

Saliva as an Easy Specimen for Diagnosis of Human and Animal Fascioliosis

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In the present study saliva was evaluated for detection of human and animal fascioliosis. Both saliva and serum samples were collected from 12 *Fasciola* infected patients, 17 cattles and 20 sheep harboring *Fasciola* eggs only in their faeces. Samples from negative non-infected hosts were also collected. Experimental infection by *F. gigantica* in rabbits was carried out for determination of the first time appearance of anti-*F. gigantica* antibodies (AFAb) and circulating *F. gigantica* antigen (CFAg). This was carried out by indirect and sandwich ELISA using purified antigen (26-28 KD) and monoclonal antibodies. AFAb were detected in saliva of naturally infected patients, cattles and sheep, the sensitivity of the assays reached 66.6%, 64.7% and 65% respectively, while the sensitivity using serum samples was 91.66, 94.11 & 100% respectively. In the contrary AFAb in saliva was more specific (100%) than that in serum as it was 100%, 92.0% and 96.0% in humans, cattle and sheep respectively. CFAg showed higher sensitivity in diagnosis using saliva in comparison with AFAb as it was 83.3, 76.47 & 85% in patients, cattles & sheep respectively. Similarly, the specificity of CFAg in saliva was higher than that recorded using serum samples as it was 100%, 96.0% and 96.0% in the three groups respectively. AFAb and CFAg were detected in serum of experimentally *F. gigantica* infected rabbits at the end of the first week post infection, and in saliva at the 15th and 18th day post infection. These data introduce saliva as an easily collected sample that can be used for diagnosis of zoonotic fascioliosis.

Fascioliosis is an emerging/re-emerging vector-borne zoonotic disease that infects approximately 17 million people around the world. The disease is regarded as one of the most important parasitic infections causing major economic and health problems affecting ruminants, other animals and man (De Almeida *et al.*, 2007). Since *Fasciola* eggs appear in feces after 76-100 days post infection (Hillyer, 1988). The needs for accurate early method for diagnosis still have special interest in development of sustainable strategies for controlling the disease. In this respect several serological techniques were adopted by different authors (Hillyer *et al.*, 1988, Mohamed *et al.*, 2004 & Espinoza *et al.*, 2005) for improving accuracy of techniques depending on detection of anti-*Fasciola* Ab. in sera using purified protein fractions. These tests are of limited diagnostic values as antibodies remain at detectable levels after cure, moreover, serological tests are negative during the first 1-4 weeks post infection. Besides, collection of blood

samples and separation of serum still need some precautions as well as specific equipments. Detection of circulating *Fasciola* antigens is an alternative diagnostic procedure. This was found in patient's sera (Espino & Finlay, 1994), in sheep (Guobadia & Fagbemi, 1996), as well as in cattles (Dumenigo *et al.*, 1996).

In the present study new approach for *Fasciola* diagnosis was initiated depending on detection of AFAb and CFAg in saliva rather than in serum. Saliva is considered as a mirror of the body which can reflect virtually the entire spectrum of normal and disease status. These include tissue levels of natural substances and large variety of molecules introduced for therapeutic purposes, hormonal status; immunological status nutritional and metabolic influences (Mandel, 1993). Antibodies detection in saliva can be used for diagnosis of many diseases, but the level of antibodies are usually higher in serum than in saliva (Sreebny, 2000). As a clinical tool, saliva has many advantages over serum.

Saliva is easy to collect, store, and ship, and it can be obtained at low cost in sufficient quantities for analysis. For patients, the non-invasive collection techniques dramatically reduce anxiety and discomfort and simplify procurement of repeated samples for longitudinal monitoring over time. For professionals, saliva collection is safer than blood tests, which could expose health-care providers to HIV or hepatitis virus. Saliva is also easier to handle for diagnostic procedures, since it does not clot, thus lessening the manipulations required. Saliva-based diagnostics is therefore more accessible, accurate, less expensive, and presents less risk to the patient than current methodologies (Li *et al.*, 2005). In this aspect Pinho *et al.* (1998), used saliva instead of serum for diagnosis of *Trypanosoma cruzi*., While Santos *et al.* (2000), used it for diagnosis of *Schistosoma mansoni* infection and Singh *et al.* (2005), used it for detection of anti-*Toxoplasma* IgG and IgM in diseased patients.

