Assiut Vet. Med. J. Vol. 43 No. 85, April 2000

Dept. of Parasitology
Fac. Vet. Med. Cairo Univ.

SOME SEROLOGICAL AND BIOLOGICAL CONTROL STUDIES ON HETEROPHYES HETEROPHYES AND PROHEMISTOMUM VIVAX IN EXPERIMENTALLY INFECTED RATS

(With 2 Tables and 2 Figures)

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(Received at 3/2/2000)

بعض الدراسات السيرولوجية والتحكم الحيوي في فئران مصابة معمليا بديدان الهيتروفس هيتروفس والبروهيمستومم فيفاكس

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تم عمل عدوى معملية لفئران باستخدام الطور المعدي لديدان هيتروفس هيتروفس والبروهيمستومم فيفاكس كلا على حدة وذلك لتقييم مدى حساسية وتخصصية نوعين من المولدات المضادة في تشخيص الإصابة بالدودتين موضع الدراسة بواسطة استخدام الاختبار المناعيّ الانزيمي المترابط (الاليزا). وقد وضحت الدراسة أن المولد المضاد الخام المحضر من الدودة البالغة أكثر حساسية وتخصصية من المولد المضاد المحضر من الطور اليرقي المعدي (ميتا سركاريا المتحوصلة). وأيضا في هذة الدراسة تم اختبار ثلاث مواد حيوية طبيعية (زينجيبيراسى – لمياسى – أمرانثاسى) للقضاء على الطور اليرقي المعدي لدودتى هيتروفس هيتروفس والبروهيمستومم فيفاكس معمليا وعمليا. وقد بينت الدراسة أن مادة زينجيبيراسى هي الافضل حيث تم القضاء على حيوية الطور اليرقى المعدى للدودتين موضع الدراسة بنسبة 100% عند استخدامها بتركيز 7, ميكروجرام في حين أعطت مادتي اللمياس والأمرانثاسى نفس النتيجة بتركيز 1,4 ، 2,8 ميكروجرام بالتتابع.

SUMMARY

The present experiment was designed to infect rats separately with *Heterophyes heterophyes* and *Prohemistomum vivax,* to study the sensitivity and specificity of crude adult worms as well as encysted metacercarial (EMC) antigens of *H. heterophyes* and *P. vivax* by ELISA.

The present study demonstrated that the crude *H. heterophyes* and *P. vivax* antigens gave the highest sensitivity and specificity values than the EMC antigens by ELISA. Also, the present study revealed that *Curcuma longa* (Zingiberaceae) had a more potent lethal effect on *H. heterophyes* and *P. vivax* EMC than *Mentha longifolia* (Lamiaceae) and *Amaranthus spinosus* (Amaranthaceae). 0.7 mg *Curcuma longa* inhibited the viability of *H. heterophyes* and *P. vivax* EMC completely for one hour exposure. While, 1.4 and 2.8 mg *Mentha longifolia* and *Amaranthus spinosus*, respectively gave the same results in the same period of exposure.

Key words: *Heterophyes Heterophyes and Prohemistomum Vivax*

INTRODUCTION

Fishes are considered as one of the most valuable nutritive protein for man and animals, but they carry numerous parasites which may be harmful to man and other fish eating animals. (Taecher and Clark, 1978).

Human infection with several heterophyid parasites of fish were reported from Egypt (Wells and Randall, 1956; Tadros and El-Mokaddem, 1983 and Mahmoud et al., 1990) and from the Far East (Sindermann, 1989; Chai and Lee, 1990 and Yu et al., 1994). On the other hand, Nasr (1941) recovered *Prohemistomum vivax* from the small intestine of a dead man in Alexandria, Egypt.

Intensive researches were carried out to study this problem from the parasitological point (Mahdy, 1991), clinical findings (Lily et al., 1990; Ana et al., 1991 and Ahmed, 1996) and histopathological changes (Shalaby, 1993 and Mahdy et al., 1995), but very little attention has been given to the serodiagnosis of the two parasites under study.

