



**HAL**  
open science

# RAINBOW TROUT COMPLEMENT FIXATION USED FOR TITRATION OF ANTIBODIES AGAINST SEVERAL PATHOGENS

M. Dorson, Corinne Torchy, Christine Michel

► **To cite this version:**

M. Dorson, Corinne Torchy, Christine Michel. RAINBOW TROUT COMPLEMENT FIXATION USED FOR TITRATION OF ANTIBODIES AGAINST SEVERAL PATHOGENS. *Annales de Recherches Vétérinaires*, 1979, 10 (4), pp.529-534. hal-00901230

**HAL Id: hal-00901230**

**<https://hal.science/hal-00901230v1>**

Submitted on 11 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## RAINBOW TROUT COMPLEMENT FIXATION USED FOR TITRATION OF ANTIBODIES AGAINST SEVERAL PATHOGENS

M. DORSON, Corinne TORCHY and C. MICHEL

*Institut National de la Recherche Agronomique, Laboratoire d'Ichtyopathologie,  
78850 Thiverval-Grignon (France)*

### Résumé

UTILISATION DE LA FIXATION DU COMPLÉMENT DE TRUITE ARC-EN-CIEL POUR LE TITRAGE D'ANTICORPS DIRIGÉS CONTRE DIVERS AGENTS PATHOGÈNES. — Devant l'impossibilité de fixer le complément (C) de cobaye avec des anticorps de Truite Arc-en-Ciel (*Salmo gairdneri*) alors que cette possibilité existe avec les anticorps de Truite Fario (*Salmo trutta*), un test de fixation du C a été réalisé qui utilise l'hémolysine et le C de Truite Arc-en-Ciel. L'injection de Globules Rouges de Mouton (GRM) lysés aux Truites a permis d'obtenir des anticorps hémolytiques (qui sont des IgM), et le sérum de Truite normale (STN) est utilisable comme source de C à des dilutions où a disparu l'activité hémolytique « naturelle » que présente le STN pour des globules rouges de nombreuses espèces d'homéothermes. 1/2 h de chauffage à 37 °C entraîne la disparition des trois quarts de l'activité complémentaire, et celle-ci disparaît complètement à 40 °C. La fixation de 3 à 4 unités hémolytiques 50 % (CH<sub>50</sub>) a été réalisée dans un test court (2 incubations de 1 h à 20 °C) dans les systèmes antigène-anticorps suivants : 2 antigènes bactériens (*Aeromonas salmonicida* et *Vibrio anguillarum*), 2 virus (Virus de la Nécrose Pancréatique Infectieuse (NPI) et virus d'Egtved). La fixation du C permet le titrage des anticorps avec une sensibilité comparable à celle de l'agglutination dans le cas de l'*Aeromonas*, avec une sensibilité très inférieure à celle de la séroneutralisation pour le virus de la NPI (ordre de grandeur de 50). La différence n'est pas si marquée pour le virus d'Egtved.

Antibody production in fish, and more particularly in salmonids, against pathogens as well as various antigens, is well documented. This subject has been extensively reviewed by Corbel (1975). Agglutination, in the case of bacteria, and neutralization, in the case of viruses, have been almost exclusively used for the titration of antibodies against those pathogens. In contrast, there is very little information concerning complement fixation. However, the existence of complement in the serum of the

eel (*Anguilla anguilla*) and the tench, (*Tinca tinca*) was reported by Nardi as early as 1938. Complement was subsequently found by Legler *et al.* (1967) in several freshwater teleosts, and by Day *et al.* (1970) in carp (*Cyprinus carpio*) and paddlefish *Polyodon spathula*. In salmonids, Chiller *et al.* (1969) pointed out the necessity of salmonid serum as a source of complement to achieve lysis of sheep red blood cells (SRBC) by rainbow trout (*Salmo gairdneri*) antibodies.

