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INVESTIGATION ON THE VARIABILITY OF HYDATID CYST FLUID AS AN ANTIGEN FOR THE IMMUNODIAGNOSIS OF CYSTIC HYDATID DISEASE

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Abstract

Cystic echinococcosis (CE) affects humans and animals worldwide. Zoonotic diagnoses depend on imaging primarily ultrasound and immunodiagnostic techniques, which are cost-effective tools for epidemiological studies.

The current study investigated the utilization of hydrated cyst fluids (HCF) as diagnostic antigens, which were collected from various intermediate hosts. Sixteen crude HCF samples from humans, sheep, gerbils, horses, and cows were evaluated for their reactivity with human sera using an enzyme-linked immunosorbent assay (ELISA). The Bradford protein assay was used to assess the total protein concentration of each sample of cyst fluid. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein contained in the HCF of the samples. Immunoblotting was used to carry out antigen recognition. The HCF of the several intermediate hosts varied in their reactivity, according to ELISA results and this variation was unrelated to the protein content in the HCF samples. HCF samples' SDS-PAGE profiles revealed variance in several proteins and existence of 12 distinct protein bands ranging in size from 8 to 148 KDa. There were 98 & 64 KDa proteins found in all HCF samples. Bovine serum albumin was the 98KDa protein. In comparison, 19% of the fluid samples included an 8KDa protein indicative by AgB, while 50% of HCF samples contained a 50KDa protein. DS-PAGE profiles' proteins didn't show Ag5. The 64KDa protein was detected with potential for application in the diagnosis of human CE, according to immunoblot results.

Keywords: Hydatid cyst fluid; ELISA; SDS-PAGE; Immunoblot; Host.

Introduction

Echinococcosis is a cestode tapeworm of genus *Echinococcus* of worldwide distributions, and more and more frequently in rural, grazing areas where dogs ingest hydatid cysts from infected herbivorous animals (CDC, 2019). Zoonotic larval echinoccocosis were reported in the Eastern Mediterranean Countries, such as Egypt (El Sayed *et al,* 2020), Gaza (Al-Hindi *et al,* 2023), Jordan (Himsawi *et al,* 2019), Kuwait (Al-Awadhi *et al,* 2021), Libya (Mohamed *et al,* 2014), Saudi Arabia (Al Malki and Ahmed, 2022), Sudan (Ahmed *et al,* 2021), Turkey (Tarladaçalışır *et al,* 2022), Yemen (Alghoury *et al,* 2010) and others.

While the domestic animals play a role in transmission of the zoonotic echinococcosis, other species use the wildlife cycles for its spreading (Woolsey and Miller, 2020). The two zoonotic *Echinococcus granulosus sensu lato* (s.l.) and *E. multilocularis* are risky to public health (Deplazes *et al*, 2017) forming cystic echinococcosis (CE) and the latter alveolar echinococcosis (AE) (Darabi *et al*, 2022). One million people globally are affected by CE, which has an estimated global impact of 184,000 disability-adjusted life years (Torgerson *et al*, 2015). Zoonotic hydatidosis often remain asymptomatic for years (Larrieu *et al*, 2019). But, before the cysts became large enough to cause symptoms in the affected organs resulting in morbidity and mortality (Belhassen-Garca *et al*, 2014).

Based on clinical symptoms and routine laboratory diagnosis, hydatidosis is more or less difficult to diagnose (Giri and Parija, 2012). Radiological imaging modalities and serodiagnosis are the two main diagnostic methods (Yoo *et al*, 2023). Computed tomography (CT) and ultrasonography (USG) are used to diagnose CE (Stojkovic *et al,* 2017). The most popular diagnostic tool among was USG. In order to identify antibodies against

E. granulosus, immunodiagnosis used various serological tests based on the whole hydatid cyst fluid (HCF) as an antigen (Jin *et al*, 2013; Iraqi, 2016). These tests' sensitivity and/or specificity restrict their ability to be used as diagnostic tools. One of the immunodiagnostic methods for identifying antibodies was accessible ELISA (Khalilpour *et al*, 2014; Wen *et al*, 2015).