Abdussalam et al. (1995) and Shaapan (1997) reported the different aspects for the control of fish-borne trematodes including prevention of contamination of fish with metacercariae (cut the life cycle) and inactivation of metacercariae in fish muscles by freezing and food processing methods.

Recently, several authors used some plant extracts as natural products with biological control properties (Mc Indoo, 1983 and Prakash et al., 1987). Hassanin et al. (1991) and Abdel Rahman et al. (1998) used some biological extracts for controlling of the

developmental stages of Fasciola gigantica, Toxocara vitulorum and some gastro-intestinal parasites.

The present study was carried out to study the evaluation of crude Heterophyes heterophyes and Prohemistomum vivax adult worms and their encysted metacercariae (EMC) antigens for the diagnosis of experimentally infected rats with the two parasites. Moreover, the present study was designed to determine the lethal effect of Mentha longifolia Linn. Huds. (Lamiaceae), Amaranthus spinosus Linn. (Amaranthaceae) and Curcuma longa Koening non-Linn. (Zingiberaceae) extracts on the encysted metacercariae of Heterophyes heterophyes and Prohemistomum vivax, as natural products recently used for human protection against the infection with the two parasites under study.

MATERIAL and METHODS

Collection of encysted metacercariae:

A total of 90 fresh water fish (*Oreochromis niloticus)* were collected from Fayoum markets, Egypt. Macroscopic examination was carried out to detect any changes in different organs and tissues of fish. The encysted metacercariae were examined from infected *Oreochromis niloticus*, according to Mahdy et al. (1995). Metacercariae of *Heterophyes heterophyes* and *Prohemistomum vivax* were identified and isolated by tissue digestion (Paperna, 1996 and Srisawangwong et al., 1997). The isolated metacercariae were separately collected and counted to determine the number of metacercariae which will be used for experimental infection to rats (Shalaby, 1982; Mahdy, 1991 and El-Reid, 1994).

Experimental infection:

Fourty five albino rats (100-150 gm B.W.) were chosen for experimental infection. The rats were fed with balanced ration and supplied with clean water, fecal samples were examined daily for three weeks to exclude any intestinal helminth infection. The rats were divided into 9 groups, each group containing 5 rats, the first two groups were infected orally with 500 freshly collected *H. heterophyes* and *P. vivax* EMC, separately. The following 6 groups were infected orally with 500 treated *H. heterophyes* and *P. vivax* EMC. EMC were treated with 0.7 mg *Mentha longifolia, Amaranthus spinosus* Linn and *Curcuma longa* Koeining extracts for one hour as recent method for control on the

two parasites. The ninth group was kept as non infected control. Meanwhile, the fecal samples were collected daily from the infected rats to detect the first appearance of the trematode eggs as well as the fecal samples from control rats were examined routinely throughout the time of experiment. The infected and control rats were kept for 30 days post infection (p.i.).

Serological study:

Preparation of Crude adult worms and Encysted metacercariae (EMC) antigens:

Heterophyes heterophyes, *Prohemistomum vivax* adults and their EMC were obtained separately from the small intestine of experimentally infected rats and from the muscles of naturally infected *Oreochromis niloticus*, respectively. The worms and EMC were washed separately in 0.01 M PBS, pH 7.4 and then individually homogenized for 15 minutes on ice using a tefflon glass homogenizer followed by sonication for 5 minutes to disrupt remaining intact worms or EMC. The sonicated materials were centrifuged at 20000 rpm for 45 minutes at 4 °C. The protein contents of each supernatant was determined using Lowry`s method (Lowry et al., 1951). The antigens were saved at –70 °C until use.

Serum samples:

Sera from both control and experimentally infected rats with *H. heterophyes* and *P. vivax* using untreated (natural) and treated EMC with 0.7 mg *Mentha longifolia, Amaranthus spinosus* and *Curcuma longa* were collected separately every three days for a period of 30 days.