Complement fixation by rainbow trout antibodies was first mentioned by Saito *et al.* (1964) who obtained fixation of guinea pig complement. Complement fixing titres were low, and the technique used poorly described. More recently, guinea pig complement fixation by brown trout (*Salmo trutta*) antibodies was clearly achieved by Ingram and Alexander (1976). In contrast negative results were obtained when using rainbow trout antibodies (Ingram, personal communication). Our first complement fixation tests (CFT) were in agreement with this last finding. Therefore, we had to design a CFT using trout complement as well as trout haemolysin in order to titrate antibodies from the same species. Zicht (1976) had previously undertaken a similar work, and pointed out the strong natural haemolytic activity of rainbow trout serum for SRBC. Immunization of trout with SRBC strongly enhanced haemolysin activity, but Zicht was unable to obtain any complement fixation. He pointed out that guinea pig complement could not replace trout C in the Trout anti-SRBC haemolysin system and inversely that trout C could not substitute for guinea pig C in the rabbit anti SRBC system.

**Material and Methods**

**1. Antigens**

The preparation of *Aeromonas salmonicida*, the causative agent of furunculosis of salmonids, for agglutinin titration in microplaques has been described (Maisse and Dorson, 1976). A freshly prepared suspension of approximately  $10^9$  bacteria per ml was washed in saline, freeze dried and subsequently used as antigen for complement fixation. For titration of anti *Vibrio anguillarum* antibodies, a bacterin (Hivax vaccine, from Tavelek inc. Seattle) was kindly supplied by Dr Amend.

Production and titration of Infectious Pancreatic Necrosis Virus (IPNV) have been described (Dorson and De Kinkelin, 1974), as well as production and titration of Egtved Virus, causative agent of Viral Haemorrhagic Septicaemia of Trout (De Kinkelin and Scherrer, 1970). Egtved Virus used in this study was produced on carp cultured cells (EPC, Fijan, unpublished).

IPN virus was concentrated by overnight incubation at 4 °C with 10 % polyethylengly-

col (PEG, Carbowax 6,000). Egtved Virus was concentrated according to the same procedure using 7 % PEG and 3 % NaCl. Both viruses were resuspended in PBS to a final titre of  $5 \times 10^8$  pfu.

Anti-complementary activity was sometimes found in *Aeromonas* and rarely in Egtved virus preparations. These were then discarded.

**2. Animals, bleeding and immunization**

150 to 400 g trout were purchased from a

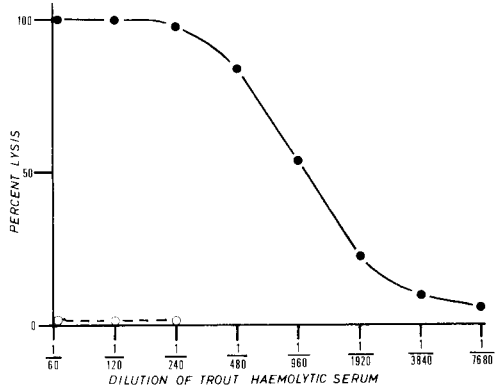


Fig. 1. — Haemolysis curve of a trout anti SRBC immune serum heated 30 min at 45 °C in presence (●) or in the absence (○) of fresh normal Trout serum (final dilution 1/100).

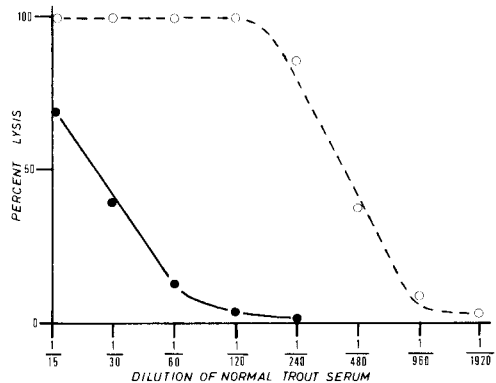


Fig. 2. — Haemolytic activity of normal trout serum in the presence (○) or in the absence (●) of Trout anti SRBC immune serum (heated 30 min at 45 °C, final dilution 1/240).

trout farm regularly controlled by the laboratory, and free of any viral disease. They were maintained in recirculated units (temperature 10 to 15 °C). Blood used as a source of antibody was drawn by the cardiac route, and sera harvested after clotting (overnight at 4 °C) and stored at - 20 °C.

