Fluorescent Antibody Technique for the Serodiagnosis of Trichinosis

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Abstract. A modification of the indirect, immunofluorescent antibody reaction (IFR) for use in the serodiagnosis of trichinosis is described. For this reaction a stable antigen was prepared from dried, but not previously frozen, larvae of *Trichinella spiralis*. 12 confirmed cases of trichinosis in man and 4 rabbits experimentally infected with larvae of *Trichinella spiralis* were examined in the IFR and the hemagglutination test (HA). We found our modified method of the IFR at least as sensitive as the HA test. An oral infection with various doses of *T. spiralis* larvae was traced in rabbits. The production of demonstrable antibodies was found to be directly related to the number of larvae.

Immunofluorescent techniques for the demonstration of antibodies are now frequently used in the diagnosis of parasitic infections for being sensitive, specific and easy to perform. The IFR was first used in 1962 in the serodiagnosis of trichinosis. Most authors compare the IFR with other serological methods and concentrate on the preparation of a stable antigen, which could retain its activity and specificity for some length of time.

SADUN et al. (1962) and SADUN (1963) obtained excellent results with larvae of *T. spiralis* fixed in 10 % formol — 0.5 % bovine serum albumin (BSA) solution. This antigen was usable for two weeks. SULZER (1965), modifying this procedure by adding only 0.5 % BSA, found nonspecific reactions to develop on the 2nd and 3rd day of storage. He therefore used cuticles of *T. spiralis* larvae, fixing them in the same way. This antigen could be stored in the refrigerator for several months without losing its specific value. KOZAR et al. (1966), using Sulzer's first modification of the antigen, studied also other fixation methods. In keeping with the method described by BARATAWIDJAJA et al. (1963), he fixed liberated larvae of *Trichinella spiralis* to a slide by warming it over a moderate flame.

Some authors studied the bond of conjugated antibodies on the body of Trichinellae. JACKSON (1959) tested fluorescence of various organs in the Trichinella body and the formation of a fluorescent precipitate during larval reaction with a hyperimmune serum on viable larvae and on sections of frozen tissue from infected rats. In none of his observations he found a fluorescent cuticle. For the same purpose BARATAWIDJAJA et al. (1963) used either pure cuticles or sections from the tissue of infected rats, in both instances finding fluorescence of the cuticle and, in addition to that, fluorescence of the digestive tube on the sections. Fluorescence of the larval cuticle was also observed by BRZOSKO et al. (1965) and SULZER (1965). CHROUST et al. (1966) observed in the direct fluores-

cent antibody test the origin of a fluorescing precipitate on the cuticle, on the oral and on the anal pore.

Our work has been mainly concerned with the preparation of a stable antigen for the IFR with the possibility of using this reaction in the serodiagnosis of trichinosis and of comparing this reaction with the hemagglutination test.

MATERIAL AND METHODS

1. Maintenance of the *Trichinella spiralis* strain. Young laboratory rats, approximately 150 g in weight, were selected as hosts to maintain the strain of *T. spiralis*. Each rat was fed with infected pieces of musculature (tongue, diaphragm) containing encysted Trichinella larvae. Before use the presence of larvae in the musculature was confirmed under the microscope.

2. Isolation of larvae. Rats that had been infected with *T. spiralis* larvae about 4—5 weeks previously were killed with ethyl ether. After skinning and eviscerating the rat carcasses the musculature was examined for the presence of larvae. Then the carcasses were ground in a meat mincer and the larvae liberated by digestion in artificial digestion fluid containing 15 g pepsin (10,000 units) and 21 ml HCl (spec. w. 39,465) to 3,000 ml tap water for each 70 g of minced meat. The mixture was left to stand for 14—16hrs (best over night) at a temperature of 37 °C, stirring it occasionally. Then the digested material was filtered through a linen cloth into a 3 l glass funnel with a test tube attached to its bottom. Within about one hour under occasional light stirring of the filtrate in the funnel, the larvae settled on the bottom of the tube.

3. Preparation of the antigen. The larvae concentrated in the sediment were thoroughly washed by repeated decantation in phosphate—buffered saline solution (PBS), pH 7.2. The rinsed larvae were used either for infecting experimental animals and obtaining hyperimmune sera or for preparing an antigen to be used in the IFR. In a test tube, a thick suspension was prepared of the washed, whole larvae and transferred into lyophilisation ampules (0.2—0.4 ml). Without previous freezing the larvae were dried in the exsiccator by exposure to P_2O_2, constant vacuum 0.01 mm Hg. After finishing the drying process the ampules were evacuated and sealed. The antigen was stored in the refrigerator at +4 °C.