The HCF is a combination made up of a wide variety of proteins with both parasite and host origins (Aziz *et al*, 2011; Wang *et al*, 2015; Garcia *et al*, 2018). The most prevalent and immunogenic HCF antigens are Antigen 5 (Ag5) and Antigen B (AgB), which have only partially studied for their involvement in the cestode's life cycle (Díaz *et al*, 2016). In the Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, Ag B, a thermostable lipoprotein, generates three subunits at 8 or 12, 16, & 20 or 24 kDa (Mamuti *et al*, 2006). Ag 5 is a thermolabile glycoprotein that exhibits 53 kDa and 64 kDa bands in SDS-PAGE in non-reducing conditions and 38 kDa and 24 kDa bands under reducing conditions (Sadjjadi *et al*, 2007).

This study aimed to examine the antigenicity of hydatid cyst fluid from different batches of cyst fluid of same host species and differences hosts by using ELISA to determine variations, and identifying characteristics of hydatid cyst fluid for accurate diagnosis.

Materials and Methods

Antigens collection and preparation: Sixteen crude HCF samples were aspirated from humans, sheep, gerbils, horses, and cow's hydatid cysts. Fluid-filled tubes were centrifuged at 2000g for two minutes, and the supernatant was then preserved at -20°C as hydatid fluid antigen. Many protoscolices were packed together in the sediment, which was then sonicated, rinsed with isotonic saline, and stored at -20°C as a crude protoscolex antigen. The cyst wall antigen was created by removing its laminated and germinal layers, homogenizing them, and kept at-20^{O}C (Aref *et al*, 2012). Bradford assay evaluated different samples' protein content (Bradford, 1976).

Serum collection: Antigen samples were tested using positive pooled human serum samples from patients with hydatid and negative pooled human serum samples from patients who had clinically negative results. Anti-human Alkaline Phosphatase Conjugate (Sigma Aldrich UK) was used as a detection antibody. Prior to conjugation, ELISA assessed the anti-human IgG's specificity. The conjugation is only specific for human IgG and human IgG, Fc fragment. ELISA is also used to assess the antibody-conjugate's cross-reactivity. Conjugate exhibited no reactivity with human IgG, Fab fragment, human light chains, human IgA, human IgM, mouse IgG, or rat IgG (Datasheet A9544 Sigma Aldrich UK).

Human echinococcosis in sera by ELISA: Pooled hydatid sheep antigen was coated on the micro-titration plates in 100μl of bicarbonate buffer (BCB) at 1:100 dilutions, and the plate was titrated down to 1:800 dilutions by doubling the dilution, using half the plate for positive sera and half the plate for negative sera. The plate's two wells had no antigen in them. The plate spent the night being incubated at 4°C while being covered in cling film. By soaking the plate in 0.1% TWEEN20-PBS (T-PBS) for 1 minute, the excess antibody was washed off the plate. The plate was then washed, 200μl of 0.3% T-PBS 5% milk solution was added, and the incubation period was 1 hour at room temperature. Plate was washed three times as before after incubation period. The positive and negative sera were added to all wells after the washing procedures, with the exception of the blanks. 100μl of positive human serum was added to the top of the first column and diluted 1:100 in 0.3% T-PBS 5% milk solution before being titrated across the plate in a doubling dilution to 1:204, 800 dilutions. Cling film was put over the plate, which was then let to sit at room temperature (RT) for an hour. After washing plate,

