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# A *Cryptobia salmositica* (Kinetoplastida: Sarcomastigophora) species-specific DNA probe and its uses in salmonid cryptobiosis

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ABSTRACT: A *Cryptobia salmositica* DNA probe (Cs-V1), of approximately 1.2 kilo base-pairs (kb), was developed from an avirulent strain of the pathogen. Nuclear DNA of *C. salmositica* was isolated, cleaved with *Hind* III, cloned, and labelled with non-radioactive digoxigenin. Cs-V1 hybridized specifically with *C. salmositica* DNA and there were no cross-hybridizations between the probe and DNA from *C. borreli*, *C. bullocki* and *C. catostomi*. A potentially useful identification/diagnostic technique for *C. salmositica* was developed using blood dried on filter paper. The dried blood DNA dot blot (DBDB) technique distinguished *C. salmositica* from *C. catostomi*, *C. bullocki* and *C. borreli* and it was also used for the diagnosis of *C. salmositica* infection in fish. The technique does not require DNA purification and the probe does not cross-hybridize with rainbow trout blood DNA. Less than 20 µl of fish blood (which contains approximately 100 000 organisms $ml^{-1}$) is required using the DBDB technique.

KEY WORDS: *Cryptobia salmositica* · DNA probe · Cryptobiosis · Diagnosis · Rainbow trout

## INTRODUCTION

*Cryptobia salmositica* is a pathogenic haemoflagellate of salmonids in western North America (Woo 1987, 1994). It causes cryptobiosis in experimentally infected rainbow trout *Oncorhynchus mykiss* (see Woo 1979). *C. salmositica* was presumed to be non-host-specific by some workers and hence it has been reported from all species of *Oncorhynchus* in North America and from many other species of freshwater teleosts (Becker & Katz 1965, Katz et al. 1966, Bower & Margolis 1984). However, Woo (1987) suggested that some *Cryptobia* in non-salmonid fishes on the west coast of North America may not be *C. salmositica*. Unfortunately, *C. salmositica* is morphologically similar to other *Cryptobia* spp. To better understand the epizootiology of cryptobiosis, it would be useful to have a relatively rapid and simple technique to distinguish *C. salmositica* from other *Cryptobia* spp. The purpose of the present study was to develop a *C. salmositica*-specific DNA probe to distinguish *C. salmositica* from other *Cryptobia* spp. and perhaps also to use it for diagnosis of infections in fishes.

One method to develop a DNA probe is by cloning highly repetitive species-specific nuclear DNA fragments (Kirchhoff & Donelson 1993). In most trypanosome species, the species-specific repetitive DNA fragments constitute up to 9% of nuclear DNA (Gonzalez et al. 1984, Kirchhoff & Donelson 1993). Thus, such repetitive specific DNA fragments are likely to be cloned and be used as species-specific probes (Massamba & Williams 1984, Kukla et al. 1987). In the present study, a *Cryptobia salmositica*-specific DNA probe was developed by cloning repetitive nuclear DNA fragments from a cloned vaccine strain of *C. salmositica*.

## MATERIALS AND METHODS

**Species of *Cryptobia*.** The pathogenic *Cryptobia salmositica* used in the present study was initially iso-

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lated from its leech vector and maintained in rainbow trout (Woo 1978). It was subsequently isolated from the blood of one of these trout and maintained in Minimum Essential Medium (MEM) at 10°C (Woo & Li 1990, Li & Woo 1991) for no more than 8 wk at which time it would still be pathogenic to trout (Woo & Thomas 1991). The pathogen was attenuated by continuous *in vitro* culture and used as a vaccine (Woo & Li 1990), which was cloned as described (Gradoni et al. 1983). Briefly, the number of parasites in a 2 wk culture was determined using a haemocytometer (Archer 1965) and diluted with the sterile culture medium until there were approximately 170 parasites $ml^{-1}$ (i.e. 1 parasite per droplet of 0.006 ml). A single droplet was dispensed into each of 7 wells (in a row) of a sterile 96-well (U-shape) culture plate which was covered and kept on ice. The droplets were examined carefully at 0, 24, 48 and 72 h using an inverted microscope (100 magnifications). Ice-cold fresh medium (0.2 ml) was added at 0 h to each of those wells which contained a single parasite. The 3 rows of wells on both sides of these 7 wells (with parasites) were filled with sterile distilled water to maintain a high humidity. Two weeks later, each of the established cloned *C. salmositica* vaccine strains was transferred to a Falcon culture flask (25 $cm^2$) containing 10 ml of the medium and incubated at 10°C as described earlier (Li & Woo 1991). Smears were made from each cloned parasite, stained with Giemsa's stain and examined using a compound microscope. All strains of *C. salmositica* were maintained continuously in MEM at 10°C.

