

Application of the Fluorescent Complement Staining in the Serodiagnosis of Trichinosis

M. UHLÍKOVÁ, J. HÜBNER

Institute of Epidemiology and Microbiology, Prague

Abstract. A description is given of the modified fluorescent complement staining (FCS) for application in the serodiagnosis of trichinosis.

Swine conjugate against guinea pig complement labelled FITC was prepared for the test. Lyophilized guinea pig serum served as a source of the complement. *Trichinella spiralis* desiccated over P_2O_5 without previous freezing were used for the antigen.

The FCS method was employed for examining sera from experimentally infected animals (rabbit, hamster, rat, mouse, cat) and sera from 11 persons with spontaneous infection. The FCS method appears to be a highly sensitive and specific serological test.

Fluorescent complement staining (FCS) has been used in various modifications in virology, bacteriology and cytology (MATUHASHI et al. 1966, GOLDWASSER et al. 1958, RÜMKE et al. 1967, KLEIN et al. 1959a, b, MAESTRONE et al. 1961, COFFIN et al. 1962, GILLISEN 1963, JENTSCH 1964).

In protozoology, KELEN et al. (1962) used the FCS for the detection of toxoplasma antibodies but were not satisfied with the results obtained. By contrast KRAMÁŘ (1967) considered this method to be very suitable, finding it to be as sensitive as the indirect fluorescent antibody test (IFAT) and more sensitive than the complement fixation test (CFT).

In helminthology, this method has been employed only by CRANDALL et al. (1966) for the serological diagnosis of experimental trichinosis. It was tested on experimentally infected rabbits, rats and mice. The results of the examination of the rabbits were compared with the IFAT and found to be more sensitive than the latter.

Although the IFAT has been used successfully for the diagnosis of trichinosis (UHLÍKOVÁ et al. 1968) its disadvantage is the preparation of a special conjugate for each animal species. This is unnecessary when using the FCS: with the same conjugate—against the guinea pig complement—it is possible to test sera of all animal species. Our paper presents the techniques of the FCS and our results in experimental and spontaneous trichinosis.

MATERIAL AND METHODS

The maintenance of the strain of *Trichinella spiralis* and the collection of larvae for preparing the antigen has been described in an earlier paper (UHLÍKOVÁ et al. 1968).

Antigen: The preparation of the antigen for the FCS is analogous to that for the IFAT using unfixed and unfrozen larvae of *Trichinella spiralis* desiccated over P_2O_5 in a vacuum; its standard properties are retained for at least 20 months.

Conjugate: the experimental batch—swine globulin against guinea pig complement labelled FITC (SwAGp C'/FITC), prepared by the Institute of Sera and Vaccines in Prague on the basis of the paper by CRANDALL et al. (1966).

Complement: the complement for immunofluorescent tests was obtained from sera of guinea pigs fed with vitamin-enriched food. The blood taken from the guinea pigs was left to separate from the serum. After centrifugation the serum was placed in ampules (1 ml per ampule), immediately frozen, lyophilized and stored in the refrigerator at $+4^\circ C$; there, it could be kept for roughly one year. Lyophilized complement keeps its standard properties better than frozen complement.

Sera: The animal sera under consideration were obtained from rabbits, hamsters, albino rats, white mice and cats infected orally with various doses of viable larvae of *T. spiralis*.

Rabbits: According to the number of larvae used for infection, the animals were divided into 4 groups:

group 1	4 rabbits, weight 1.75—1.85 kg	—20 larvae
group 2	2 rabbits, weight 1.85—2.10 kg	—200 larvae
group 3	3 rabbits, weight 2.15—2.35 kg	—2000 larvae
group 4	3 rabbits, weight 2.15—2.40 kg	—20 000 larvae

Blood was taken from all rabbits prior to infection. Following infection, blood was collected from the heart at certain previously fixed intervals up to a period of one year. The sera were kept in the deep freeze at $-15^\circ C$.

After the final blood collection the animals were killed and their muscles inspected for *Trichinella* larvae.

Hamsters: 4 hamsters (*Mesocricetus auratus*) were infected orally with 20 and 200 larvae respectively taking always one young and one old animal. Blood was taken from the retrobulbar venous plexus at fixed intervals. Postmortem examination of the animals was performed with trichinoscopy.

Rats: 5 albino rats were infected orally with 200, 1000, 1500, 3000 and 6000 larvae. For serum collection and tracing of *Trichinella* larvae the same technique as that described for the hamsters was used.