Enzyme linked immunosorbent assay (ELISA):

ELISA was performed according to Iacona et al. (1980). The optimal reaction conditions regarding sensitizing antigen concentration, antibody and conjugate dilutions were chosen for use with micro-ELISA after preliminary checker board titration. In the present study, the optimum conditions were 5 µg/ ml coating buffer antigen concentration, 1:50 serum dilution, 1:1000 peroxidase labelled protein-A (Sigma company) as conjugate and ABTS solution, one component (Sigma) as substrate. All incubation steps were carried out at 37°C in a moist chamber. The positively threshold value was determined as double fold the mean cut off value of negative sera.

Effect of some biological extracts on the viability of *H. heterophyes* and *P. vivax* EMC:

H. heterophyes and *P. vivax* EMC were collected from naturally infected *Oreochromis niloticus*, washed twice by water and counted . Each 600 EMC were exposed separately to the different dilutions (0.17, 0.35, 0.7, 1.4, 2.8 mg) in *Mentha longifolia* Linn. Huds. (Lamiaceae) (Horse-mint), *Amaranthus spinosus* Linn. (Amaranthaceae) (Spiny) and *Curcuma longa* Koeining non-Linn. (Zingiberaceae) (Turmeric) extracts for one hour. After exposure to the different dilutions, the EMC were carefully washed in water and examined under the microscope to count the number of non viable EMC as protocol of Shapaan (1997). The obtained data were tabulated and subjected to statistical analysis based on the work of Polo-Pc (Robertson et al., 1980) to determine the LD50 values.

RESULTS

The sensitivity and specificity of crude *H. heterophyes* and EMC antigens using sera from experimentally infected rats with *H. heterophyes* and *P. vivax* separately were evaluated by ELISA. The analysis of the obtained ELISA data illustrated in Fig. (1) showed significant antibody levels in sera collected from experimentally infected rats with untreated *H. heterophyes* EMC comparing with negative control group at 9 and 18 days p.i. with crude adult and EMC antigens, respectively. The antibody levels increased gradually till 21 days p.i. and nearly remain in constant levels till 30 days p.i.(the end of experiment). Also, fig. (1) cleared that the sera collected from experimentally infected rats with treated *H. heterophyes* EMC has no significant antibody levels through all the time of experiment. The Optical density (O.D.) value in treated groups with *M. longifolia* and *A. spinosus* was slightly higher than the O.D. value of control sera but still within negative range, while the O.D. value in treated group with *C. longa* appeared as that of control group with crude and EMC *H. heterophyes* antigens. On the other hand, the two antigens were unable to detect the antibodies in serum samples from rats experimentally infected with *P. vivax* throughout the experiment.

Also, the sensitivity and specificity of crude *P. vivax* and EMC antigens using sera from experimentally infected rats with *P. vivax* and *H. heterophyes* were evaluated by ELISA. The obtained data cleared in Fig. (2), the antibody levels in sera collected from experimentally

infected rats with *P. vivax* comparing with negative control group at 6 and 15 days p.i. with crude and EMC antigens, respectively. The antibody levels increased gradually till 21 days p.i. and decreased gradually till 30 days p.i. (the end of experiment) but still within positive range. Also, Fig. (2) cleared that the sera collected from experimentally infected rats with treated *P. vivax* EMC has no significant antibody levels through all the time of experiment. The Optical density (O.D.) value in treated groups with *M. longifolia* and *A. spinosus* was slightly higher than the O.D. value of control sera but still within negative range, while the O.D. value in treated group with *C. longa* appeared as that of control group with crude and EMC *P. vivax* antigens. On contrast, the two antigens were unsuitable to detect the antibodies in sera from rats experimentally infected with *H. heterophyes* till the end of experiment.