When blood was used as a source of complement, it was chilled in an icebath as soon as drawn. The serum was harvested 1 h later, and used soon after or freeze dried.

Immunization doses (0.5 ml intraperitoneally) were as following :

— *Aeromonas salmonicida* :  $5 \times 10^8$  bacteria killed by 0.6 per cent formalin. (commercial formalin containing 30 % formaldehyde). One injection.

— *Vibrio anguillarum* : Hivax vaccine. Two injections (4 months interval).

— *IPN Virus* :  $4 \times 10^8$  plaque forming units (pfu) inactivated by formalin 1/200 24 h, then dialysed against PBS to eliminate excess formalin. Three injections (6 months interval).

— *Egtved Virus* :  $10^8$  pfu inactivated by  $\beta$  propiolactone 1/6000. Three injections at time 0, 9 and 16 months.

— Sheep Red Blood Cells (SRBC) were injected in the form of membranes after lysis by distilled water and subsequent washings. Each injection (time 0, 6 and 8 months) corresponded to 2.5 ml of packed whole SRBC (approximately  $5 \times 10^{10}$ ), with 0.1 ml of Complete Freund's adjuvant.

### 3. Antibody titration

The agglutination of *Aeromonas salmonicida* cells in microplaques has been previously described (Maisse and Dorson, 1976).

The sero-neutralisation of IPN and Egtved viruses by the plaque number reduction technique on RTG<sub>2</sub> cells monolayers has also been described (Dorson and De Kinkelin, 1974).

The complement fixation test was adapted from the classic procedure (Williams and Chase, 1977), and carried out in 5 ml test tubes according to the following schedule :

1. 0.1 ml antigen ;
2. 0.1 ml immune serum (previously heated 30 min at 45 °C) ;
3. 0.1 ml complement (C).  
Incubation 1 h at 20 °C.
4. 0.1 ml trout anti-SRBC serum (previously heated 30 min at 45 °C).
5. 0.2 ml of a 0.8 % SRBC suspension.

Incubation 1 h at 20 °C.

6. 1.5 ml of chilled citrate buffer ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ , 0.075 M, NaCl 0.12 M, adjusted to pH 7.3 by citric acid).

Centrifugation (5000 g, 10 min).

Reading of optical density at 413 nm.

All classic controls (particularly for anti complementary activity of antigens and sera) were systematically included in the tests. Antisera were not pre-incubated with SRBC since it was noticed that all natural activity against SRBC was destroyed after heating and was not restored by C. Dilutions were calculated in volumes 1 to 3 for C.F. activity, in volumes 1 to 5 for haemolytic activity. The reagents were diluted in Mayer's buffer (Institut Pasteur Production) with 0.1 per cent bovine serum albumin (Calbiochem). SRBC, guinea pig complement and rabbit anti SRBC haemolysin were supplied by Institut Pasteur.

### 4. Gel filtration

Purification of trout immunoglobulins by gel filtration through Sepharose 4B has been previously described (Dorson, 1972).