The aim of the present study was to investigate the value of saliva in comparison with sera for diagnosis of *Fasciola* infection. AFAb and CFAg were diagnosed in selected groups of naturally infected patients, cattles, and sheep as well as in *F. gigantica* experimentally infected rabbits. Specific anti-*Fasciola* monoclonal anti-body via antigen capture enzyme linked immunosorbant assay (ELISA) was used for diagnosis.

Material and Methods

Collection of blood and saliva samples

a-From Patients

According to Sobaniec *et al.* (2007), blood samples and un-stimulated saliva were taken at the same time in the morning from 12 fasting *Fasciola* infected patients and 25 healthy individuals. These patients were of 20-40 years old and all demonstrated infection by 2-3 *Fasciola* egg/gram stool. They were selected from patients referred to the outpatient clinic of the Research Institute of Tropical Medicine-Cairo.

Blood samples were taken using vein puncture needle, while saliva was collected in sterile screw capped wide mouth plastic cups. In the laboratory, saliva samples were drawn by needless syringes then transferred to test tubes containing two times volume of phosphate buffer saline (PBS). The samples were filtered using disposable syringe-less filter glass microfiber, 0.45mm (Fiser, catalog no. 09-919) to remove the undesirable particulate matters.

b- From animals

In private farm at El-Fayoum Governorate, 17 cattles and 20 sheep harboring *Fasciola* eggs only in their feces were selected for the study, after repeated fecal examination using fluke finder (Welch *et al.*, 1987). Additional, 25 healthy cattle and sheep free from any parasitic infection were also selected as control. Jugular blood was collected and left to clot, and then serum samples were separated.

Collection of saliva from animals

A suitable piece of cotton was firmly warped around the proximal end of toothed forceps. Early in the morning, water was introduced to animal, after they drink; the cotton piece was inserted under the tongue after control of their mouth. The forceps was kept under the tongue for 3-5 min. before it was drawn out. The cotton piece was transferred directly to wide mouth screw capped plastic cups containing 20 ml of phosphate buffer saline (PBS). The cotton piece was immersed in the fluid. The cup was firmly covered and transferred to laboratory in ice box. After good mixing of the cotton piece with the buffer, it was squeezed enough, and transferred from the solution. The final volume was estimated and the degree of saliva dilution was calculated.

Production and separation of *F. gigantica* antigen

F. gigantica ES antigens were extracted from living flukes collected from freshly condemned buffalo's livers according to River Marrero *et al.*, (1988). The antigens were fractionated using SDS-PAGE with the aid of modified 1.5mm, three wells comb (one wide for the sample and 2 small for the molecular weight protein standards, MW) according to Laemmli (1970). Ten percent polyacrylamide gel slabs in Tris-glycine buffer, pH 8.3 (Sigma chemical Co.), low and high MW standard (Sigma SDS-100B) were employed. Longitudinal gel strip containing MW standard and part from *F. gigantica* fractionated Ag was cut out, fixed and stained by Commasi-blue stain (Tsai & Frasch, 1982). After identification to the level of each protein fraction in the gel strip, transverse gel strips containing the 26-28 KD protein fraction (El-Bahy *et al.*, 1992), was cut out horizontally across the whole gel. The

protein content was extracted from the selected gel strip by electro-elution. Elution tube membrane 6-8 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060) in PBS (pH 7.4) was used in Bio-Rad elution unit at 10V, 100mA over night at 4 °C according to Katrak *et al.* (1992). The protein content was increased by dehydration using polyethyleneglycol in the above tube membrane. The protein content of the eluted concentrated antigens was measured (Lowry *et al.*, 1951) then stored at -70 °C until use.