*Cryptobia bullocki*, a pathogenic haemoflagellate of the summer flounder *Paralichthys dentatus*, was maintained in MEM at 10°C (Woo 1987) and *C. catostomi*, a nonpathogenic haemoflagellate of white suckers *Catostomus commersoni*, was isolated from an infected white sucker and maintained in the modified TDL-15 medium at 10°C (Li & Woo in press). *C. borreli*, a pathogenic haemoflagellate of the carp *Cyprinus carpio*, was maintained in infected carp at 24°C. The pathogen was kindly provided by Prof. W. B. van Muiswinkel, Wageningen Agricultural University, The Netherlands.

**Isolation of nuclear DNA.** The method for nuclear DNA isolation was modified from Lynn & Sogin (1988). Briefly, *Cryptobia bullocki*, *C. catostomi* and the 5 strains of *C. salmositica*, each consisting of approximately $100 \times 10^6$ parasites (determined using a haemocytometer; Archer 1965) in 20 ml of medium were collected by centrifugation at $2600 \times g$ for 20 min at 4°C in a Hitachi automatic high speed refrigerated centrifuge (Model CR20B2, Hitachi Koki, Tokyo, Japan). Parasites were suspended in 1 ml of STE buffer (0.1 M NaCl, 0.01 M Tris and 0.001 M EDTA, pH 8.0) and then lysed with additional sodium dodecyl sulfate (SDS) at 1% (w/v) of final concentration. Nuclear DNA was separated from kinetoplast DNA by centrifugation at 54 000 $\times g$ for 1 h (Fairlamb et al. 1978). Nuclear DNA was extracted, precipitated and resuspended in TE buffer (0.01 M Tris-HCl and 0.001 M EDTA, pH 8.0) (Sambrook et al. 1989). The amount of the nuclear DNA was determined using a Gene Quant RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd, England).

Fish blood (1 ml) with *Cryptobia borreli* was collected from an infected carp (infected intraperitoneally with 1 000 000 *C. borreli*) by a caudal vein puncture at 3 wk post infection. The blood was suspended in 10 ml of TE in a centrifuge tube and let stand for 30 min at room temperature. The supernatant (7 ml) containing approximately 20 000 000 parasites and 65 000 fish blood cells determined using a haemocytometer were transferred to another centrifuge tube. *C. borreli* with fish blood cells was collected by centrifugation as described earlier

**Preparation of a Cs-V1 probe from *Cryptobia salmositica* nuclear DNA.** The purified nuclear DNA (5 µg) of the cloned vaccine strain (Cs-c) of *C. salmositica* was cleaved with a restriction endonuclease *Hind* III (10 U $\mu g^{-1}$ DNA; Promega, Madison, WI, USA) and fragments were separated using electrophoresis in 0.8% agarose gel at 100 v for 2 h (PowerPac 300; Bio-Rad, Melville, NY, USA). A distinct DNA band (approximately 1.2 kilo base-pairs; kb) stained with ethidium bromide was sliced and extracted from the agarose gel by centrifugation (Heery et al. 1990). The DNA fragment named Cs-V1 was cloned using a plasmid vector (Pbluescript, SK, 2.9 kb; Stratagene, La Jolla, CA, USA), amplified in *Escherichia coli* (DH5αF; GIBCOBRL, Burlington, ON, Canada) and isolated as described (Sambrook et al. 1989). The Cs-V1 fragments were labelled with non-radioactive digoxigenin-11-dUTP (DIG) according to the manufacturer's instructions (Boehringer Mannheim, Laval, PQ, Canada).