4. Sera. Human sera were collected from 12 well-documentated human cases of trichinosis infection (eastern Slovakia 1965). Immune animal sera were prepared by experimentally infecting rabbits with pure, isolated larvae of *T. spiralis*. Each rabbit was given a dose of 20—70,000 larvae via stomach-tube. Previous to the infection all rabbits were examined in the IFR. After the infection, blood was taken by heart puncture at beforehand determined regular intervals. The sera were stored frozen at —15 °C.

5. Immunofluorescent staining. In the IFR we used commercially produced conjugates (Institute of Sera and Vaccines, Prague): rabbit globulin against human gamma-globulin and goat globulin against rabbit gamma-globulin labeled FITC. For the reaction both sera and globulins were diluted with PBS pH 7.2. Titration of the conjugates had to be performed in such a way as to obtain in reactions with the known positive serum constant antibody titres, but not to evoke a positive reaction with a negative serum. The most suitable dilution of the rabbit conjugate was 1 : 4, of the goat conjugate 1 : 2.

6. Optical equipment and photomicrography. The Soviet microscope ML-2 was used with FS-1, BS-8-2 and SZS-7-2 exciter filters and a ŽS-18-2 barrier filter. Optics: Objective ×10, ocular ×10 with monocular adapter. For photomicrography we used the camera Zorki 4, cine-film Orwo 27 DIN.

7. Performance of the IFR. After opening the ampules containing the antigen, 1 ml of PBS was added to every 0.1 ml of the original volume of live larvae, allowing them to soak for at least 1/2 h before using them in the test.

The examined, non-inactivated sera were diluted in geometric series, using four-fold dilution (1 : 4, 1 : 16, 1 : 64 etc). The reaction was performed in agglutination test tubes (80 × 8 mm) by placing a drop of suspended antigen containing approximately 100 – 150 *Trichinella* larvae into each test tube and adding 0.1 ml of prediluted serum.

After an incubation period of 1/2 h, during which the tubes were shaken constantly on the electric shaker, the larvae were washed 3-times by decantation in PBS pH 7.2. After the last rinse the PBS was sucked off, 0.1 ml of titrated conjugate was added and the larvae were incubated on the shaker for another 1/2 h at room temperature. Then they were washed again 3-times in PBS, which was sucked off after the final rinse, adding to each tube one drop of buffered glycerin (glycerin without autofluorescence diluted with PBS pH 7.2 – 9 parts of glycerin to 1 part of PBS). The reaction could be read immediately or on the following day after keeping the tubes over night in the dark at +4 °C, which has proved the better way.

Parallelly with these reactions the following control tests were performed:

A. For each serum: the same serum diluted at 1 : 4 with antigen without adding the conjugate·
B. For the complete reaction: 1) Control with positive serum – the positive serum of a known titre value was diluted and treated in the same way as the examined sera; 2) Control with negative serum – the definitely negative serum was treated in the same way as the examined sera. 3) Control of the conjugate – a mixture of antigen and conjugate without serum. 4) Control of the antigen – only pure antigen without serum and conjugate.

The results were read from a small drop extracted from each test tube, and placed on a slide and covered with a coverslip.

8. Evaluation of fluorescence:

Negative = no traces of fluorescence in the antigen

    + = weak, distinctly green fluorescence of larval outlines

    ++ = strong, green fluorescence of larval outlines

    +++ = brilliant green fluorescence of larval outlines; pale green fluorescence of the surface of larvae.

RESULTS

A. At first we confirmed the stability of some antigens used by other authors. Test tube method: the liberated larvae of *T. spiralis* were fixed in 4% and 10% formol and in 10% formol- 5% bovine albumin. The antigen remained usable for a very short time. Nonspecific fluorescence started to develop on the third day, making an accurate evaluation of the IFR very difficult.—Slide method: we tested the fixation of isolated larvae to a slide by using egg albumin, blood serum, agar, gelatine, starch and water glass.

a) viable larvae suspended in distilled water were placed on the fixation layer on the slide and dried at room temperature, under a current of warm air or in a heated histological oven (50—60 °C).

b) a thick layer of the same suspension of viable larvae was placed on a straight slide and left to dry at 37 °C. The dry coating was cut with a razor blade into small blocks (length of side approxim. 3 mm), removed from the slide and transferred to another slide, coated with fixation. In the same way were treated the larvae, previously fixed in 4% formol.

c) viable larvae in a suspension of H_2O were dried on a slide over a moderate flame.