the alkaline phosphatase conjugated anti-human IgG (Sigma Aldrich, Abingdon, UK) was added to all wells except blanks and diluted 1:10, 0000 in 0.3% T-PBS (no milk). The plate was then incubated for 1 hour at room temperature. Diethanolamine buffer pH 9.8 and PNPP tablets (Sigma Aldrich, UK) were added to all wells, including the blanks, after being cleaned as before. Plate was allowed to develop for 10 minutes on the bench before absorbance measurements at 405 nm were taken using an Ascent plate reader from Thermo-fisher Scientific UK. Serum concentration was graphically displayed against positive optical density readings to find the best dilutions (in terms of reactivity) to use for the next assays. Using the negative optical density values, this procedure was performed again (Muoz *et al*, 1986). Using human positive and negative serum samples at a ratio of 1:100 and HCF samples with protein concentrations of either 5 g/ml or 1 g/ml, ELISA was carried out in accordance with the optimum procedure.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) & immunobloting: According to molecular weight, proteins from hydatid cyst fluid samples were separated using SDS-PAGE. Using pre-stained molecular weights (MW) protein marker 16-270 kDa, each of the hydatid cyst produced antigens was independently fractionated in four 12% SDS-PAGE (GeneDirex, USA). One gel was stained with Coomassie Brilliant Blue R-250 dye following SDS-PAGE, and the results were analyzed using a gel documentation system (Laemmli, 1970). Others were blotted onto 0.45 nitrocellulose membranes (Towbin, 1979). In a nutshell, positive naturally infected sera at dilution 1:50 in TBS/0.5% bovine serum albumin (BSA) were incubated overnight against all prepared antigens on blocked membranes with 1% dry skimmed milk/Tris-buffered saline (TBS). Protein-A peroxidase conjugate, diluted to 1:2500 in 0.5% BSA/TBS solution, was used to probe the nitrocellulose sheets for one hour.

To generate the immunogenic bands, substrate solution (1-chloronaphthol, Sigma-Aldrich, USA) was utilized for 20min. Then, the nitrocellulose membranes were captured on camera and analyzed using the Invitrogen I-blot system according to a standard manufacturer's methodology.

Results

ELISA showed that 1:100 dilutions of both serum and antigen were the best for use in subsequent experiments because they produced the highest levels of reactivity. Negative sera didn't exhibit a high absorbance for any antigen or sera concentration indicating a lack of reactivity clearly variation in reactivity between the various intermediate hosts investigated as well as within the same host species used for the two different dilution sets. This variation was more pronounced for sheep species, where reactivity for sheep 10 is nearly twice that of sheep 7. Sample human 2 didn't show any difference between positive and negative sera, indicated that the antigen was bonded to the anti-human IgG directly in absence of sera.

A standard curve of known protein concentration determined total protein content in each fluid sample. Standard curve represented graphically as an O.D. measurement at 620nm vs. protein concentration in mg/ml. Total protein concentration was utilized to generate an HCF dilution with fixed protein concentrations of 1g/ml & 5g/ml to be used in ELISA against positive and negative human sera. Due to the variance among proteins found in HCF, there were differences in reactivity. Each sample of cyst fluid used was processed on an SDS-PAGE gel under reducing conditions to separate the proteins.

SDS- PAGE evaluated the quality of protein alterations. At molecular weights of 148, 98, 64, 55, 50, 46, 45, 34, 31, 26, 24, & 8 kDa, were 12 distinct protein bands, with 8 kDa subunit one. Big band 148 kDa in sample Ger2 may indicate that a protein was not completely reduced, or represented an unknown protein. For three species, She 10, Hor, and Ger1, an 8 kDa band were detected.

Since She 10 and Hor both displayed high absorbance values of 0.6305 and 0.4954 in ELISA test at 1:100 dilutions, the existence of this antigen was connected to strong reactivity observed by ELISA for these two substances. Cyst fluid sample, despite the presence of 8kDa subunit in Ger1 species, didn't exhibit a particularly high absorbance in the ELISA test (0.4229). All samples have a 64KDa protein band that was extremely faint than the three She1, She9, or Hum1.

Sheep showed only two bands 55KDa. Four of HCF samples contained a band of 31KDa, indicating that this protein may be crucial for HCF different types. But, many sheep HCF samples contained minor, flimsy bands: 34KDa, 41KDa, 45KDa, & 46KDa. A faintly weak band at 24KDa was identified in one sheep that was considered as a minor, faint band, established that under reducing conditions, AgB & Ag5 separate into 24Kda subunits.

Nitrocellulose membrane was effecti-vely blotted easier in assaying proteins. A positive band at 98 kDa was found in all samples She 2, 3, 5, 6, and Ger2, a positive band at 64 kDa was present in She1 and She7, and a positive band at 50 kDa was in only She5. The presence of a 98KDa band on the SDS-PAGE for all samples but only specific samples in the immunoblot suggests poor transfer. However, this could also be due to low protein levels and thus low reactivity or that not all 98KDa bands are the same protein and only certain ones are positive in the serum and thus detected during immunoblot.