**Southern blot hybridizations.** Nuclear DNA (0.5 µg) from *Cryptobia salmositica*, *C. borreli*, *C. bullocki* and *C. catostomi* was cleaved with *Hind* III, and were separated by electrophoresis in 1.0% agarose gel at 24 V for 12 h. DNA fragments (target DNA) in the gel were denatured in a denature solution (1.5 M NaCl, 0.5 N NaOH) and neutralized in a neutralization solution (1.0 M Tris-HCl and 1.5 N NaCl, pH 8.0). The denatured target DNA fragments were transferred from the gel onto a nylon membrane (Boehringer Mannheim) using a VacuGene XL blotting unit at 40 millibars (Pharmacia LKB Biochrom Ltd, England) and were fixed onto the membrane at 80°C for 2 h (Southern 1975). DNA hybridization with the DIG-labelled Cs-V1 probe was performed according to the manufacturer's instructions (Boehringer Mannheim).

**Dried blood DNA dot blot (DBDB) technique.** *Cryptobia bullocki*, *C. catostomi* from cultures and the pathogenic *C. salmositica* (with trout blood) from an infected rainbow trout and *C. borreli* (with carp blood) from an infected carp were concentrated as described earlier and added to ice-cold heparinized naive trout blood which was preheated at 45°C for 30 min to inactivate the complement (Sakai 1981). The short term heat treatment did not cause visible changes (e.g. haemolysis) to the blood. The number of parasites was estimated using a haemocytometer and was diluted with trout blood until there were approximately 1 000 000 parasites $ml^{-1}$ of blood. A volume of 0.02 ml blood and parasites from each dilution was dispensed onto a Whatman filter paper (No. 5), air-dried and stored in a sealed plastic bag at –20°C until used.

A disc (6 mm diameter) of the filter paper with dried blood and parasites was cut with a paper puncher and the disc was incubated with 0.2 ml of TE in a microcentrifuge tube at 24°C for 1 h on a shaker (60 cycles $min^{-1}$). Each disc was rinsed with another 0.2 ml of TE. The DNA in the pooled elute (0.4 ml) was extracted and precipitated (as described in Sambrook et al. 1989). Each DNA sample from 1 disc was resuspended in 0.005 ml of TE, blotted onto the nylon membrane and air-dried at 24°C. The DNA was denatured by laying the nylon membrane on a Whatman filter paper (No. 5) saturated with a denature solution for 10 min and then on another filter paper saturated with a neutralization solution for another 10 min. The DNA was fixed onto the membrane and hybridized with the Cs-V1 probe as described earlier. Similarly, purified nuclear DNA (see 'Isolation of nuclear DNA') of pathogenic *Cryptobia salmositica* (7.5 ng), *C. bullocki* (9.5 ng), *C. catostomi* (8.9 ng), and crude DNA of *C. borreli* (9.0 ng) and fish blood DNA (6.7 µg) were also blotted on filter paper and hybridized with the probe.

**Standardization of the DBDB technique.** The cloned vaccine strain of *Cryptobia salmositica* (Cs-c) from culture was washed once in STE and added to rainbow trout blood. The number of *C. salmositica* was determined using a haemocytometer and this was subsequently diluted with trout blood so that the final concentrations ranged from 1000 to 1 000 000 *C. salmositica* $ml^{-1}$. Blood at each dilution was examined for parasites using the haematocrit centrifuge technique (HCT; Woo 1969, Woo & Wehnert 1983). Also, each blood dilution (0.02 ml) was dispensed onto Whatman filter paper (No. 5) and air-dried. The DBDB technique was applied to the dried blood as described earlier.

**Diagnosis of *Cryptobia salmositica* in experimentally infected fish using the DBDB technique.** Four rainbow trout (226.3 ± 12.7 g) were tagged and each was infected intraperitoneally with 100 000 pathogenic *C. salmositica* from an infected trout. The fish were maintained in continuous fresh well water with aeration at 10 ± 2°C. Blood samples (0.4 ml $fish^{-1}$) were collected weekly by caudal vein punctures before and after the infection (Li & Woo 1991). Parasites were detected using the HCT and counted using a haemocytometer. A volume of 0.02 ml of blood sample was dispensed onto Whatman filter paper (No. 5), air-dried and stored in sealed plastic bags at –20°C until the DBDB was performed.

## RESULTS

In the cloning experiment, 3 of 7 wells contained a single *Cryptobia salmositica*, the other 4 wells either contained 2 parasites (in 3 wells) or no parasites (in 1 well). The 3 cloned vaccine strains were named Cs-c, Cs-d and Cs-e, and they were morphologically similar to the uncloned vaccine strain. The generation time of the cloned strain was approximately 48 h during the first 3 d after cloning.