Rabbit hyper-immune serum (RHI)

F. gigantica ES antigen (FESAg.) was used for vaccination of rabbit according to Osorio *et al.* (1998). Rabbits were subcutaneously injected with 8 mg of protein emulsified in Freund's complete adjuvant. Three subsequent injections in Freund's incomplete adjuvant were given at 1-week intervals. Antibody titer was roughly estimated using agar precipitation test before bleeding of the vaccinated rabbits at 7 days post the last injection. The collected sera were stored at -20°C until used.

Monoclonal antibody

Monoclonal antibody M2 D5/D5F10 (F10) specific for the 26-28 KD *F. hepatica* (MAb F10) was kindly supplied via personal communications from the Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA.

AFAb and CFAg in saliva and serum of experimentally infected rabbits

In order to determine the first time of appearance of AFAb and CFAg in saliva and serum, ten, (1.8-2) kg white New-Zealand rabbits were allocated into two groups each of five. One was kept as control, while the others were experimentally orally infected by 25 *F. gigantica* encysted metacercariae (EMC) of ten days old. The EMC were obtained after light exposure to naturally infected *Lymnaea caillaudi* snails. The snails were kept according to El-bahy *et al.* (1992), in clean de-chlorinated water in cellophane lined Petri-dish. The shed cercariae were left to encyst naturally on the cellophane, and then they were collected and kept in refrigerator under water surface till use. The infected rabbits were kept under observation, fed dry herbage during the period of experiment (2 months) at the Research Institute of Ophthalmology animal house. Water was provided at labium. Blood samples were collected from ear vein. Saliva samples were collected as before using suitable cotton pieces. The samples were collected every 3 days post infection for two successive months.

Enzyme linked immunosorbent assay technique (ELISA)

The test was done as described by Espino *et al.* (1997). High binding flat-bottom 96-wells micrometer plates were used. Each plate was coated with the eluted concentrated 26-28 KD antigens (2 µg / ml in coating buffer) which adjusted after checkerboard titration. After overnight incubation at 4°C, the plate was washed then blocked with 0.1% bovine serum albumin fraction V (Sigma), 100µl/well. Saliva was tested at 1:2 dilutions in PBS, while serum samples were used at 1:50 dilution. RHI sera was used as reference positive control. Horseradish peroxidase conjugated (HRPC) rabbit anti-bovine, anti- human and anti-sheep IgG and IgM, {Heavy and light chains (H & L)} (Sigma) as well as HRPC goat anti-rabbit IgG (whole molecule) (Sigma) were used at 1:2000 dilution. Orthophenylenediamidine (OPD) was added at a concentration of 340 µg / ml substrate buffer. Absorbency was read at 490 nm using full automated Titerteck multiskan ELISA reader. The cut off points were set as 2SD above the mean of control negative samples.

Two-antibodies Sandwich ELISA (Antigen capture assay)

Fasciola circulating antigens in saliva and serum were detected using two anti-bodies sandwich ELISA according to Osori *et al.* (1998). Using checkerboard titration the optimal dilutions of the sera and enzyme conjugate were determined. High binding flat-bottom 96-wells micrometer plates were coated with 100 µl / well of MAb (20 µg / ml) diluted in 0.1 m carbonate buffer (pH 9.6). The plates were incubated overnight at 4°C. After washing two replicate of each serum (1:50) and saliva (1:2) samples were incubated for 3 hours at 37° C. After washing 100 µl / well of rabbit (HIS) previously prepared versus FESAg (20 µg / ml) diluted in PBS and incubated at room temperature for 2 hours. After washing, 100 µl of HRPC goat anti-rabbit IgG, diluted 1:1000 with blocking buffer was added to each well and incubated for 1 hour. After washing 100 µl of the OPD substrate was added to each well. The reaction was allowed to proceed for 15 minutes at room temperature in the dark and was stopped by addition of 50 µl / well of 1 M H₂SO₄. Color changes were measured as before. Validity of the test was estimated according to Sadjjadi *et al.* (2007) by calculating the mean of sensitivity and specificity.

