to its mean cut off values for each antibody.

Total human IgG had the highest mean OD values against

**Table 1.** Mean OD values of human IgG class, subclass and IgE responses against mice HCF antigen

| Ab | OD | SD | Minimum | Maximum | Number | Range | Cut off |
|---|---|---|---|---|---|---|---|
| IgG | 0.71 | 0.14 | 0.49 | 0.99 | 30 | 0.5 | 0.06 |
| IgG2 | 0.14 | 0.02 | 0.12 | 0.17 | 30 | 0.05 | 0.12 |
| IgG3 | 0.12 | 0.004 | 0.11 | 0.12 | 30 | 0.01 | 0.16 |
| IgG4 | 0.29 | 0.36 | 0.07 | 0.97 | 30 | 0.9 | 0.04 |
| IgE | 0.12 | 0.01 | 0.1 | 0.14 | 30 | 0.04 | 0.11 |

(P = 0.001, F = 725.38).

mice HCF antigens while IgG3`and IgE the lowest one (Table 1). As the mean OD values for all antibodies in response to mice antigen were higher than that of cut off values, all the human sample sera are regarded as positive indicating an antibody reaction by the human sera and against mice HCF antigens. ANOVA analysis showed that the difference between the mean OD values of human antibodies against mice hydatid cyst crude antigens was statistically significant (P < 0.001). In other words human immune responses against mice hydatid cyst crude antigens are strongly detectable. While the sensitivity and specificity of ELISA for human IgG2 against mice HCF antigens was 90 and 100% respectively this ratio were 70 and 100% for the human IgE against the same antigen respectively. At the same time another parameter was calculated as “OD ratio” by dividing mean OD value of each antigen to its cut off which was about 12 for human total IgG and about 7.5 for human IgG4 while about 1 for human IgE.

Though the strength of the human antibody response was different for each antibody, this difference was statistically significant using ANOVA (P < 0.001). Post Hoc analysis also showed significant differences between different antigens based on human antibody responses (Table 2).

## Sheep antibody response to mice HCF antigens

The mean OD value of the sheep total IgG against mice HCF antigens was 0.36 and the OD ratio 4 compared to the cut off value. The sensitivity and specificity of ELISA for sheep total IgG was 100 and 91.7%, respectively. In other words sheep had IgG antibody response against the mice HCF antigens at significantly a higher rate compared to the cut off point indicating that sheep can recognize the mice HCF antigens strongly. Post Hoc analysis showed that there was a significant statistical difference between the sheep IgG responses to different HCF antigens when HCF of mice origin was compared to that of human and sheep along with B compartment of human HCF in case group (Table 3) while such a difference was not demonstrated for the control group (results are not shown).

## Mice antibody responses to the mice HCF antigens

The best antibody response of mice sera against the mice hydatid cyst fluid antigen belonged to the IgGAM, IgG2b and IgG total respectively (Table 4) but the strongest OD ratio for mice antibody response compared to the cut off values was seen for IgE with 13 and IgGAM with 10 respectively. The sensitivity and specificity of ELISA for mice IgGAM, IgG, IgG2b and IgE against the mice HCF was 100%. Post Hoc analysis showed that there was a significant statistical difference between the mice IgG responses to different HCF antigens when HCF of mice origin was compared to that of human and sheep along with B compartment of human HCF in case group (P < 0.01) (Table 5) while such a difference was not demonstrated for the control group (results are not shown).

## Results of SDS PAGE

SDS PAGE showed the following bands (MW in KDa) from human sheep and mice HCF antigens (Figure 1):

20, 24, 26, 34, 37, 42, 48, 66

Similar bands of HCF antigens from different host were obtained by which the reactivity of sera was assessed and compared more efficiently. This finding made it sensible to compare the strength of responses amongst different hosts.