One distinctive DNA band (approximately 1.2 kb) cleaved with *Hind* III was consistently found only in *Cryptobia salmositica* nuclear DNA (vaccine and pathogenic strains) but not in other *Cryptobia* spp. (Fig 1). The DNA was cloned, amplified, isolated and labelled with non-radioactive DIG to produce the Cs-V1 probe.

The Cs-V1 probe hybridized with *Hind* III cleaved nuclear DNA fragments from all 5 strains of *Cryptobia salmositica* (Southern blot hybridization). The hybridization band was intense with 1.2 kb DNA fragments (Fig. 2). Another *C. salmositica* DNA fragment (approx-

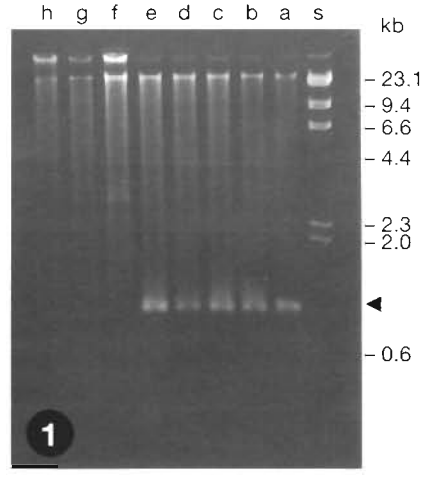


Fig. 1. *Cryptobia* spp. Electrophoresis (1.0% agarose) of *Hind* III cleaved nuclear DNA fragments. Lane a: pathogenic strain of *C. salmositica*; lane b: vaccine strain of *C. salmositica*; lanes c to e: 3 *C. salmositica* vaccine clones (Cs-c, Cs-d and Cs-e); lane f: *C. borreli*; lane g: *C. bullocki*; lane h: *C. catostomi*; lane s: DNA standard size markers (arrow indicates 1.2 kb DNA fragment)

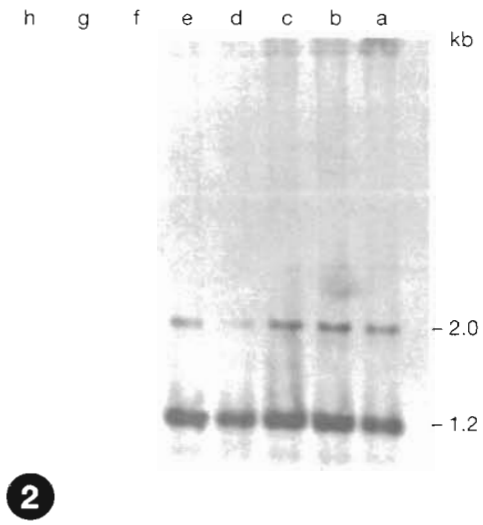


Fig. 2. *Cryptobia* spp. Hybridization of cleaved nucleic DNA fragments with DIG-labelled Cs-V1 probe. Lane a: pathogenic strain of *C. salmositica*; lane b: vaccine strain of *C. salmositica*; lanes c to e: 3 *C. salmositica* vaccine clones (Cs-c, Cs-d and Cs-e); lane f: *C. borreli*; lane g: *C. bullocki*; lane h: *C. catostomi*

imately 2.0 kb) also hybridized with the Cs-V1 probe but the band was not as intense as with the 1.2 kb DNA fragments. There were no cross-hybridizations between the Cs-V1 probe and cleaved DNA from *C. catostomi*, *C. borreli*, or *C. bullocki*.

Using the DBDB technique, the Cs-V1 probe hybridized only with homologous target *Cryptobia salmositica* DNA, which was either purified nuclear DNA (of parasite from cultures) or crude DNA (parasites in fish blood). There were no cross-hybridizations between the Cs-V1 probe and DNA from other *Cryptobia* spp. which were either purified nuclear DNA or crude DNA. There was also no cross-hybridization between the Cs-V1 probe and a massive amount of fish blood DNA (Figs. 3 & 4). *C. salmositica* (in the experiment on standardization of the DBDB technique) was detected using the HCT when there were about 1000 to 1 000 000 parasites $ml^{-1}$ in fish blood. However, parasite DNA (in not more than 0.02 ml of dried fish blood) from filter paper was detected using the DBDB technique when there were approximately 100 000 parasites $ml^{-1}$ of fish blood (Fig. 5).