Mice: 10 albino mice were infected with 20 larvae each. Blood was taken every week until the 9th week after infection inclusive.

Cats: 3 adult cats were fed with muscles harbouring numerous encysted larvae of *Trichinella spiralis*. Blood was taken from the heart. The sera of two cats were examined for 13 1/2 months, these of one cat for 12 months.

Human sera were collected from persons with verified trichinosis from an epidemic outbreak in eastern Slovakia in 1965. These were the same as the sera examined previously with the IFAT (UHLÍKOVÁ et al. 1968).

The test proper. The sera inactivated in a water bath at $56^\circ C$ for 30 min were diluted in test tubes with buffered saline (PBS—phosph. pH 7.2 M/15) in geometric series from 1 : 2 up to the necessary dilution, omitting always one dilution (1 : 2, 1 : 8 etc.). 0.1 ml of prediluted serum was mixed with 0.1 ml of complement diluted with PBS with regard to the results of previous titration. By adding the complement we obtained the final dilution of the sera, i.e. 1 : 4, 1 : 16 etc. One drop of suspended antigen containing approximately 100—150 larvae of *Trichinella spiralis* which had been placed at least 30 min before using them in the test in PBS to swell, was added to each tube. The tubes were shaken and then incubated in a water bath at $+37^\circ C$ for 30 min under occasional light stirring. After this period the larvae were washed three times by decantation in PBS, then the PBS was sucked

off and 0.1 ml of titred conjugate was added. Incubation for 20 min was repeated under the same conditions and followed by 3 rinses in PBS. After the last rinse and the sucking off of the PBS one drop of buffered glycerine (glycerine without autofluorescence diluted in PBS at 9 : 1) was added to each test tube. The tubes were kept over night in the refrigerator at +4 °C and the test was read on the next day.

Simultaneously we performed control tests for:

a) each serum: the tested serum diluted at 1 : 4 + complement + antigen

b) for the test proper:

1. examination of the positive serum—the positive serum of a known titre value is diluted and treated the same as the sera under examination,
2. examination of the negative serum—the definitely negative serum is treated the same as the sera under examination,
3. examination of the conjugate—a mixture of antigen with conjugate without complement,
4. examination of the complement—a mixture of antigen with complement without conjugate,
5. examination of the antigen — pure antigen without conjugate and complement.

The results were read by placing a small drop from each tube on a slide, covering it with a coverslip and examining it in the fluorescent microscope (the Soviet microscope ML2 with FS-1, BS-8-2 and SZS-7-2 exciter filters and ŽŠ-18-2 barrier filter. Optics: objective 10×, ocular 10× with monocular adapter.

Evaluation of the fluorescent intensity:

††† = brilliant green fluorescence of larval outlines; pale green fluorescence of larval surface

†† = strong green fluorescence of larval outlines

† = feeble but discernable green fluorescence of larval outlines

(†) = feeble green fluorescence of the outlines

neg. = no traces of fluorescence in the antigen

RESULTS

Figs. 1—4 give the results of the FCS in experimentally infected rabbits. According to the number of larvae administered a heavier infection is responsible for the earlier outset of antibody production and for increased titre value reaching its peak in the 8th week of infection (only in group 3 the peak was reached in the 12th).

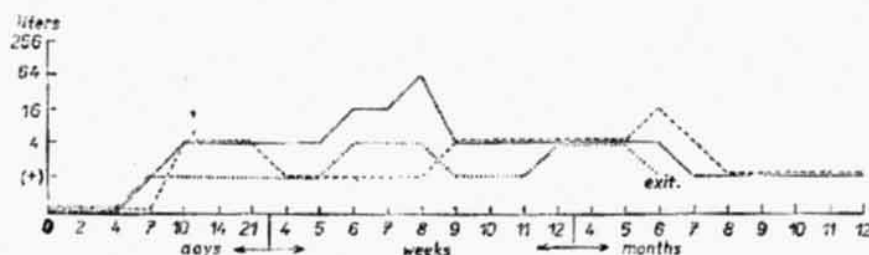


Fig. 1. Group 1. The course of the serological curve of the FCS in 4 rabbits infected with 20 larvae of *T. spiralis*.

Antibodies of low titres remained present until the end of the experiment in all animals except one animal of group 1.

Fig. 5 illustrates the course of the serological curve of the FCS in the hamsters. Antibody production started within the first week of infection with great variations in the titre values; in two animals antibodies disappeared within the 9th and 12th

week but their production increased abruptly after this period. Also in this experiment antibodies were present until the end of the experiment (i.e. for one year in all animals). We found relatively low antibody levels in the hamsters; their titres never surpassed 1 : 16.