The lethal effect of the three biological extracts at 5 concentrations on the viability of *H. heterophyes* and *P. vivax* EMC were studied in vitro. Table (1) showed *Mentha longifolia, Amaranthus spinosus* and *Curcuma longa* had lethal effect on EMC with LD_{50} values were 0.025, 0.04 and 0.023 mg for *H. heterophyes* and 0.036, 0.039 and 0.025 mg for *P. vivax*, respectively. The highest lethal effect on *H. heterophyes* and *P. vivax* EMC (100 % non-viable) was observed at 0.7, 1.4 and 2.8 mg for *Curcuma longa, Mentha longifolia* and *Amaranthus spinosus*, respectively.

The experimental infection of rats by treated *H. heterophyes* and *P. vivax* EMC with 0.7 mg *M. longifolia, A. spinosus* and *C. longa* comparing with non treated EMC as control were done to study the lethal effect of these three extracts on the viability of EMC in vivo. The fecal egg counts from experimentally infected rats were done every 3 days. Table (2) cleared the *H. heterophyes* and *P. vivax* eggs began to appear in the fecal samples from control group after 6 and 3 days p.i., respectively and gradually increased till reach to the maximum number at 12 and 6 days p.i. (793 and 245 eggs / gram), respectively. The same result was observed with rats experimentally infected with treated *H. heterophyes* and *P. vivax* EMC by 0.7 mg *M. longifolia* and *A. spinosus* but the number of eggs was very low (56, 80, 11 and 16 eggs / gram feces), respectively. On contrast, the eggs did not appeared in experimentally infected rats with treated *H. heterophyes* and *P. vivax* EMC by 0.7 mg *C. longa* through all the time of experiment.

DISCUSSION

In the present study, crude and EMC *H. heterophyes* antigens were used in an ELISA to capture specific antibodies against *H.heterophyes* in sera from experimentally infected rats. The antibody levels of collected sera appeared at 9 and 18 days p.i. with both crude and EMC antigens, respectively. These data were in agreement with these mentioned by El-Ganayni et al. (1989) who reported that IgG and IgE were detected in sera from human naturally infected with heterophyiasis by single radial immuno-diffusion plates. On contrary, Abou-Zakham et al. (1987) reported that the antibodies appeared in experimentally infected rats, 2 weeks p. i. This variation might be related to the test used for diagnosis, who used intra-dermal test, CIEF and IFAT, while the present study used ELISA. The ELISA was the most suitable technique for diagnosis of several helminth infections (Gamble and Graham, 1984 and Smith, 1987).

Also, the present study crude and EMC *P. vivax* antigens were used in an ELISA to capture specific antibodies against *P. vivax* in sera from experimentally infected rats. The antibody level of collected sera appeared at 6 and 15 days p.i. with both crude and EMC antigens, respectively. These data were recorded for the first time concerning the available literatures.

It is difficult to control *H. heterophyes* and *P. vivax* EMC by chemotherapy, salting and freezing methods. This is due to undesired side effects beside these methods are not completely killing the EMC. Fahmy et al., (1980) reported that 20 % salting or frozen the fish for two days was suitable method for controlling of *H. pumilio* and *P. vivax* EMC. Recently, several authors used the biological materials in the parasitological fields for controlling of different helminth infections instead of the application of anthelmintic compounds. In the present study, three biological extracts belonging to *M. longifolia, A. spinosus* and *C. longa* were used for controlling of *H. heterophyes* and *P. vivax* EMC. The present study revealed that the three biological extracts had lethal effect on EMC depending on the non-viability test (Shapaan, 1997). These effect (100 % degeneration of all EMC) was observed at 0.7, 1.4 and 2.8 mg for *C. longa*, *M. longifolia* and *A. spinosus*, respectively. The three biological materials were used for controlling of *H. heterophyes* and *P. vivax* EMC for the first time. These extracts were used previously by Mc Indoo (1983) and Parakash (1987) who applied

these materials for controlling of cotton aphid and larvae of grain moth. From the previous discussion, we can conclude that the *C. longa* is more effective on *H. heterophyes* and *P.vivax* EMC than *M.longifolia* and *A. spinosus*. These biological materials are safe for public health (Abdel Rahman et al., 1998).

