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A latex agglutination test for the field determination of abnormal vitellogenin production in male fishes contaminated by estrogen mimics

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Abstract

Estrogen mimics are pollutants present in the aquatic environment. These compounds induce abnormalities in the reproductive system of male fishes, which lead to a total or partial male feminization, or to their demasculinization. Ultimately, these alterations could lead to a disappearance of the total contaminated fish population. Moreover, these toxic substances possess the capacity to mimic endogenous estrogens and to induce the abnormal production of vitellogenin (VTG) in male and immature fishes. The purpose of this research was to develop an easy, specific, cheap and fast method for diagnosing the contamination of male fishes by estrogen mimics, using VTG as biomarker. The selected method is based on a reverse latex agglutination test (rLAT), developed with monoclonal antibodies specific of this biomarker. The development of this VTG-rLAT has involved, firstly, the purification of carp VTG to produce monoclonal antibodies, specifics of this protein. One of these antibodies was selected to recover latex particles (diameter: 1 μm). Finally, the immunoreactivity of the VTG-rLAT was verified with different fish plasma samples from males treated with 17β-estradiol and non-treated males or females in vitellogenesis.

1. Introduction

Immunoassays based on particle agglutination have been used in clinical chemistry for many years [1–6], particularly for diagnosing diseases presented in rural areas [7–10]. This is an easy, highly selective, cheap and fast method to diagnose the abnormal presence of an antigen or an

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antibody in the biological fluids (urine, blood . . .). The performance of these tests does not require any specific laboratory material and electric appliance; in fact, they were appropriated for fieldwork. The agglutination reaction involves *in vitro* aggregation of latex particles. This aggregation is mediated by a specific reaction between antibodies and antigens.

Some pollutions in wildlife were evaluated by immunoassays like the estrogen mimics impact in fish populations. In this case, the pollutants responsible for this phenomenon included a large variety of different man-made chemicals, such as PCBs (polychlorobiphenyls), phthalates, alkylphenols, pesticides, and also exogenous natural estrogens (mycoestrogens, phytoestrogens and animal estrogens) or synthetic estrogens (17α-ethinyloestradiol). These toxic substances induce abnormalities in the reproductive system of male fish which lead to a total or partial feminization of immature and juvenile males, or just a destructuring of the testis in adults [11–18]. Ultimately, these alterations could lead to the disappearance of the total contaminated fish populations.

Moreover, these toxic substances possess the capacity to mimic endogenous estrogens and to induce the abnormal production of vitellogenin (VTG) in male and immature fishes. This abnormal production was used as a bioindication of xenoestrogenic impact [19–21]. Usually, estrogens induce VTG synthesis only in the liver of female oviparous vertebrates. Male and immature fish possess the VTG gene but, normally, they do not synthesize enough estrogens to induce its expression [19]. Estrogen mimics have the capacity to bind to the estrogen-receptors [22–25], inducing the transcription of the VTG gene in male and immature fish [26–29].

Actually, in order to assess the estrogenicity potential of these and their estrogenic effects on reproduction, different radioimmunoassays (RIA) [30] and enzyme-linked immunoassays (ELISA) against VTG have been developed for various teleost fish species. However, these immunoassays were not appropriate for fieldwork.

The aim of this study is to develop a reverse latex agglutination test (rLAT), adapted for fieldwork, using monoclonal antibodies specific for VTG, in order to detect its abnormal production for diagnosing the contamination of male and immature fish by estrogen mimics.

2. Materials and methods

2.1. Vitellogenin purification

2.1.1. Induction of vitellogenin synthesis. Ten mirror carp *Cyprinus carpio*, with an average mass of 1 kg, were purchased from a commercial fish farm (Saulnoy GAEC pisciculture, France). They were reared in two 500 l aquaria provided with running fresh tap-water at about 20 °C. The fish were held on an 8 h light/14 h dark photoperiod, with oxygenation and fed on commercial food.

VTG synthesis was induced in five carp by intraperitoneal injections of 10 μg g^{-1} body weight of 17β-estradiol (Sigma), dissolved in 100 μl methanol:chloroform (v:v). Two injections were given at ten-day intervals. The other five carp received two intraperitoneal injections of only methanol:chloroform (non-treated carp). A week after the last injection, blood samples were collected from the caudal sinus and rapidly centrifuged at 1500 g, 4 °C, for 15 min to separate blood cells, and plasma was drawn off and stored at −20 °C before purification. After blood collection, the fish were killed and dissected in order to determine the sex by histological observations.

