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Adaptation of an immunodot assay for multiple prey identification of squid paralarvae in field trials

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An optimized method, using polyclonal antibodies in an immunoassay, for prey detection in the diet of paralarvae of South African *Loligo reynaudii* is described. The study has increased the specificity of the antisera by determining the optimum antiserum dilutions and the detection limits of the antisera. Unfed laboratory-hatched paralarvae (negative control) were exposed to antisera and showed cross-reactions with polychaete antiserum.

INTRODUCTION

As is the case for other loliginids, the survival of paralarvae of *Loligo reynaudii* from South African waters probably greatly depends on the capability to catch their prey items (Chen et al., 1996). However, diet studies on field collected paralarvae remain problematic because of the maceration of stomach contents, making visual identification virtually impossible. Venter et al. (1999) developed a multiple detection system for the simultaneous identification of five putative zooplankton preys (*Calanus agulhensis*, euphausiids, cladoceran, polychaetes and fish larvae) in the guts of paralarvae of *Loligo reynaudii* by using an immunodot assay with polyclonal antibodies. Application of this method detected multiple predation (*C. agulhensis*, euphausiids and polychaetes) in six paralarvae. In Venter et al. (1999) it was proposed to increase specificity and to simplify the method in several ways to prepare it for large-scale field application, which is the aim of the present study.

MATERIALS AND METHODS

Detection limits and optimum antiserum dilution

Nitro-cellulose membranes were cut to the size of a 280 template, washed for 5 min in de-ionized water at room temperature and air dried before coating with antigens (antigens towards which antisera were produced are abbreviated in this study as X-Ag, and antisera that were raised against antigens are abbreviated as RbtαX). Every antigen amount was added to the membrane in duplicate or quadruplicate (dot volume=0.5 μl), starting with 1 μg per dot and halving the amount in every subsequent step by diluting the antigen homogenate with Tris buffered saline (TBS) (pH 7.4). The membrane was air dried to bind the antigens.

Every coated membrane was blocked by incubation in 30 ml blocking buffer for 1 h. Antigen coated membranes were incubated in 15 ml antiserum dilution (diluted in antibody buffer) for 2 h under constant shaking. Different membranes with the same antigen coatings were exposed to decreasing antiserum dilutions starting with the dilution where cross-reaction was found (1:1000 for RbtαClad, RbtαC.a and RbtαEuph; 1:100 for RbtαCop). For removal of low affinity bindings, the membranes were washed for 5 min, three times, in 20 ml Tween 20 washing solution. The membranes were then incubated in a 1000 fold dilution of monoclonal anti-rabbit IgG (γ-chain specific) peroxidase conjugate for 1 h under constant shaking, after which the membranes were again washed for 5 min, three times, in Tween 20 washing solution.

In order to make reactions between antisera and antigens visible, membranes were developed by incubation in substrate solution (0.06 g 4-chloro-1-naphthol dissolved in 20 ml of cold methanol added to 100 ml of TBS with 60 μl of H_2O_2). The membranes used in the optimizing experiments were incubated overnight. The membranes used in the screening of paralarvae were incubated in substrate solution until all the target antigens, added on the first row of the membrane, were clearly visible. Rinsing the membrane with de-ionized water terminated the development.

Unfed paralarvae as a negative control

Eggs of *Loligo reynaudii* were kept in an aquarium without food. Newly hatched paralarvae were removed from the aquarium and stored in liquid nitrogen. Eighteen paralarvae were dried and weighed at 10-min intervals. When the mass did not change in two subsequent intervals, the paralarvae were dried sufficiently. The dry mass of the paralarvae was determined to the nearest μg after which they were homogenated in an Eppendorf tube with 200 μl saline (0.9% NaCl). After homogenization, 300 μl saline was added and the homogenate was then centrifuged for 1 h in an Eppendorf centrifuge. The supernatant was transferred to a cryotube and stored at −70°C.

Paralarval homogenates were added to the membrane using a micropipette with a dot volume of 0.5 μl. On the membranes the first row consisted of undiluted paralarval homogenate, which was diluted, two times in every subsequent step, ending in an eight times diluted paralarvae

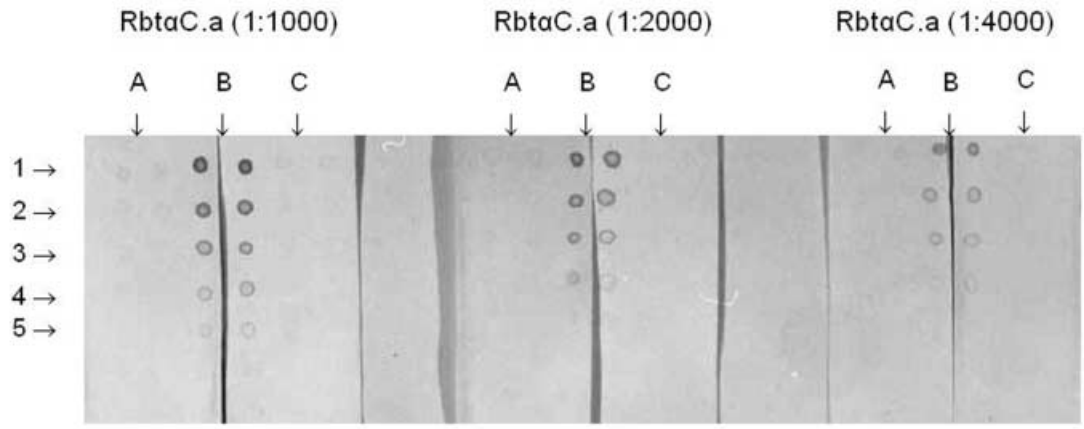

Figure 1. Antiserum Rbtα*C.a* at three different dilutions (1:1000; 1:2000 and 1:4000) screened against: (A) cross-reacting antigens Euph-Ag; (B) target antigen *C.a*-Ag; and (C) Pol-Ag. Antigens were added in duplo with amounts of (1) 1 μg/dot; (2) 0.5 μg/dot; (3) 0.25 μg/dot; (4) 0.125 μg/dot; (5) 0.0625 μg/dot.