## Results

### 1. Titration of trout anti SRBC haemolysin and trout complement.

At the beginning of this study, no precision could be obtained about the C concentration in normal trout serum (NTS), which exhibited a noticeable spontaneous haemolytic activity against red blood cells from all the various homeotherms tested : sheep, horse, rabbit, man, chicken. This natural activity was destroyed by heating (30 min at 40 °). So, when checking sera from trout immunized against SRBC, these were heated (30 min at 45 °) and NTS was used as C at the first 1/2 dilution which had no natural haemolytic activity. The first tests were performed 3 months after the first injection of SRBC into 10 table size trout, and low but significant induced haemolytic activities were recorded. One individual reached a haemolytic titre of 1/300 (dilution at which 50 % haemolysis was recorded). From 1 to 4 months after the last booster high titres (up to 1/3000) were obtained. It must be stressed that only 4 animals in 10 responded well to injection. In others, titres remained low throughout the course of immunization. A typical titration curve of induced haemolytic

activity is shown in figure 1. Haemolysis assay on fractions obtained after gel filtration of immune trout serum on Sepharose 4B demonstrated that haemolytic activity was associated with IgM peak. For subsequent experiments, trout haemolysin was used in excess (at least 10 times the concentration providing 50 % haemolysis). Thereafter, complement could be precisely titrated, and figure 2 demonstrates such a titration. It was verified that C activity of NTS was completely abolished by Ethylenediamine Tetraacetic acid (EDTA) 0.03 M, as well as guinea pig C tested with rabbit anti SRBC. The sensitivity to heating of trout C was measured (fig. 3) : it was completely destroyed after 30 min at 40 °C, and 45 °C was chosen for inactivation of sera. A 50 per cent decay of C titer was noticed after overnight storage at 4 °C, which led to the use of the short complement fixation test described in material and methods. This test worked from 10 to 25 °C, and 20 °C was chosen as the optimum temperature.

2. Complement fixation tests with trout antibodies.

All the tests were performed with 3 to 4 CH<sub>50</sub> units (amount of C providing a 50 % haemolysis), for the two bacterial and the two viral antigens.

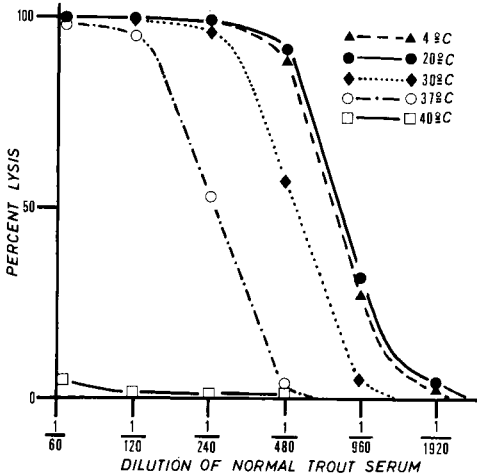


Fig. 4. — Complement fixation by trout anti *Aeromonas salmonicida* sera with *A. salmonicida* antigen. Trout complement concentration was 3 C H<sub>50</sub> units.

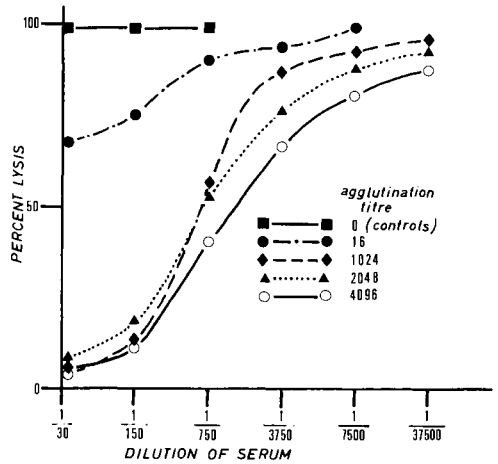


Fig. 3. — Complement titration of normal trout serum incubated 1/2 hour at the indicated temperatures before adding trout anti SRBC immune serum and SRBC, and allowing hemolysis 1 h at 20 °C.