2.1.2. Chromatography. The VTG purification method was based on a high performance liquid chromatography (HPLC) technique, using a 600S Waters Controller Chromatography System (Waters, France). The system consists of several units, including a 626 Pump (Waters),

a photodiode array detector 996 (Waters), at two wavelengths: 254 and 280 nm. The system was piloted by a computer running with Millenium[32] software. The system was connected to a Model 2128 Fraction Collector (Bio-Rad).

VTG was purified in a one-step by ion-exchange chromatography, using a 0.75 cm × 5 cm DEAE column (301, VHP 575P, VYDAC). The column was equilibrated with 25 mM tris-HCl buffer, pH 8.5. Proteins were eluted by a discontinuous gradient of NaCl with steps of 12.5 mM per 4 min from 0 to 0.5 M NaCl. The flow-rate of elution was 1 ml min^{-1} and the running time was 56 min. Fractions were collected every 2 ml and stored at −20 °C before the protein analysis [31].

The purification of proteins was controlled by measuring the absorbency at 254 and 280 nm (AU), temperature (celsius), pressure (psi) and flow-rate (ml min^{-1}) continuously.

After the chromatographic step, purified fractions containing vitellogenin were pooled and concentrated to a final volume of 1 ml, by centrifugal ultrafiltration at 3000 g and 4 °C, for 20–30 min, using Vivaspin 6 Falcons with a 50 kDa molecular mass cut-off membrane (Vivascience, France).

2.1.3. Electrophoresis. Native discontinuous polyacrylamide gel electrophoresis was carried out in slabs of 0.75 mm thickness, using a Mini Protean II (Bio-Rad). The resolving gel contained 6% acrylamide solution and the stacking gel contained 4% of the same solution, according to Laemmli (1970) [32]. Samples and protein markers were diluted in 0.5 M tris-HCl, pH 6.8, 40% glycerol and 0.02% bromophenol blue. Migration was performed under 200 V, for 45 min.

The gels were transferred in an acetic acid:ethanol:water fixation solution (1:4:4; v:v:v), for 20 min with shaking. Then, the gels were stained with a 0.15% Coomassie blue solution overnight [32]. After two water washings, the gels were destained in an acetic acid:ethanol:water mixture (1:4:4; v:v:v) and finally air-dried (Bio-Rad Gel Air Dryer).

2.2. Monoclonal antibodies production

2.2.1. Immunization. Female BALB/c mice (Janvier, France) were immunized with purified carp VTG solution. Three injections of VTG solution (200 μg diluted in 150 μl tris buffer, pH 7.4) mixed with the same volume of Freund's incomplete adjuvant (AIF, Sigma) were applied intraperitoneally in two-week intervals. One week after the last intraperitoneal injection, the blood was collected from the tail vein of each mouse and the immunological response against VTG of the serum was determined by direct ELISA. Four days before the sacrifice, the mice with the highest anti-VTG responses were then given an intravenous injection of 20 μg of VTG suspended in 50 μl of physiological serum (0.9% NaCl). Hybridomas were produced according to the method of Galfré and Milstein [33].

2.2.2. ELISA screening and cloning. Direct ELISA was performed to select the hybridoma anti-VTG antibodies producers after fusion and cloning, using VTG plasma samples from E2-treated and non-treated male and female carp as antigen.

Hybridoma cells, whose culture supernatants were positive in the ELISA screening, were then subsequently cloned two-fold. Two techniques were used: by limiting dilution fractionation and by cloning in semisolid 3% agar noble medium, respectively [33].

2.3. Adsorption

A specific carp VTG monoclonal antibody (VTG-mAb) was used to recover blue dyed latex particles (diameter: 1 μm, Polysciences—Biovalley, France) by adsorption according

to the methods of Ramadass [2], Molina-Bolívar [34] and the manufacturer's instructions (Polysciences, USA).

Latex beads were washed in borate buffer, 0.1 M, pH 8.5, twice by centrifugation at 8000 g for 6 min each time. The final latex beads suspension (1% w:v) was mixed with an equal volume of VTG-mAbs solution (470 μg ml^{-1} of beads). This mixture was incubated at room temperature overnight with constant shaking. The sensitized beads were centrifuged at 8000 g, for 10 min. The pellet was resuspended in borate buffer added to 10 mg ml^{-1} bovine serum albumin (BSA) in order to obtain a 1% bead suspension (w:v). After incubation for 30 min at room temperature with constant shaking, the mixture was centrifuged at 8000 g, for 6 min. The aim of this step was to block the aspecific sites. This blocking step was repeated twice. Finally, the latex beads were centrifuged as before and the pellet resuspended as a 1% in PBS (phosphate buffer saline), pH 7.4, containing 1% of BSA (w:v) and 0.1% of sodium azide (w:v) and 5% of glycerol (v:v) and stocked at 4 °C.