Details were given in tables (1 & 2) and figures (1, 2, 3, 4, 5 & 6)

Table 1: Summary of SDS-PAGE results from different intermediate host species and same species.

Sample	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
She1	98	(poss 64 faint)	-	-	-	-
She 2	98	64	50	34	26	-
She3	98	64	50	41	-	-
She4	98	84	64	46	-	-
She5	98	64	50	45	-	-
She6	98	64	55	-	-	-
She7	98	64	-	-	-	-
She8	98	64	55	50	34	24
She9	98	(poss 64 faint)	-	-	-	-
She10	98	64	50	31	8	-
Hum1	98	(poss 64 faint)	-	-	-	-
Hum2	98	64	-	-	-	-
Ger1	98	64	50	31	8	-
Ger2	148	98	64	50	31	-
Bov	98	64	-	-	-	-
Hor	98	64	50	31	8	-

Table 2: Immunoblot results from different intermediate host species and same species.

Sample	Band 1 (KDa)	Band 2 (KDa)	Band 3 (KDa)
She1	-	-	-
She 2	98	64	
She3	98	64	-
She4	64	-	-
She5	98	64	50
She6	98	64	-
She7	-	-	-
She8	64	-	-
She9	64	-	-
She10	64	-	-
Hum1	64	-	-
Hum2	64	-	-
Ger1	64	-	-
Ger2	98	64	-
Bov	64	-	-
Hor	64	-	-

Discussion

Generally speaking, larvae of *Echinococcus granulosus* causing hydatidosis was apparently a problem since the ancient Egyptian time as hydatid cysts were detected in some Egyptian mummies (Tapp, 1984), however, EC still remains one of the health problems worldwide (WHO, 2021). *Echinococcus granulosus* serological and immunological assays helped in initial diagnosis of infection in humans and animals, and post-surgery and/or medical treatment (Torgerson and Deplazes, 2009), but with different sensitivity and specificity that the immunodiagnosis remains a major problem (Ahn *et al*, 2015).

In the present study, initially HCF and serum concentrations were examined to ascertain the best ELISA reactivity. It was decided to use HCF and serum concentrations of 1:100 and 1:400 for all samples. There was significant variation in OD readings at these concentrations, pointing to different reactivity's across samples taken from the same species and those taken from various hosts. Shirazi *et al*. (2022), who utilized several dilutions to measure each well's optical absorbance, a concentration of 0.5g/ml and a serum dilution of 1:400 was the ideal one.

The present study showed that the total protein level in the HCF samples was available for the various intermediate hosts with differences between the hosts as well as within the same species. However, without detailed information of the HCF samples, it was challenged to infer the source(s) of the results changes. Total protein concentration variations could result from a variety of factors, including the intermediate host's immunological profile, cyst stage, location, fertility, size, and total protein concentration. It is impossible to make assumptions about the parameters that contributed to the obtained results for the HCF samples under study in the absence of any information on these parameters. This agreed with Mero and Abdullah (2012), they claimed that there was no variation in the protein concentration of the HCF between cysts taken from the liver and lung of sheep. This might be brought on by variations in study methodologies or in the classification of cysts and infection stages (Zhang *et al*, 2008). Due to the possibility of proteins being digested by the parasite, the study's protein concentration was low. This may be the case because the parasitic infection may cause changes in metabolism to aid the parasite's survival, whether in terms of the persistence or prosperity within their hosts (Shea-Donohue *et al*, 2017). Curiously, heterogeneity in relativities was still found between samples even when protein concentration was standardized at 1μg/ml or 5μg/ml. Although, there was no difference between positive and negative serum at1μg/ml, the variation decreased when protein concentration was lowest, therefore this could be because of the low concentration. These results indicated that the difference in reactivity between HCF samples was not primarily due to protein content. Nevertheless, the variation observed might be brought on by specific proteins present in some samples, which might increase their susceptibility to detection by human serum and, consequently, their reactivity.