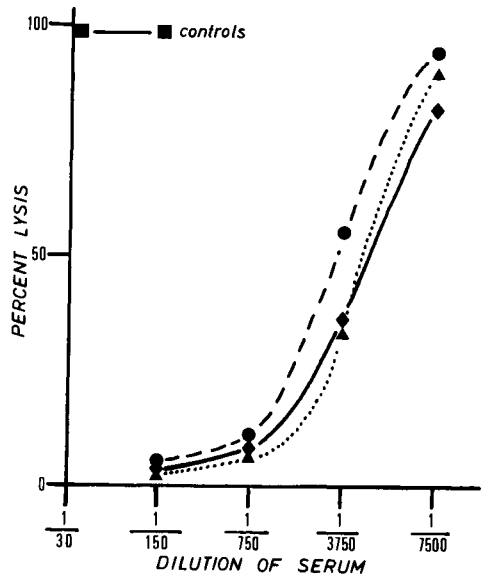


Fig. 5 — Complement fixation by three trout anti IPN virus and three non immunized controls sera with IPN virus as antigen. Trout complement concentration was 3 CH<sub>50</sub> units.

Complement fixation curves were established for several trout immunized against *A. salmonicida* and bled two months later (fig. 4).

The maximum titre (50 % inhibition of haemolysis) was 1/2000, and there was a good correlation between complement fixation and agglutination titres.

In the case of *V. anguillarum*, complement fixation titres reached 1/300 1.5 month after the first injection, and 1/1000 1.5 month after the second one, in the six immunized fishes. There was no CF activity in non-immunized controls.

In the case of IPN, antibodies came from 3 hyperimmunized trout (fig. 5). Titres were 1/3000 - 1/4000 in CFT, and 1/200000 - 1/250000 in seroneutralization.

Finally, the test was carried out with Egtved Virus. The first CFT was performed one week after second injection and gave negative results. Four weeks later significant titres were obtained (from 1/30 to 1/80). This was parallel to a weak neutralizing activity of Egtved virus (30 to 50 % neutralization at 1/20 dilution). Titres then decreased and all fish were negative three months after second injection. One month after third injection, titres increased again (up to 360 in CFT and from 20 to 100 % neutralization at 1/40 dilution). Again, as for anti SRBC immunization, several of the 14 immunized trout developed very poor titres. Non immunized controls remained negative throughout the experiment.

## Discussion

After immunization of rainbow trout against SRBC and obtainment of trout haemolysin, trout complement could be titrated and some of its properties have been demonstrated. EDTA sensitivity of trout C indicates the same requirement for divalent cations as for other known complements. Trout C is extremely

heat sensitive, and very labile. CFT has been achieved with the four antigens used. The sensitivity of CFT is comparable to that of agglutination in the case of *A. salmonicida*.

As expected, in the case of IPN, neutralization was far more sensitive than CFT. But in the case of Egtved Virus, to which it is always difficult to elicit neutralizing antibody response (Vestergard-Jorgensen, 1971), the difference was not so marked. It must be stressed that, until now, CFT has not enabled us to detect anti Egtved Virus antibodies in the absence of any neutralizing activity. On the other hand, we are now able to add known amounts of C in sero-neutralization tests, and to check for the role of C in virus neutralization. This aspect is now under investigation. Of course all the reagents can be used in a microtechnique, but the technique described in this paper is more precise and appeared more suitable for this preliminary work, which only aimed to demonstrate that CFT could be used with rainbow trout for antibody titration.

A puzzling situation is represented by the fact that in some salmonids (Brown Trout), guinea pig complement can work in a CFT, while in others (Rainbow Trout) it cannot. This fosters further investigation, especially on the purification of Salmonid C components, and the examination of the factors which determine their interaction with different antigen-antibody complexes.

*Accepted for publication September 22nd 1978.*

## Acknowledgements

This work was supported by the Conseil Supérieur de la Pêche. We are grateful to Dr Amend, from Tavolek inc., who kindly supplied Hivax vaccine, and to the Neuville Trout farm for experimental fish.