Also these slide antigens revealed some disadvantages. The relatively best fixation medium for larvae was egg albumin, but also here nonspecific fluorescence was found on the larval surface even in the negative serum. The same applies to the antigen fixed over a moderate flame. All other methods of fixation to a slide proved completely unsuitable because, when rinsing the larvae, they were washed off either completely or partly from the slides. For these reasons we used only antigen from dried larvae for our serological tests.

Table 1. The results of HA and IFR in 12 human sera (reciprocal values of titres)

Patient no.	After 3 months		After 12 months	
	HA	IFR	HA	IFR
1	negat.		negat.	negat.
2	20 000		300	16
3	5 000		5 000	64
4	1 200		1 200	64
5	2 500		1 200	250
6			40	negat.
7	nonspec.		160	64
8	10 000		300	16
9	. 600		negat.	4
10	1 200		300	16
11			negat.	16
12			1 200	16

B. We tested the possibility of using the IFR in the serodiagnosis of infection in man on sera collected from 12 patients suffering from trichinosis (Tab. 1). The sera were collected twice. All samples from the first collection were tested only for HA reactions; in samples from the second collection a comparison was made between the values of the HA and IFR titres. (The second collection of serum samples coincided roughly with the time, when the IFR was introduced to our laboratory and therefore, we used these only because of the too long storage of the first samples.) At present the use of the IFR has been generally accepted for testing clinically suspect cases in our laboratory.

C. From experimentally infected rabbits we tried to obtain some information on the dependence of the height of titres on the number of administered larvae. The onset of antibody production seemed to be roughly proportionate to the number of administered larvae — in weak infections after approximately 4 weeks,

Table 2. The origin and course of serological response in experimentally infected rabbits

Rabbit no.	Weight kg	Number of larvae	Weeks						Months											
			1	2	3	4	6	8	3	4	5	6	7	8	9	10	11	12	18	24
I	2.35	20	—	—	—	—	250	250	250	64*										
II	2.30	200	—	—	—	—	—	4	16*											
III	2.65	1 500	—	4	4	4	64	1 000	250	250	250	64	64	64	64	64	64	16	16	16
IV	2.60	20 000	—	—	4	16	64	1 000	4 000	250	250	64	64	16*						
V	3.45	30 000	—	—	4	64	250	1 000	250	250	1 000	250	250	250	250	250	250	64	64	64
VI	3.40	54 000	—	—	4	16	250	1 000	250	250	250*									
VII	3.65	70 000	—	4	4	4	250	1 000	250	4 000	1 000	1 000	1 000	1 000	1 000	250	250	250	250	64

* = exitus

in massive infections after 1—2 weeks. The antibody level reached its peak between the 8th and 12th week, becoming reduced to lower values after this period. Antibody values in weak infections were low (1 : 16); in heavy infections medium values of 1 : 64 were retained for many months or probably years.

For Tab. 2 we selected infected animals with a typical curve of serological response to *T. spiralis* infection.
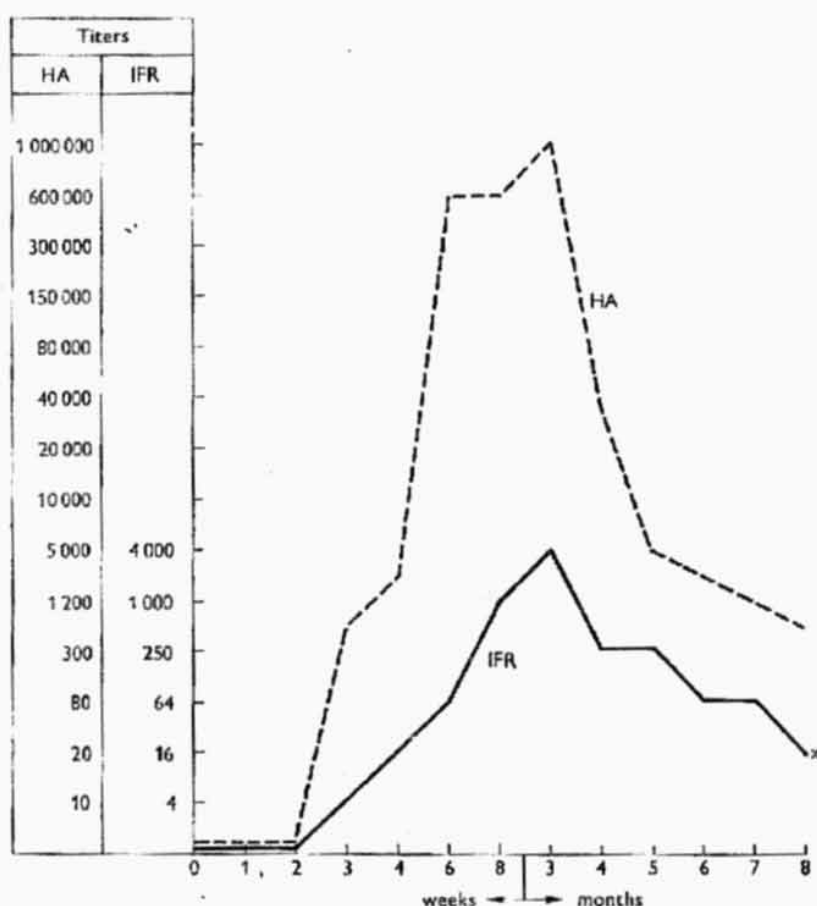