2.4. Reverse latex agglutination assay

The reverse latex agglutination assay (rLAT) was performed on glass slides. Before utilization, the bead suspension was washed by centrifugation at 8000 g, for 10 min. The pellet was resuspended in the same volume of PBS, pH 7.4, containing 0.1% of sodium azide (w:v). 25 μl of immunized particle suspension (1%, w:v) and 25 μl of carp plasma sample (E2-treated and non-treated male or vitellogenic female) were mixed and manually shaken slowly for 5 min. At the same time, a test was realized with non-immunized particles as a control. Agglutination only with immunized particles was considered to be positive.

3. Results

3.1. Vitellogenin purification

To produce anti-VTG mAbs, it was necessary to purify this protein. A new purification method of carp VTG was developed in order to produce, in a single step, high quantities of non-denatured VTG, from E2-treated fish plasma [31]. This new method was based on a high performance liquid weak anion-exchange chromatography technique, using a discontinuous elution gradient of NaCl with steps of 12.5 mM/4 min from 0 to 0.5 M.

Protein elution profiles on weak anion-exchange chromatography, from non-treated and E2-treated fish plasma showed the presence of three peaks only in E2-treated fish (male or female), which were eluted at 19–20, 23–24, and 27–28 min, respectively (figures 1(A) and (B)). These protein fractions were analysed by native-PAGE electrophoresis in order to estimate the protein composition and the presence of VTG (figure 2). The results obtained, with Coomassie blue gel colouration, revealed the presence of a pure and non-denatured protein of about 370 kDa, in E2-treated fish fractions only. These results show that this protein possesses electrophoretic characteristics consistent with fish VTG [21]. The amino acid sequencing confirmed that this protein is carp *Cyprinus carpio* VTG. Consequently, this purification method permitted us to obtain a pure and non-denatured carp VTG solution. In addition, the high quantities of VTG obtained were sufficient to produce monoclonal antibodies specific of this protein.

3.2. Vitellogenin specific monoclonal antibodies production

After the fusion of SP2/O myeloma cells and spleen cells of mice immunized with pure VTG, about 500 hybridomas were screened by ELISA using E2-treated fish plasma samples as antigen

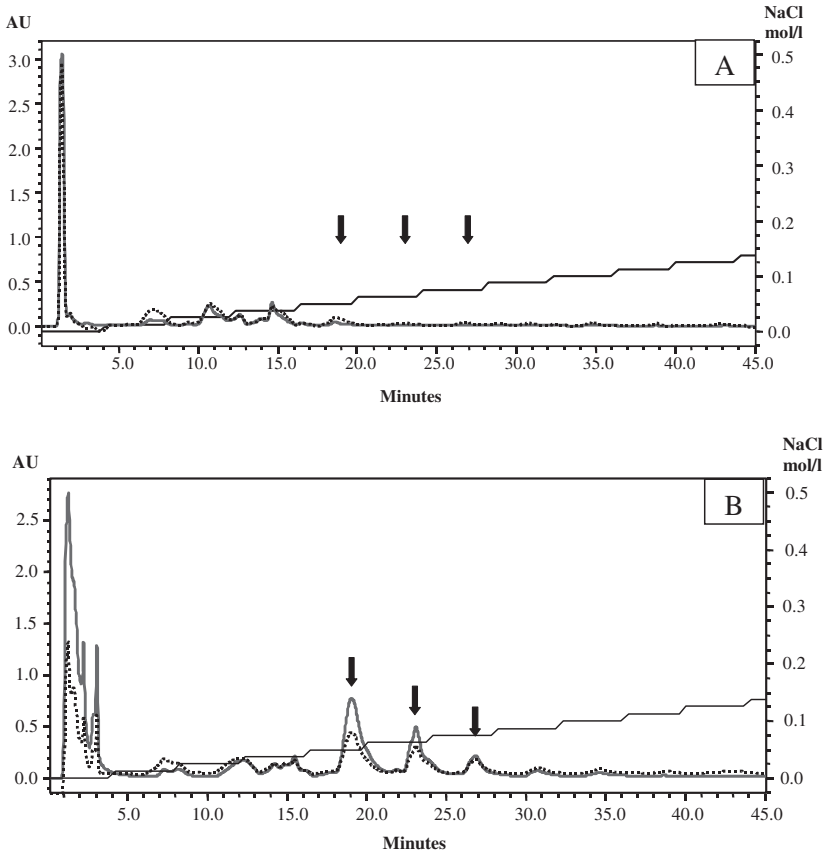

Figure 1. Elution profiles of plasma proteins from non-treated carp (A) and treated carp (B), at 254 nm (dotted curve) and 280 nm (solid curve), purified by weak anion-exchange chromatography, with a discontinuous gradient of NaCl from 0 to 0.5 M in steps of 12.5 mM/4 min (thin curve). The arrows represent the VTG peaks at 19–20, 23–24 and 27–28 min.

source and non-treated fish plasma samples as controls. Finally, ten hybridomas were obtained by cloning. One of them (G10/5/1) was selected for its cell culture efficiency according to ELISA test responses against carp VTG.