Statistical analysis

The slope of regression "b" of the log values response titer curve were obtained from respective standard preparation calculated according to Spiegel (1981). The concentration of the tested samples was calculated by

linear equation of Snedecor (1971), all values were presented as the mean + SE and the significant differences were determined by analysis of variance.

Results

Indirect ELISA was used to diagnose anti-*Fasciola* antibodies (AFAb) both in saliva and serum samples. The data in (Table 1) evidenced presence of AFAb in serum as well as in 1:2 diluted saliva of *Fasciola* infected humans and animals. Generally, AFAb in saliva appear less sensitive but high specific for diagnosis of infection in comparison with that in serum. The sensitivity of the test is

high using serum (91.66, 94.11 & 100%) in human, cattle and sheep respectively. The sensitivity using saliva was (66.6, 64.7 & 65%) in the same groups respectively.

Examination of negative samples revealed 2 cattles and one sheep reacted falsely with serum. This decrease the specificity of AFAb to (92% & 96%) in cattles and sheep serum samples respectively, while absolute specificity (100%) was recorded using human sera. In the same time absolute specificity (100%) was recorded in all cases using saliva.

Table 1. Anti-*Fasciola* antibodies (AFAb) in saliva and serum samples from infected humans and animals

Examined samples	No. examined	Serum samples				Saliva samples				Validity (%)
		No. +Ve	Sensitivity %	Specificity %	Validity (%)	No. +Ve	Sensitivity %	Specificity %	Validity (%)	
Human	<i>Fasciola</i> infected	12	11	91.66	95.83	8	66.66	100	83.3	
	Control (- Ve)	25	0.0	100		0				
Cattle	<i>Fasciola</i> infected	17	16	94.11	93.06	11	64.7	100	82.4	
	Control (- Ve)	25	2	92.0		0				
Sheep	<i>Fasciola</i> infected	20	20	100	98	13	65	100	82.5	
	Control (- Ve)	25	1	96.0		0				

Validity = mean sensitivity + mean specificity/2

The diagnostic ability of saliva was improved using antigen capture ELISA (Table 2). The sensitivity of CFAg in saliva samples was increased up to 83.33, 76.47 & 85% in human, cattles and sheep respectively. In the same time, absolute diagnostic sensitivity (100%) was recorded for CFAg in serum of all cases.

Concerning specificity of these antigens in saliva, high specificity was recorded for CFAg in saliva in comparison with that in serum samples. It was 96% in cattles' and sheep saliva corresponding to 92.0% in serum of the same animals. Specificity was 100% in case of human sample using saliva or serum samples.

Table 2. *Fasciola* circulating antigen (CFAg) ($\mu\text{g}/\text{ml}$) in saliva and serum samples from infected humans and animals

Examined samples	No. examined	Serum samples				Saliva samples			
		No. +Ve	Sensitivity %	Specificity %	Validity (%)	No. +Ve	Sensitivity %	Specificity %	Validity (%)
Human	<i>Fasciola</i> infected	12	12	100	100	10	83.33	100	91.66
	Control (- Ve)	25	0.0	100		0	100		
Cattle	<i>Fasciola</i> infected	17	17	100	96	13	76.47	96.0	86.24
	Control (- Ve)	25	2	92.0		1	96.0		
Sheep	<i>Fasciola</i> infected	20	20	100	96	17	85.0	96.0	90.5
	Control (- Ve)	25	2	92.0		1	96.0		

After experimental infection for the two groups of rabbits, examination of saliva and serum was performed. The data as in (Table 3) evidenced that AFAb and CFAg could be diagnosed in serum at the 7th day post infection (d.p.i), while CFAg could be detected in saliva at the 15th (d.p.i). Anti-*Fasciola* Ab was diagnosed in saliva at the 18th (dpi). The obtained results cleared the presence of significant difference ($P < 0.05$)

for the calculated ELISA values between saliva and serum. This difference still recorded till day 21st (d.p.i.) for CFAg and day 27th (d.p.i.) for AFAb as in (Table 3). From the above days inward, no significant difference as could be detected ($P < 0.05$) between the mean ELISA values reflecting values of AFAb or CFAg till end of the experiment.