Fig. 1. Rabbit IV — weight 2.60 kg — 20,000 larvae of *T. spiralis*.
× = exitus, ———— = IFR, --------- = HA.

On Figs. 1, 2, 3, 4 a comparison is given of the titre values obtained in the IFR and HA test from 4 infected rabbits. In both tests we found the onset of antibody production and the rise and fall of the serological curve to be practically the same. Different were only the distinctly higher values of the HA titres.

To test the specificity of the IFR, hyperimmune rabbit serum was absorbed with Trichinella antigen. For absorption, the antigen was prepared from dried, powdered *T. spiralis* larvae (isolated from rats), adding to it a hyperimmune serum with a known antibody titre at a rate of 0.1 ml serum: 2 mg antigen.

The serum was absorbed for 3 days—each day for about 8 hrs at 37 °C, shaking it vigorously every 15 min. Over night the absorbed sera were kept in the refrigerator at +4 °C. Next day the serum was sharply centrifuged, the sediment removed and the same amount of fresh antigen added to the supernatant. The control antigen prepared from dried and powdered muscular tissue of healthy rats was treated in the same way. The not absorbed portion of the hyperimmune

serum, used for the control of antibody level, was kept under the same conditions. After absorption the sera were examined in the IFR. In the hyperimmune serum we found antibodies of the predicted titre value. Analogous results were obtained from the serum with the control antigen. The serum absorbed with the Trichinella antigen remained completely negative.
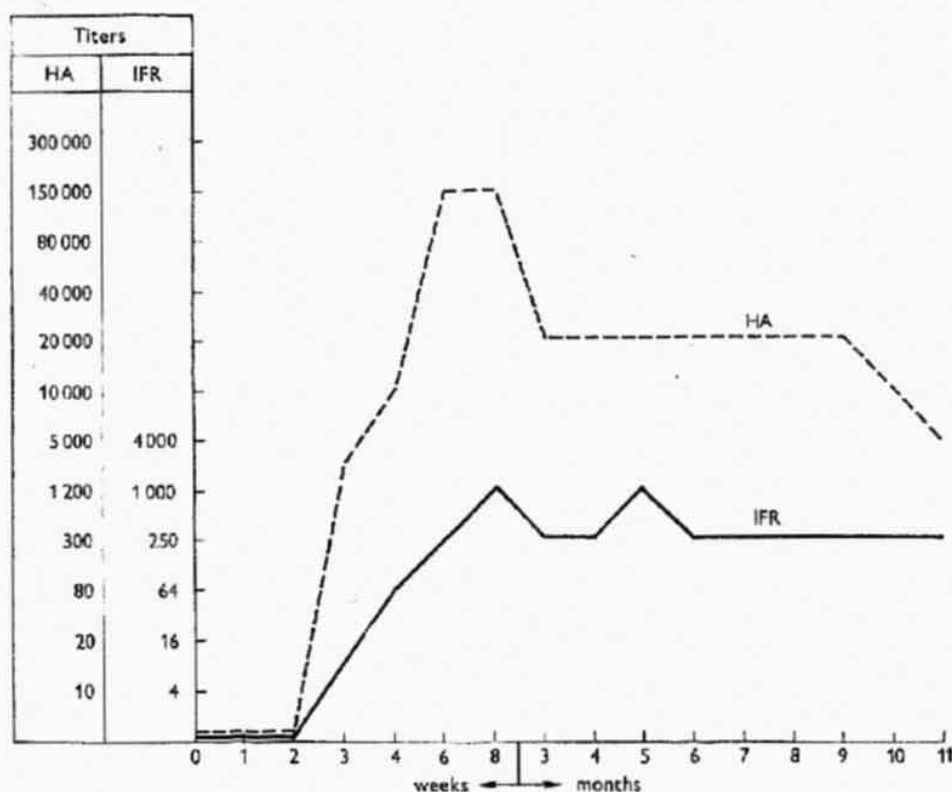

Fig. 2. Rabbit VIII — weight 3.45 kg — 30,000 larvae of *T. spiralis*.
————— = IFR, ·········· = HA.