3.3. Vitellogenin specific reverse latex agglutination test

Monoclonal antibodies from G10/5/1 were used to coat 1 μm latex particles by natural chemical adsorption. The immunoreactivity of these complexes was tested with plasma samples from E2-treated male carp, non-treated male carp and vitellogenic female carp. In addition, the same test was realized with non-immunized latex particles as a control. No agglutinations were observed with the different non-immunized latex particles (figures 3(A1)–(A3)), nor with immunized latex particles and non-treated male plasma (figure 3(B1)). By contrast, plasma samples from E2-treated male and vitellogenic female carp reacted with immunized latex particles and agglutinations were observed during the first five minutes of contact (figures 3(B2) and (B3)). Agglutinations obtained were specific between VTG present in fish plasma and mAbs coated on the surface of particles.

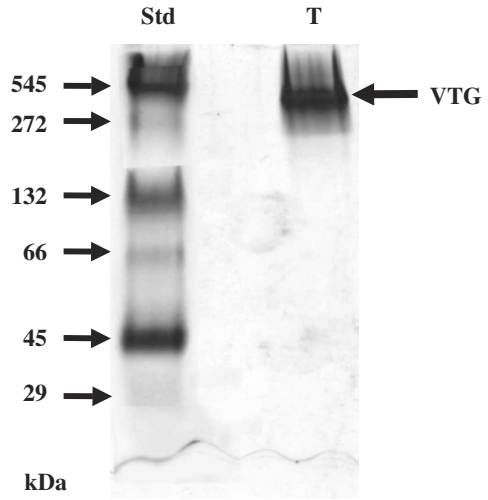

Figure 2. Protein composition analysis by native-PAGE 6% electrophoresis of VTG purified solution stained with Coomassie blue. Std: molecular mass standards; T: purified VTG solution from E2-treated fish; VTG: vitellogenin.

4. Conclusion

Environmental pollutants may be detected directly (physico-chemical methods) or indirectly by measuring the physiological modifications induced by those pollutants in organisms. In the case of aquatic pollutions by estrogen mimics, it is difficult to determine their presence in the environment by direct physico-chemical methods. In fact, this pollutant group contains molecules with different chemical characteristics: natural or synthetic estrogens and also molecules like PCBs, alkylphenols or phthalates. . .. Environmental estrogens may be measured by using semi-quantitative methods such as dip strip ELISA [35]. Nowadays, PCBs, alkylphenols, phthalates and other non-steroidic estrogen mimics are only measured by liquid or gas chromatography methods [36–38].

In that way, it is more useful to evaluate the physiological modifications induced by this heterogeneous group of molecules (steroidic or non-steroidic). The detection of the abnormal production of VTG by male or immature contaminated fish was a way to solve this problem [19–21]. VTG was initially detected by RIA [30] or ELISA [39–46]. These tests permitted the measurement of VTG production, but they needed the use of specific materials, and the transport of samples to the laboratory. In addition, these tests were expensive and time-consuming. Consequently, they were not useful for diagnosing, rapidly, the estrogen mimics pollution on the field.

The purpose of this study was to develop a general, rapid and specific method, capable of detecting abnormal VTG production in male and immature fish, which was chosen as a universal bioindicator of estrogen mimics aquatic pollutions [19, 21].

The development of a reverse latex agglutination test (rLAT) involved different preliminary steps. First, a new purification method of carp VTG was developed to obtain, in a single step, high quantities of pure and non-denatured VTG in order to produce specific monoclonal antibodies [31]. Some other authors have realized different chromatographic methods to obtain VTG, using several steps [45, 47–52] or a single step [46, 53], but in both cases the efficiency or the efficacy of these purification methods were not sufficient for optimal antibodies production.