## DISCUSSION AND CONCLUSION

Mice HCF antigens raised a detectable antibody cross-reaction by sera of mice, sheep and human indicating that such an antigen can be considered for analyzing the human and animal hydatidosis. Such similar antibody responses amongst different hosts of hydatid cyst are the first step toward a diagnostic kit for both human and animals. Even it can be claimed that such a procedure can be employed to screen either human or animals’ hydatidosis where the screening of the disease can help

**Table 2.** Multivariate analysis of different HCF antigens in response to human total IgG using Post Hoc in case group.

| (I) Ag | (J) Ag | Mean Difference (I - J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound |
| Human | Sheep | 0.25878* | 0.04703 | 0.000 | 0.1663 | 0.3512 |
| | Mice | 0.25007* | 0.05776 | 0.000 | 0.1365 | 0.3636 |
| | B | -0.32781* | 0.06173 | 0.000 | -0.4491 | -0.2065 |
| | Cattle | 0.28147* | 0.05501 | 0.000 | 0.1734 | 0.3896 |

**Table 3.** Multivariate analysis of different HCF antigens in response to sheep total IgG using Post Hoc in case group

| (I) Ag | (J) A | Mean Difference (I - J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound |
| Human | Sheep | -0.06643* | 0.01286 | 0.000 | -0.0922 | -0.0407 |
| | Mice | -0.16517* | 0.01438 | 0.000 | -0.1939 | -0.1364 |
| | B | 0.08042* | 0.01508 | 0.000 | 0.0502 | 0.1106 |
| | Cattle | 0.03762* | 0.01508 | 0.015 | 0.0074 | 0.0678 |

**Table 4.** Mean OD values of mice IgG class, subclass and IgE responses against mice HCF antigen

| Ab OD | Mean | SD | Minimum | Maximum | Number | Range | Cut off |
|---|---|---|---|---|---|---|---|
| IgG | 0.29 | 0.04 | 0.27 | 0.38 | 30 | 0.12 | 0.07 |
| IgG2b | 0.31 | 0.04 | 0.27 | 0.38 | 30 | 0.38 | 0.12 |
| IgE | 0.26 | 0.03 | 0.23 | 0.32 | 30 | 0.09 | 0.02 |
| IgGAM | 0.4 | 0.13 | 0.26 | 0.64 | 30 | 0.4 | 0.04 |

**Table 5.** Multivariate analysis of different HCF antigens in response to mice total IgG using Post Hoc in case group.

| (I) Ag | (J) Ag | Mean difference (I - J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound |
| Human | Sheep | 0.02082 | 0.03594 | 0.563 | -0.0501 | 0.0917 |
| | Mice | 0.08555* | 0.03594 | 0.018 | 0.0147 | 0.1565 |
| | B | 0.13197* | 0.04658 | 0.005 | 0.0401 | 0.2239 |
| | Cattle | 0.12162* | 0.04658 | 0.010 | 0.0297 | 0.2135 |

for decision toward the treatment or some preventive plans by the health related centers. As we were only able to use the IgG subclass for human and mice the human total IgG and IgG4 were the best human antibody response while mice IgGAM and IgG2b showed to be the best mice antibody response against mice HCF antigenas their OD value and also OD ratio were both the highest values observed during this study. These data are evidenced that some antigen are stronger than the others in raising human and/or animal sera reaction and also some IgG subclasses are preferred over others in diagnosing HCF antigens of different origins which was confirmed by the others (Sobihi et al., 1996; Grimm et al., 1998) too. The interesting point was the highest OD ratio of mice IgE (13 folds compared to its cut off) and human IgG(10 folds compared to its cut off) to the mice HCF antigens though the sheep IgG had also a good reaction against this antigen revealing that mice HCF antigens can raise a distinguishable responses from the sera of the other hydatid cyst hosts. Many studies have reported similar results for the human IgG subclasses against human HCF antigens (Wen and Craig, 1994; Dreweck et al., 1997; Grimm et al., 1998; Siracusano et al., 2004; Khabiri et al., 2006) which can show the importance of