Table 3. Kinetics of appearance of anti-*Fasciola* antibodies response ($\mu\text{g}/\text{ml}$) and antigens in serum and saliva of experimentally infected rabbits. (n=5)

Days post infection	Diagnosed Antibodies values		Diagnosed Antigen values	
	Saliva values	Serum values	Saliva values	Serum values
7 th dpi	--	0.62 ± 0.02	--	0.60 ± 0.06
15 th dpi	--	0.64 ± 0.02	0.45 ± 0.05 ^b	0.67 ± 0.08
18 th dpi	0.40 ± 0.07	0.81 ± 0.08	0.64 ± 0.06 ^b	0.86 ± 0.08
21 st dpi	0.52 ± 0.06	1.02 ± 0.09	0.71 ± 0.08 ^a	1.12 ± 0.19 ^a
24 th dpi	0.60 ± 0.08	1.22 ± 0.06	0.84 ± 0.09 ^b	1.11 ± 0.08 ^b
27 th dpi	0.88 ± 0.12 ^a	1.20 ± 0.09 ^a	0.77 ± 0.04	1.13 ± 0.05
31 st dpi	0.97 ± 0.16 ^b	1.12 ± 0.15 ^b	0.82 ± 0.04	1.18 ± 0.06
33 rd dpi	0.98 ± 0.11 ^c	1.02 ± 0.13 ^c	0.84 ± 0.09 ^c	1.12 ± 0.08 ^c
Negative control	0.24 ± 0.06	0.16 ± 0.03	0.3 ± 0.03	0.2 ± 0.05

Mean ELISA OD values have different litters (a-c) are significant different at $P < 0.05$
dpi = days post infection

Discussion

It has been reported that saliva-based diagnostics was more accessible, accurate, less expensive, and presents less risk to the patient than current methodologies (Li *et al.*, 2005). In other studies, saliva proved to be useful as a diagnostic tool rather than serum for some parasites including *Trypanosoma cruzi* (Pinho *et al.*, 1998), *Schistosoma mansoni* infection (Santos *et al.*, 2000) as well as *Toxoplasma gondii* infection (Singh, 2005). In addition, Sreebny, (2000) reported that antibodies detection in saliva, can be used for diagnosis of many diseases, but the level of antibodies are usually higher in serum than in saliva.

In the present study a trial was made to detect AFAb and CFAg in saliva of *F. gigantea* infected patients, cattles and sheep using indirect and sandwich ELISA. The study demonstrated the presence of the target antibodies at diagnostic levels in saliva of these infected cases. Generally, AFAb in saliva appears less sensitive but more specific for diagnosis of infection in comparison with that in serum using ELISA technique which has been recognized as sensitive and simple method for semi-quantitative determination of antibodies (Kaddah *et al.*, 1992) or antigen (Dumenigo & Mezo, 1999). This decrease in sensitivity and increase in specificity may be due to lower protein concentration in saliva (Cullum *et al.*, 2003). Low sensitivity of antibodies in saliva than that in serum has been previously recorded by Pinho *et al.* (1998), where they used saliva instead of serum for diagnosis of chronic infection by *Trypanosoma cruzi*, and were able to detect specific Ab in (1: 2) diluted saliva with a sensitivity 90.4% and specificity 95%. Also Santos *et al.* (2000), used saliva in comparison with serum in diagnosis of *Schistosoma mansoni* infection via ELISA technique. They mentioned that the sensitivity