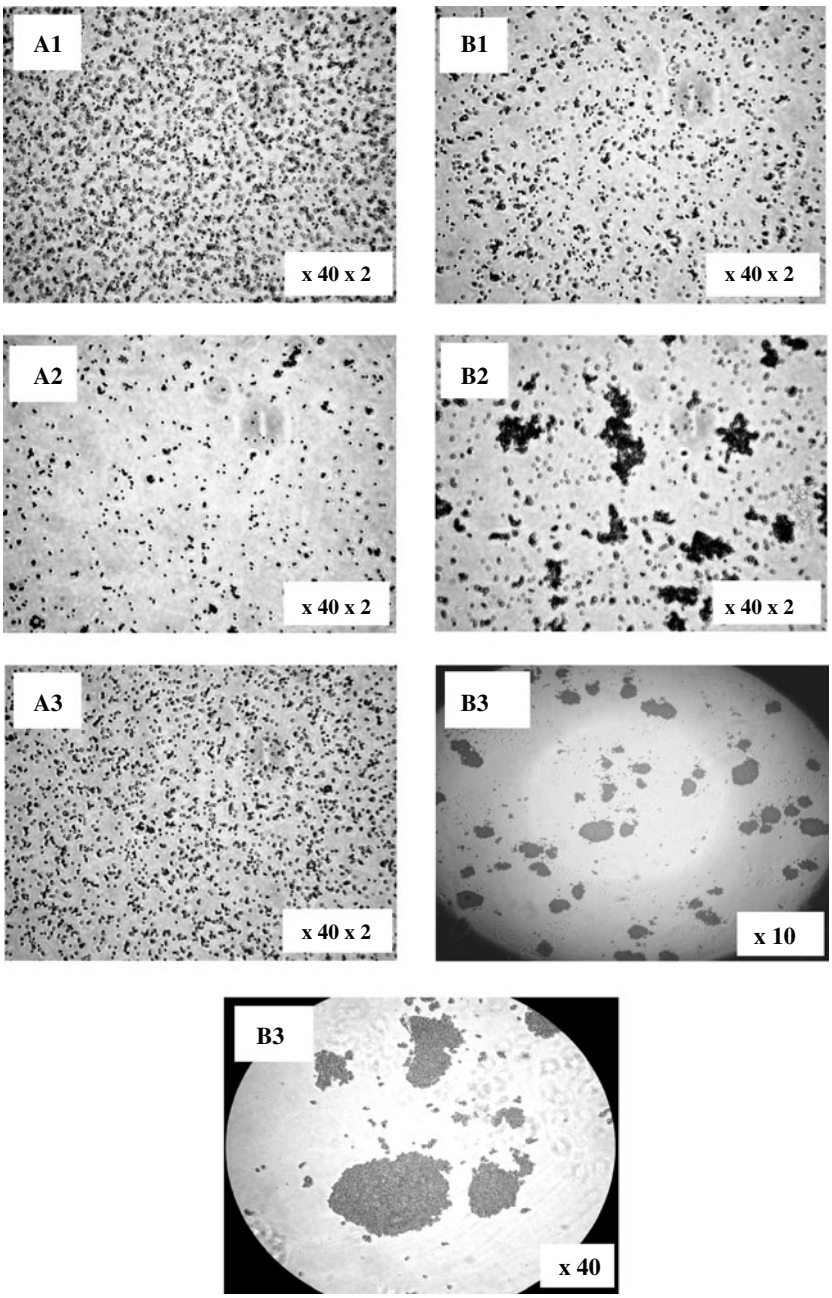

Figure 3. Vitellogenin reverse latex agglutination tests realized with non-immunized particles (A) or immunized particles (B) and carp plasma samples from non-treated male (1), E2-treated male (2) and vitellogenic female (3).

A monoclonal antibody specific to pure and non-denatured carp VTG was produced and used to recover latex particles (diameter: 1 μm). In this study, we showed that the immunoreactivity tests between VTG-rLAT and different plasma samples give only positive

responses with plasma coming from E2-treated fish and vitellogenic females (figures 3(B2) and (B3)). The reaction time was between one and five minutes, and was directly visualized. Some monoclonal antibodies were produced but only against Atlantic salmon (*Salmon salar*), Japanese medaka (*Orazias latipes*) and Rainbow trout (*Oncorhynchus mykiss*) species and were used just for ELISA testing [44, 46, 54].

Finally, diagnosis of male fish contamination by estrogen mimics, with VTG-rLAT, offers many advantages. First, it is a simple and rapid slide agglutination test, the results of which can be obtained within minutes of testing. Second, unlike traditional ELISA or RIA, the test does not require any expensive reagents, technical expertise and transport to a highly equipped laboratory, and hence was an adequate technique for fieldwork. Consequently, it will allow realizing, in the short-term, a cartography of polluted sites.

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