In the present study, SDS-PAGE profiles showed that albumin, a protein with a molecular weight of 98 KDa, was present in all of the HCF samples. This protein of 50 KDa was detected in SDS-PAGE profiles of horse samples, one gerbil, and five sheep samples, which band might be calreticulin Eg CRT because this sized protein was found in the germinal layers and protoscoleces of *E. granulosus* hydatid cysts (Cabezon *et al*, 2008). This agreed with Taherkani *et al*. (2007), they reported that the band (55KDa) in sheep HCF increased the possibility to be a species-specific molecule, that that this protein is a sheep host protein rather than a protein associated with a parasite because it did not appear in the immunoblotting test. Also, it was claimed this protein after transfer to nitrocellulose membrane and application of Anti-EgCRT, which possibility be crucial used for the early diagnosis of hyda-

tid disease, particularly given that in the current study, all samples that tested positive for the 50KDa band by SDS-PAGE also exhibited significant ELISA reactivity's. It's interesting to observe the existence of a protein at 55 kDa, a distinct band for some sheep HCF (Saleh *et al,* 2021).

In the present study, a protein with the 64KDa molecular weight was documented. This agreed with Taherkani *et al*, (2007) who reported the presence of a 66KDa protein. It is possible that this protein also exists but has a slightly different migratory pattern. The protein may have undergone post-translational modification as a result, or the buffers employed in the SDS-PAGE procedure might have slightly changed. So, the 64KDa subunit's appearance on immunoblot may be proof that it was a parasite-specific protein rather than a host-specific one. This hypothesis is confirmed by the identification of this antigenic subunit in all HCF samples from the investigated hosts. Consequently, this protein may be used to evaluate human CE.

In the present study, the three HCF samples analysed, She 10, Hour, and Ger. 1, showed that the antigenic subunits of 8 Kodak by SDS-PAGE, but not by immunoblotingtest. This agreed with Monteiro *et al*, (2012), who suggested that this subunit in particular to antigen B, must be respected, nonetheless, the antigenic profile showed that the bands of this subunit very faintly recognized. They added that there would be inadequate protein transfer onto a blot the protein, which was not detected as only one HCF for a particular host, She8, contained the antigenic subunit of 24KDa. Also, Mariconti *et al*, (2014) reported that the band (24 kDa) recognized in CE patients' sera corresponded to the primary HCF antigens, AgB, and the component (34 KDa) belonged to Ag5.

In the present study, the subunits 41KDa, 45KDa, and 46KDa were identified. This agreed with Al-Olayan and Helmy (2012), who claimed that the 45–40 kDa band cluster was one of the most often identified bands by CE cases, as not all of them detected on the SDS-PAGE were detected positively on immunoblot. There were a number of possible causes for this, including: absence of protein in the combined human serum, no reactivity to HCF antigens was anticipated, or its low concentration a fact that it was only present in species-specific HCF, but protein didn't transfer well onto nitrocellulose membranes.

Conclusion

The SDS-PAGE profiles showed that the common proteins with molecular weights were 98KDa & 64KDa. The immunoblotting tests showed that 64KDa protein possess potential to be used in human evaluation CE. But, lack of positive by immunoblot showed more exploration is a must.

The 50KDa protein in 50% of the samples suggested that it may be an essential detecting protein. Based on the available data and taking into account the study's limitations, a number of considerations were done to accurately identify cystic disease by serology.