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Table 1: The effect of three biological extracts (at five concentrations after one hour exposure) on the viability of *H. heterophyes* and *P. vivax* EMC in muscles of *Oreochromis niloticus*.

Concentration of extracts	**The percentage of non-viable *H. heterophyes* and *P. vivax* EMC**							
	M. longifolia		*A. spinosus*		*C. longa*		Conrtol EMC	
	H.h.	*P.v.*	*H.h.*	*P.v.*	*H.h.*	*P.v.*	*H.h.*	*P.v.*
0.17	70	73	80	78	85	87	0	0
0.35	73	75	85	82	90	90	0	0
0.7	75	80	87	83	100	100	0	0
1.4	100	100	95	90	100	100	0	0
2.8	100	100	100	100	100	100	0	0
LD50	*0.025*	*0.036*	*0.04*	*0.039*	*0.023*	*0.025*	-	-

Table 2: The lethal effect of 0.7 mg of three biological extracts on the development of *H. heterophyes* and *P. vivax* EMC in experimentally infected rats.

Days p.i.	Mean H. heterophyes and P. vivax egg count / gram rat feces							
	Heterophyes heterophyes				Prohemistomum vivax			
	M. longifolia	*A. spinosus*	*C. longa*	Conrtol	*M. longifolia*	*A. spinosus*	*C. longa*	Conrtol
0	0	0	0	0	0	0	0	0
3	0	0	0	0	11	20	0	168
6	30	40	0	180	35	63	0	245
9	50	77	0	680	20	33	0	205
12	56	80	0	793	16	11	0	118
15	22	39	0	245	5	9	0	61
18	7	12	0	83	0	0	0	0
21	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0

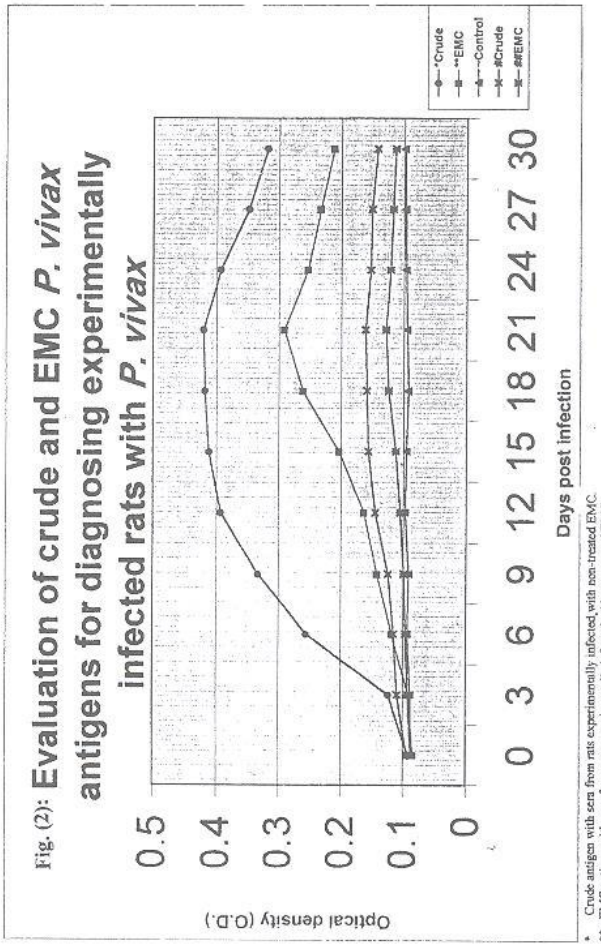

Fig. (2):

* Crude antigen with sera from rats experimentally infected with non-treated EMC.
** EMC antigen with sera from rats experimentally infected with non-treated EMC.
*** mean optical density from negative control sera.
\# Crude antigen with sera from rats experimentally infected with treated EMC.
\## EMC antigen with sera from rats experimentally infected with treated EMC.