## Summary

Since it is impossible to fix guinea pig complement (C) with Rainbow Trout (*Salmo gairdneri*) antibodies, whereas it is possible with Brown Trout (*Salmo trutta*) antibodies, a C fixation test has been designed which uses haemolysin and C from Rainbow Trout. Immunization of trout against lysed sheep red blood cells (SRBC) elicited production of haemolytic IgM. Normal trout serum (NTS) can be used as a source of C at a dilution at which its « natural » haemolytic activity (against various homeotherms RBC) has disappeared. Heating of trout C 30 min at 37 °C leads to a 75 % loss of its activity, and this one is completely abolished at 40 °C. Fixation of 3 to 4 CH<sub>50</sub>

units has been achieved in a short test (2 h at 20 °C) for the following antigen-antibody systems : 2 bacterial antigens (*Aeromonas salmonicida* and *Vibrio anguillarum*) ; 2 viruses (Infectious Pancreatic Necrosis (IPN) virus and Egtved Virus). C fixation has a sensitivity comparable with agglutination in the case of *A. salmonicida*. For IPN Virus, sero-neutralization is 50 times more sensitive than C fixation. In the case of Egtved virus, the difference is not so great.

#### Références

- CHILLER J.M., HODGINS H.O., WEISER R.S., 1969. Antibody response in Rainbow Trout (*Salmo gairdneri*). II Studies on the kinetics of development of antibody producing cells and on complement and natural hemolysin. *J. Immun.*, **102**, 1202-1207.
- CORBEL M.J., 1975. The immune response in fish : a review. *J. Fish Biol.*, **7**, 539-563.
- DAY N.K.B., GEWURZ M.D., JOHANNSEN R., FINSTAD J., GOOD R.A., 1970. Complement and complement-like activity in lower vertebrates and invertebrates. *J. Exp. Med.*, **132**, 941-950.
- DE KINKELIN P., SCHERRER R., 1970. Le virus d'Egtved. I. Développement, stabilité et structure de la souche danoise F<sub>1</sub>. *Ann. Rech. Vét.*, **1**, 17-30.
- DORSON M., 1972. La réponse immunitaire chez la truite Arc-en-Ciel (*Salmo gairdneri*) : Quelques caractéristiques des immunoglobulines produites lors d'une réaction primaire. *Ann. Rech. Vét.*, **3**, 93-107.
- DORSON M., de KINKELIN P., 1974. Nécrose Pancréatique Infectieuse des Salmonidés : enquête sérologique dans les populations domestiques et sauvages. *Ann. Rech. Vét.*, **5**, 249-256.
- INGRAM G.A., ALEXANDER J.B., 1976. The immune response of brown trout (*Salmo trutta* L.) to injection with soluble antigens. *Acta Biol. Med. Germ.*, **35**, 1561-1570.
- LEGLER D.W., EVANS E.E., DUPREE H.K., 1967. Comparative immunology : serum complement of Freshwater Fishes. *Trans. Amer. Fish. Soc.*, **96**, 237-242.
- MAISSE G., DORSON M., 1976. Production d'agglutinines anti *Aeromonas salmonicida* par la truite Arc-en-Ciel. Influence de la température, d'un adjuvant et d'un immunodépresseur. *Ann. Rech. Vét.*, **7**, 307-313.
- NARDI F., 1938. Untersuchungen über die Konstitution der Hämolyse der normalem Fischsera. *Zeitschr. Immunitätforsch*, **94**, 505-520.
- SAITO Y., OTSURU M., FURUKAWA T., KANDA K., SATO A., 1964. Studies of an infectious disease of rainbow trouts. *Acta Med. Biol. Niigata*, **11**, 267-295.
- VESTERGARD-JORGENSEN P.E., 1971. Egtved virus : demonstration of neutralizing antibodies in serum from artificially infected rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd. Canada*, **28**, 875-877.
- WILLIAMS C.A., CHASE M.W., 1977. *Methods in immunology and immunochemistry*. Vol. IV, 127-274. Academic Press, New York.
- ZICHT R.R., 1976. *Application of the complement fixation test to the study of Rainbow Trout (Salmo gairdneri) serum*. M.S. Thesis, The American university, Washington D.C.