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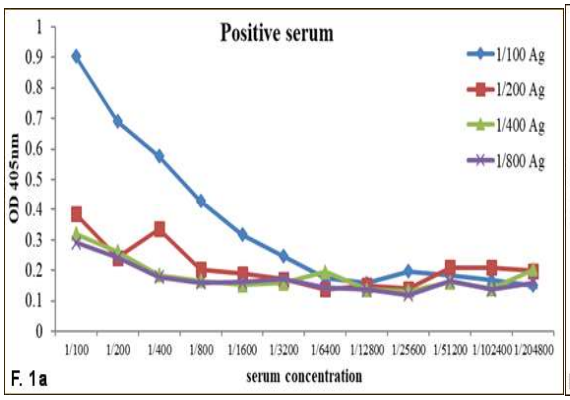
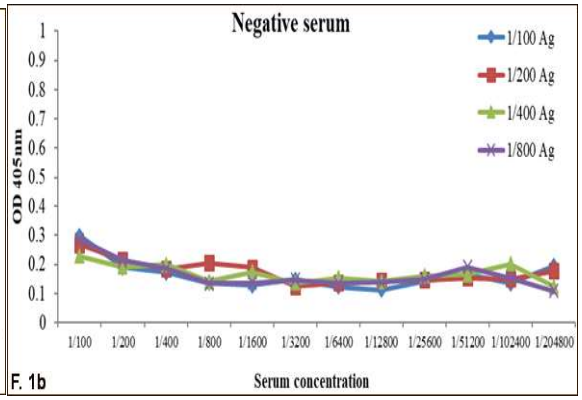
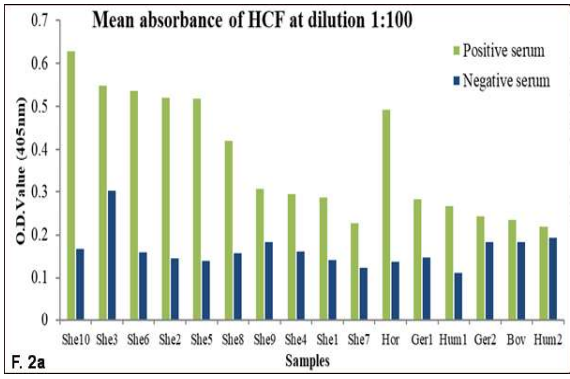
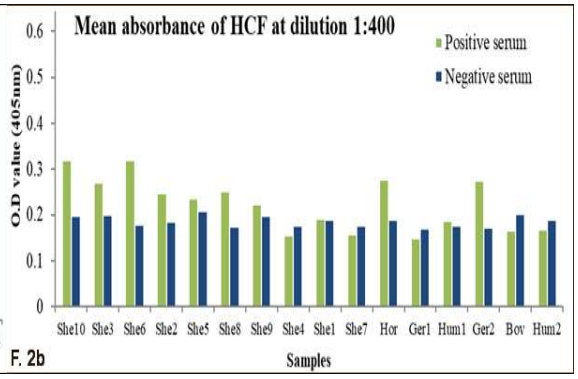
Explanation of figures

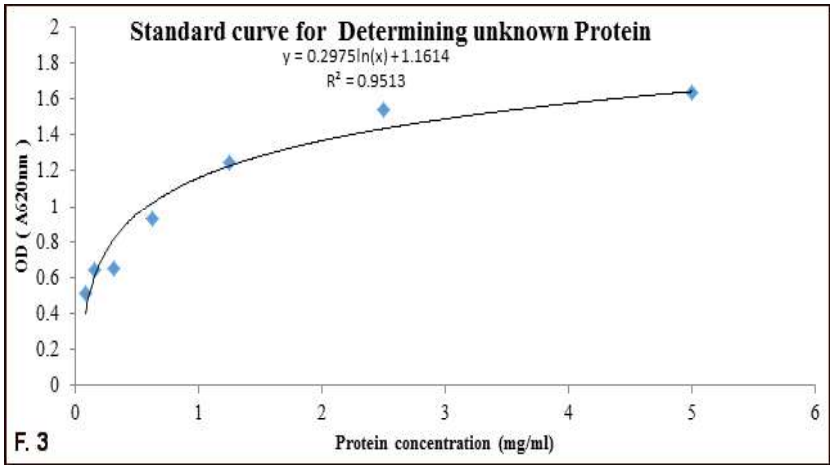
Fig. 1: Graph displaying absorbance measurements, 1A: positive serum vs. antigen; 1B: negative serum vs. antigen.
Fig. 2: Graphical representation of ELISA optical densities of serum samples, A: HCF diluted at 1:100; B: HCF at a 1:400 dilution.
Fig. 3: Standard curve obtained from Bradford protein assay.
Fig. 4: Graphical representation of ELISA optical densities of serum samples, A: standard protein concentration of 1µg/ml; B: standard protein concentration of 5µg/ml.
Fig. 5: Image of SDS-PAGE gel illustrating separation of protein bands from different intermediate hosts (lane 11-16) and same species (lane 1-10). MW of bands indicated in KDa.
Fig. 6: Analysis of Immunoblot probed with positive serum; She1-She8.