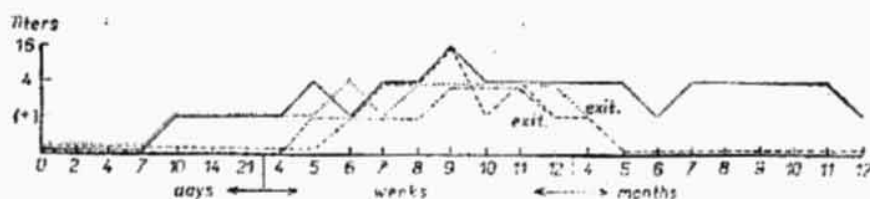


Fig. 2. Group 2. The course of the serological curve of the FCS in 3 rabbits infected with 200 larvae of *T. spiralis*.

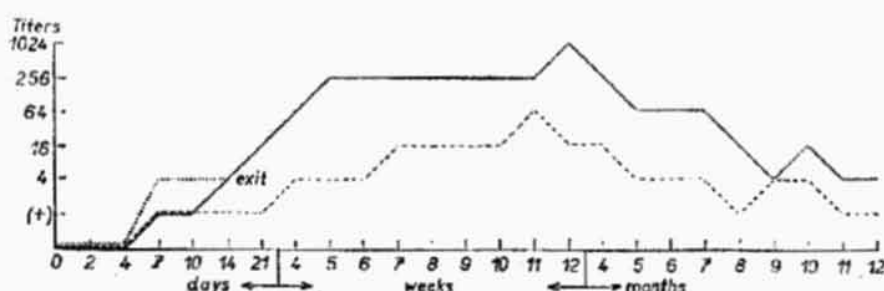


Fig. 3. Group 3. The course of the serological curve of the FCS in 3 rabbits infected with 2000 larvae of *T. spiralis*.

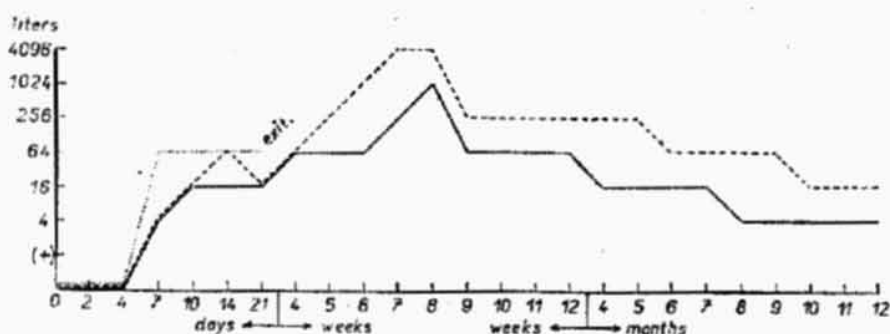


Fig. 4. Group 4. The course of the serological curve of the FCS in 3 rabbits infected with 20 000 larvae of *T. spiralis*.

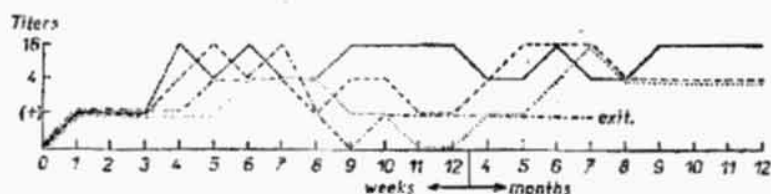


Fig. 5. The course of the serological curve of the FCS in 4 hamsters infected with larvae *T. spiralis*.

..... = 20 larvae
 = 200 larvae

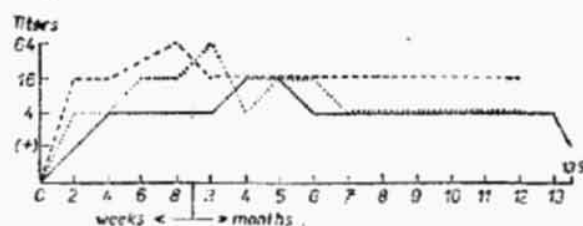


Fig. 6. The course of the serological curve of the FCS in 3 cats infected with larvae *T. spiralis*.

Also the titres of the experimentally infected cats (Fig. 6) were relatively low (maximum 1 : 64); antibody production started within two weeks, reaching its peak within 2—4 months. Again, antibody was present up to the end of the experiment, i.e. for 13 1/2 and 12 months respectively.