DISCUSSION

The described modification of the IFR is simple, sensitive and specific. The dried antigen was easy to prepare and remained usable for at least 14 months and possibly even longer. After restoring the larvae to normal size by leaving them to swell in the PBS, they were completely undamaged and, having been incubated with the positive serum, their cuticle exhibited constant fluorescence. No nonspecific fluorescence occurred on the surface of the larvae. When using our antigen the larvae did not break and there was no uneven distribution of fluorescence on the cuticle as experienced on lyophilized larvae by SADUN et al. (1962). The advantages of an antigen prepared from dried larvae became evident in a comparison with an antigen fixed in formol after SADUN (1962). In keeping with the observations by SULZER (1965) we also observed a nonspecific fluorescence of the surface of Trichinella bodies a few days after fixation.

When fixing the antigen to a slide over a moderate flame, a similar nonspecific fluorescence of the surface of Trichinella bodies was observed. This may have influenced KOZAR et al. (1966) in their evaluation of positive results up to a titre

of 1 : 500 when also observing this nonspecific fluorescence mainly in less diluted sera or in negative sera.

In all instances we observed a fluorescence of the cuticle as recorded by all authors except JACKSON (1959). Our evaluation of antibody titres was started at
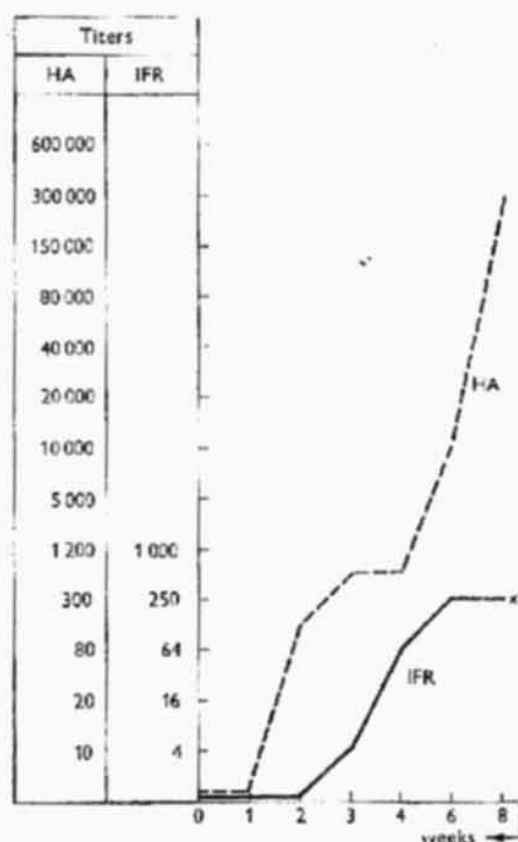

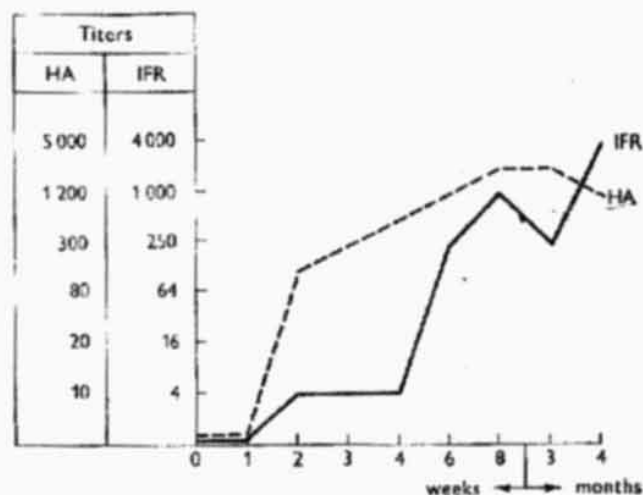Fig. 3. Rabbit IX — weight 3.15 kg — 30,000 larvae of *T. spiralis.* × = exitus, ——— = IFR, ········· = HA.