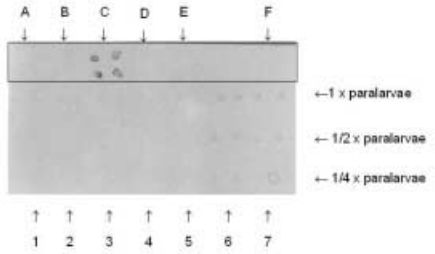

Figure 2. Unfed paralarvae screened against RbtαPol (1:1000). Target antigens added in quadruplicate, 0.1 μg per dot; (A) *C.a*-Ag; (B) Clad-Ag; (C) Pol-Ag; (D) Fish-Ag; (E) Euph-Ag; and (F) Cop-Ag. Unfed laboratory hatched paralarvae of *Loligo reynaudii* indicated by numbers 1–7.

homogenate. The membranes coated with paralarval antigens were screened according to the method described above, using the optimum antiserum dilutions. On every membrane the detection limit for the optimum antiserum dilution was added to serve as a positive control.

RESULTS

Different amounts of target antigen Clad-Ag (0.25; 0.125; 0.0625 and 0.03125 μg) and non-target antigen Euph-Ag (1; 0.5; 0.25; 0.125; 0.0625 and 0.03125 μg) were screened against four dilutions of antiserum RbtαClad (1:1000; 1:2000; 1:3000 and 1:4000). Cross-reactions between RbtαClad (1:1000) and Euph-Ag disappeared after 0.25 μg of Euph-Ag, while a reaction for target antigen Clad-Ag was still visible.

For screening of target antigen Euph-Ag and non-target antigen Clad-Ag against RbtαEuph, the same antigen amounts and antiserum dilutions were used. At a 2000-fold dilution the cross-reaction between RbtαEuph and Clad-Ag disappeared completely but the dots for target antigen Euph-Ag were clearly visible.

Rbtα*C.a* (1:1000; 1:2000 and 1:4000) was screened against target antigen *C.a*-Ag and cross-reacting antigens Euph-Ag and Pol-Ag (Figure 1). Antigen amounts were added to the membrane starting with 1 μg per dot and halving the amount per subsequent row resulting in 0.03125 μg per dot in the last row. At 0.125 μg of target antigen against a 1:2000 antiserum dilution, the cross-reactions with non-target antigens Euph-Ag and Pol-Ag completely disappeared.

Cross-reactions between RbtαCop and non-target antigens Pol-Ag, Euph-Ag and *C.a*-Ag were omitted using a Cop-Ag amount of 0.1 μg and a copepod antiserum dilution of 1:200. The cross-reaction between RbtαCop and *C.a*-Ag persisted in further dilution (1:400), although the dots for the target antigen Cop-Ag were clearer.

The optimum antisera dilutions determined in the former part were used to screen the unfed newly hatched paralarvae. Eighteen newly hatched paralarvae were screened. No cross-reactions were found between the antisera dilutions and paralarval antigens except for RbtαPol (Figure 2). For all paralarvae cross-reactions with RbtPol were found in the first row, corresponding with the undiluted paralarval homogenate. For two paralarvae the cross-reactions continued until the last row of dots, corresponding with a four times diluted paralarval homogenate.

DISCUSSION

Detection limits and optimum antiserum dilution

The detection limit is the smallest amount of target antigen detectable for the antiserum, without cross-reaction with non-target antigens. This amount of target antigen should be added to the membrane when paralarvae are screened, to serve as a positive control for the antiserum. Monospecificities towards 0.1 μg of target antigen were achieved at 1000-fold antiserum dilutions (RbtαFish and RbtαPol), and 2000-fold antiserum dilutions (Rbtα*C.a* and RbtαEuph). A 400-fold antiserum dilution for RbtαCop eliminated the cross-reaction with euphausiid and polychaete antigen but the cross-reaction with Rbtα*C.a* persisted. Cross-reaction between RbtαClad and Euph-Ag persisted at all dilutions, although dots that emerged at a 2000-fold dilution for target Clad-Ag were brighter than those for the cross-reaction with Euph-Ag. In future screening, these are the optimum antiserum dilutions and they will render most specific reactions with target antigens. The cross reactions that could not be omitted may be the result of the superior protein binding capacity of the nitrocellulose membrane since no cross-reactions were observed for the antisera when PVDF

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membranes were used at higher antigen concentrations and lower antiserum dilutions (Venter et al., 1999).

Unfed paralarvae as a negative control

Eighteen unfed paralarvae were screened against all six antisera using the optimum antiserum dilutions. Only RbtαPol showed cross-reaction with paralarval homogenate. The significant cross-reaction of RbtαPol with paralarval antigens was unexpected since both groups of organisms belong to different phyla and cross-reaction of an antiserum with a non-target antigen may indicate a phylogenetic relation (Feller et al., 1985). Cross-reaction is either a genuine property of the antibodies or is caused by antibodies produced against some unfolded protein contaminating the native protein used for immunization (Leder et al., 1994). A possibility, which needs additional experimentation, is that cross-reactions between paralarvae and RbtαPol were the result of yolk still present in the digestive system of the paralarvae. The paralarvae used in this study were newly hatched and most probably still had large amounts of yolk in their system.

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