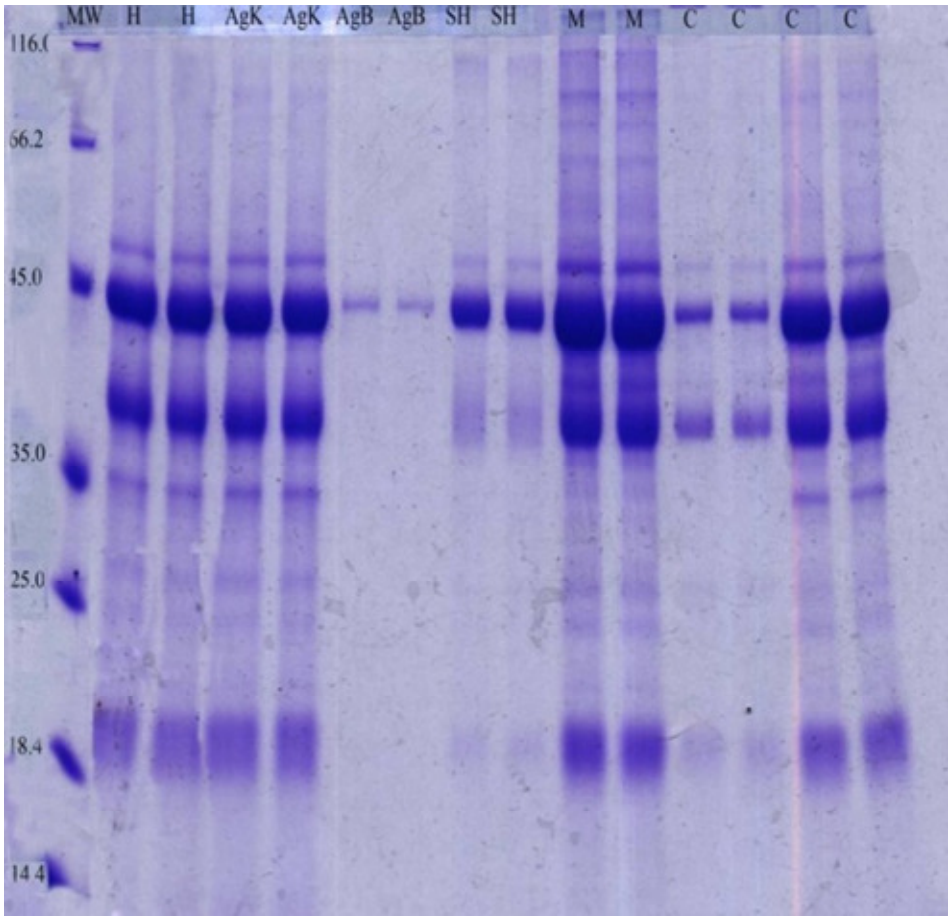


**Figure 1.** SDS PAGE showing different fractions of human and animal HCFs. MW=molecular weight, H=human, B=antigen B, AgK=antigen hydatid kit, SH=sheep, M=mice, C=cattle.

the current study that had employed the responses of human, sheep and mice against the mice HCF antigens which is different from the findings of the above researchers. As a result it can be mentioned that the majorty of studies carried out on hydatidosis so far have worked on human immune sera while the current study focused on both the human and animal immunesera usingt mice HCF antigens. As the aim of the current study was to find the best antibody response which is reactive against the mice HCF antigens we were able to obtain a clear understanding of immune responses against the mice HCF antigens by different hosts.

Generally, speaking when ELISA was carried out using human and animal IgE or IgG class and subclasses, the high OD value and strong OD ratio was found for either hosts sera against the mice HCF antigens indicating that the mice HCF antigen is a good candidate to elicit the antibody responses. Cross reaction strongly exists between human and animals antibody responses against HCF antigens of mice origin. IgG antibody can be seen in sera of human and animal against the HCF antigens of mice which is a sign of humoral response and can be used for designing a diagnostic kit in animal and human.

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