Fig. 4. Rabbit VII — weight 3.65 kg — 70,000 larvae of *T. spiralis.* ——— = IFR, ········· = HA.
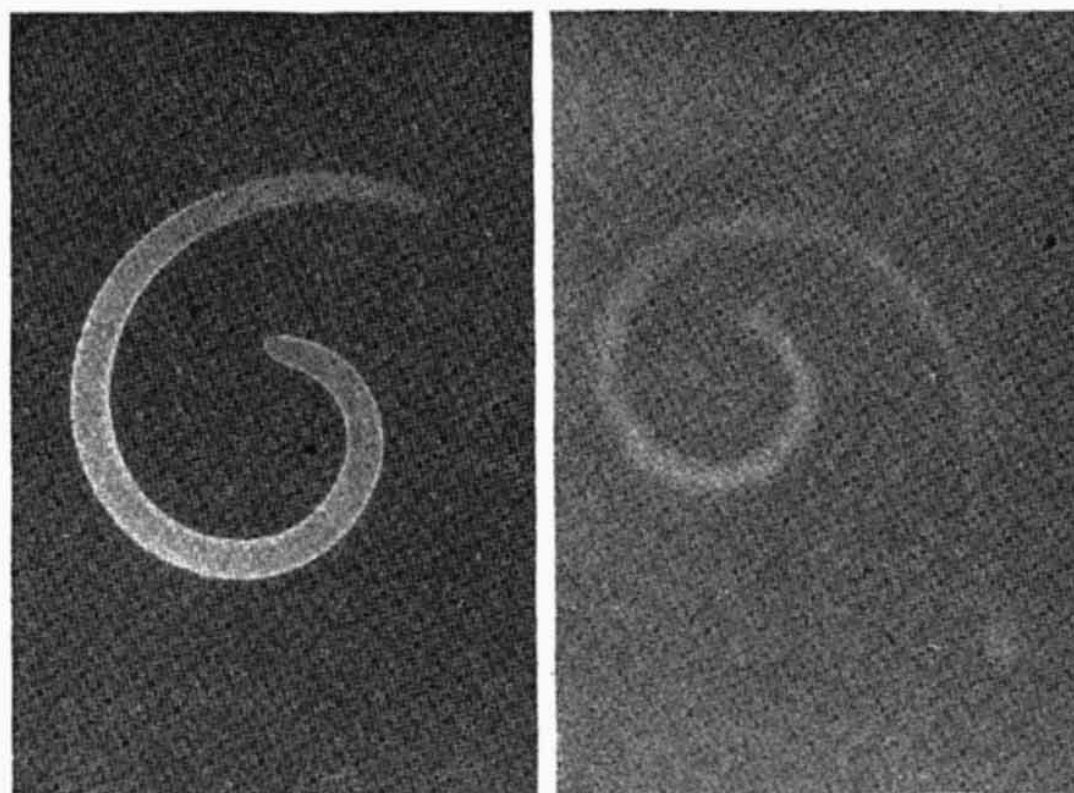

Fig. 5. Larva of *Trichinella spiralis.* Left — flourescence of cuticle in reaction to positive serum; Right — reaction with negative serum, no fluorescence of cuticle (× 100)

a dilution of 1 : 4, the highest titres being 1 : 4,000. These values were also recorded by SADUN (1963), BARATAWIDJAJA (1963) and LABZOFFSKY (1964). In none of our experimental infections we obtained titres of such high values as given by KOZAR et al. (1966) (up to 1 : 128, 000). The phenomenon of the prozone recorded by SADUN (1962) was neither observed by KOZAR et al. (1966) nor by us.

In the IFR we observed the onset of antibody production between the 7th and 28th day; on the 7th day in rabbits experimentally infected with 50,000 larvae; on approximately the 14th day when medium doses (1,500—30,000 larvae) were used; on the 21st to the 28th day when using small doses (20—200 larvae). An earlier onset of antibody production in more massive infections was also recorded by SADUN on the 7th day of infection. LABZOFFSKY (1964) observed antibody production following an infection with approximately 6,000 larvae as early as on the 4th day. In experiments with our antigen LABZOFFSKY's results could not be confirmed.

The specificity of the reaction was tested with a complete absorption of antibodies in hyperimmune serum with a homologous antigen. We succeeded in saturating the serum up to a complete loss of titre value. The use of the IFR has proved most satisfactory in standard serological diagnostics and in the serology of experimental infection in rabbits.

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