Although the results of examination of the group of rats infected with different doses of larvae showed a shortening of time of the outset of antibody production in dependence on the number of larvae administered—from 8 weeks (200 larvae) up to 2 weeks (6000 larvae), the titres remained very low (maximum 1 : 4) even in rats infected with doses of 3000—6000 larvae. Only in these animals antibodies were present until the end of the experiment, i.e. for 6 months.

The infected mice were inspected for 9 weeks p.i. One out of a group of 10 mice died at the beginning of the experiment, 3 died after 3 weeks. In 6 mice, antibody production started within 1—2 weeks, in 3 mice within 4—5 weeks. In all surviving animals antibodies were present until the end of the experiment. The maximum antibody titre in the mice was 1 : 4.

A comparison of the FCS and the IFAT, both used in 11 cases of spontaneous infection of man, is given in Table 1. The sera tested had been collected 3 months and 11 months p.i. respectively.

DISCUSSION

Our results indicate that the FCS technique is very satisfactory for serological examination of trichinosis both in animals and man. Although this test involves additional labour in comparison with the IFAT—the dilution and addition of

Table 1. The results of IFR and FCS in 11 human sera (reciprocal values of titres)

Serum No.	1st blood taken		2nd blood taken	
	IFR	FCS	IFR	FCS
1	4	16	64	64
2	8	4	4	4
3	8	64	256	256
4	4		64	64
5	(+)	4	16	16
6	4	16		
7	4	16		
8			16	64
9			16	16
10			16	64
11			256	256

complement—its advantage is that it does not require a special conjugate for each animal species and, moreover, that it is as sensitive as the IFAT.

Most important is the preparation of a suitable conjugate. Initially we used the commercially produced rabbit gamma globulin labelled FITC against guinea pig serum. However, the results revealed considerable differences in the individual batches of conjugate. Therefore, the conjugate described in the text was prepared in cooperation with the Institute of Sera and Vaccines in Prague. Its properties were satisfactory and, therefore, we recommended it for commercial production.

Lyophilized complement was used in the test, because it keeps its properties unchanged for at least one year when stored at $+4^{\circ}\text{C}$. The complement has to be titred together with the conjugate; a surplus of complement gives an unspecific higher fluorescence, its shortage decreases the intensity. Most suitable was a dilution of 1 : 10.

The antigen used in the test is very satisfactory; we confirmed that it keeps its standard properties for at least 20 months.

The FCS has proved to be very suitable for detecting *Trichinella* antibody in all species of infected animals. The sensitivity of this test has been confirmed in a group of rabbits, mice, hamsters and cats infected with 20 larvae only. Antibody could be detected in all of them.

In other groups of experimentally infected animals, the immunological response was related to the number of larvae administered; only in the group of rats infected with different doses, antibody production remained very low. In this case we were unable to demonstrate the dependence of the height of the titre on the number of larvae administered.

The course of the serological curve, i.e. the outset of antibody production, its peak and persistence is practically the same as that of the IFAT.

The results of the FCS in examining sera of persons with spontaneous trichinosis were very satisfactory. We used both tests, the IFAT and the FCS for examining two samplings of blood, one taken 3 months, the other 11 months p.i. The titres of the sera from the first blood collection were higher in the FCS than in the IFAT in spite of the fact that at the time of examination these sera had been stored frozen for a relatively long time. The titres from the second collection were of the same value in both tests. It is interesting that, in both tests, the titres of the sera from the second collection were mostly higher than those from the first collection.

The specificity of the antigen in the FCS test has been confirmed by a single saturation of *Trichinella* antibody in immune serum with a homologous antigen until the complete loss of titre. In the same way we tried to saturate *Trichinella* antibody with the antigen of *Ascaris lumbricoides*, *Echinococcus granulosus*, *Taeniarrhynchus saginatus*, *Fasciola hepatica* and also with pure rat protein. In none of these instances a reduction occurred in the titre of *Trichinella* antibody in comparison with the control group.

We also tried to affect in the FCS test *Trichinella* antigen with rabbit hyperimmune sera of *Ascaris lumbricoides*, *A. suum*, *Echinococcus granulosus*, *Cysticercus bovis*, *Toxocara cati* and with human immune sera (from patients with bilharziosis and ascariasis). The reaction was negative in all cases.

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M. U., Ústav epidemiologie
a mikrobiologie,
Praha 10, Šrobárova 48, ČSSR