of the test was 100% using serum samples while it was 94.6% using saliva. The specificity was 100% with serum and it was 91.7% using saliva, but it reached to 100% using oral transudation. They concluded that the use of oral fluids for diagnosis of *S. mansoni* infection was equivalent to sera with respect to test efficacy, offering an alternative to blood collection. Also Singh (2005) succeed in detection of anti-*Toxoplasma* IgG and IgM in saliva of patients suffering from immunodeficiency virus with sensitivity of 64% and 81.25% respectively, while specificity was 94.67% and 85.71% respectively. Concerning validity percentage (Sadjjadi *et al.*, 2007) using AFAb in diagnosis of infection after examination of saliva or serum, the validity is high (93.06 – 98%) using serum than that recorded using saliva (82.4 – 83.3%).

Different serological techniques have been used for the diagnosis of *Fasciola* infection depending on detection of antibodies in sera (De Almeida *et al.*, 2007). Antibodies in sera could be diagnosed from the 2-3 weeks post infection. This is considered as a good tool for early diagnosis in comparison with detection of eggs in feces. However, detection of antibodies is of no value in chronic infection or after treatment of the parasite as it could be present for more than 6 months post parasite eradication.

Determination of CFAg appears more specific and sensitive for diagnosis of infection in both saliva and serum in comparison with previous determination of AFAb. These facts came in agreement with Osorio (1998) as he mentioned that diagnosis of fascioliosis via detection to the parasite circulating antigen is considered to be more accurate and firmly associated with the vital presence of active parasite. The data clearly demonstrated that determination of CFAg improved the validity using saliva, as it varied

from 96 to 100% using serum samples and 86.24 to 91.66% using saliva samples.

In this study, the used Monoclonal Ab.(Mab F10) has been previously developed from the protein fraction (26 kd) of *F.hepatica*, and proved to have high specificity in capturing *Fasciola* antigens in infected faecal samples as early as 6 weeks post infection (Abdel-Rahman *et al.*, 1998) and (Dumenigo & Mezo, 1999). Experimental infection of rabbits by *F.gigantic* proved that AFAb and CFAg could be diagnosed in serum at the 7th day post infection (d.p.i), while the circulating *Fasciola* antigens could be detected in saliva at the 15th (d.p.i.).Anti-*Fasciola* Ab were diagnosed in saliva at the 18th (d.p.i). Diagnosis of CFAg in rabbit during the second week post infection was previously mentioned by Dumenigo *et al.* (2000) and Viyanant *et al.* (1997).

It was worthy to mention that the level of CFAg and AFAb in saliva and serum at 21st and 27th days post infection (d.p.i.) respectively were parallel. This relation supported the view that saliva samples could replace serum samples for diagnosis of *Fasciola* infection in human and animals after 3 weeks post infection. In the contrary, Fagbemi *et al.* (1997) detected CFAg from the 3rd (w. p. i.) In the author's opinion, the delayed diagnosis of CFAg and AFAb in saliva may be related to the dilution nature of saliva, which means that the antigen and antibodies could be present but in undetectable amount.

The authors agreed with Santos *et al.* (2000) and Singh, (2005) as the use of oral fluids for diagnosis of infection was equivalent to sera with respect to test efficacy. It has been reported by McDevitt (2007) that, saliva with its slimy mix of proteins, hormones and antibodies, can tell a lot about a person's health and it is much easier and less painful to collect than blood, but until now the

medical community lacks the technologies to perform large-scale salivary diagnostics.

In conclusion, the present study demonstrated the utility of saliva as an alternative specimen for diagnostic of *Fasciola* infection. This type of sample has more advantage over serum. The sensitivity and specificity of the used test could be improved in the future after characterization of special solvent that is more accurate in de-solving different contents of saliva.

Conflict of Interest

This work was done via the research activities of faculty staff in Department of Zoonoses, college of Veterinary Medicine Cairo University and Department of Parasitology, Research Institute of Ophthalmology, El-Giza province, Egypt. There is no conflict of interest with any other body or agencies in both departments concerning publication or future development of this work.

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