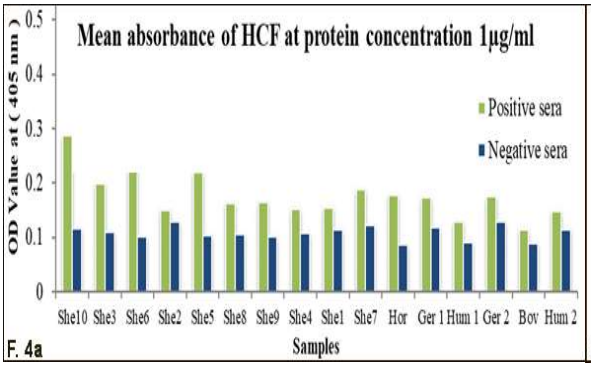


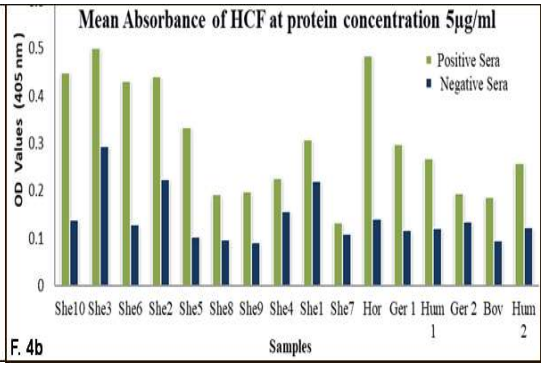


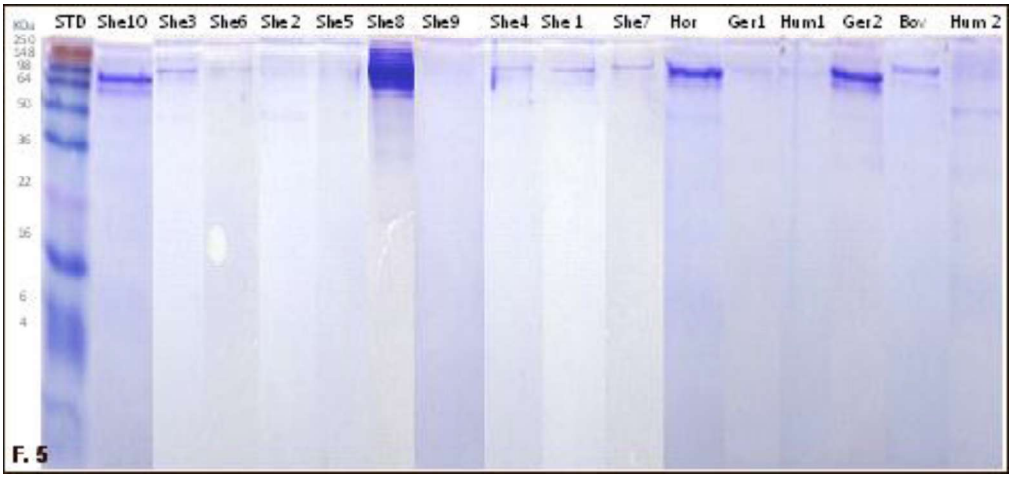







Mean absorbance of HCF at protein concentration 1µg/ml
OD Value at (405 nm)
0.5
0.4
0.3
0.2
0.1
0
Positive sera
Negative sera
She10 She3 She6 She2 She5 She8 She9 She4 She1 She7 Hor Ger 1 Hum 1 Ger 2 Bov Hum 2
Samples
F. 4a


Mean Absorbance of HCF at protein concentration 5µg/ml
OD Values (405 nm)
0.5
0.4
0.3
0.2
0.1
0
Positive Sera
Negative Sera
She10 She3 She6 She2 She5 She8 She9 She4 She1 She7 Hor Ger 1 Hum 1 Ger 2 Bov Hum 2
Samples
F. 4b


KDa
STD
She10
She3
She6
She2
She5
She8
She9
She4
She1
She7
Hor
Ger1
Hum1
Ger2
Bov
Hum 2
F. 5


F. 6