Using the HCT (Table 1), parasites were detected at 2 wk post-infection in 3 experimentally infected fish (Nos. 2 to 4) and at 4 wk post-infection in the remaining fish (No. 1). However, *Cryptobia salmositica* DNA was first detected using the DBDB technique at 3 wk post-infection in 3 fish (Nos. 2 to 4; when there were 1 200 000 to 1 600 000 parasites $ml^{-1}$ blood) and at 5 wk post-infection in Fish No. 1 when there were


Fig. 3. *Cryptobia* spp. DNA hybridization in dried blood on filter paper (DBDB technique); each dot was from 2 filter paper disc (3 replications). Lane a: *C. salmositica* crude DNA; lane f: *C. borreli* crude DNA; lane g: *C. bullocki* crude DNA; lane h: *C. catostomi* crude DNA; lane i: naive trout blood DNA

900 000 parasites $ml^{-1}$ of blood. Subsequent determinations up to 11 wk post-infection showed very similar results using the 2 techniques. Fish No. 4 from 8 wk post-infection had low numbers of parasites and they were detected using the HCT but were negative using the DBDB technique.

## DISCUSSION

The present results suggests that the *Hin*d III restriction Cs-V1 fragments are highly repetitive and are *Cryptobia salmositica*-specific. One distinct *C. salmositica*-specific DNA fragment (approximately 1.2 kb) was successfully cloned from nuclear DNA after it was cleaved using *Hin*d III. The fragment (Cs-V1) after amplification was used to develop a DNA probe.

The Cs-V1 fragments hybridized specifically with purified or crude homologous target *Cryptobia salmositica* DNA. There were no cross-hybridizations with purified or crude nuclear DNA from *C. borreli*, *C. bul-*

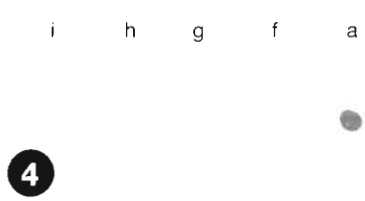


Fig. 4. *Cryptobia* spp. DNA dot blot hybridization with Cs-V1 probe. a: *C. salmositica* nuclear DNA (7.5 ng $dot^{-1}$); f: *C. borreli* crude DNA (9.0 ng $dot^{-1}$; mixed with carp DNA); g: *C. bullocki* nuclear DNA (9.5 ng $dot^{-1}$); h: *C. catostomi* nuclear DNA (8.9 ng); i: fish blood DNA (6.7 µg $dot^{-1}$)

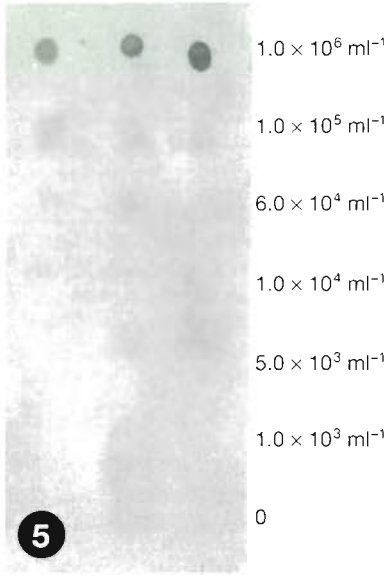


Fig. 5. Standardization of DBDB technique using trout blood with known numbers of *Cryptobia salmositica* dried on filter paper; each dot was from 1 filter paper disc (3 replications)

*locki*, *C. catostomi* and fish blood DNA. This indicates that the Cs-V1 fragment can be used as a DNA probe to distinguish *C. salmositica* from other species of *Cryptobia*. Similar results were also found in some species of mammalian trypanosomes; the highly repetitive species-specific DNA fragments constitute up to 9% of the total nuclear DNA (Gonzalez et al. 1984, Kirchhoff & Donelson 1993). Hence, such highly repetitive DNA fragments are likely to be cloned and subsequently used as species-specific DNA probes (Massamba & Williams 1984, Kukla et al. 1987).

Table 1. Detection of *Cryptobia salmositica* and parasite DNA in the blood of experimentally infected rainbow trout using the haematocrit centrifuge technique (HCT) and the dried blood DNA dot blot (DBDB) technique. For HCT, parasitaemias are given as positive (+) where just detected or as × 1 000 000 parasites $ml^{-1}$ blood where measurable. –ve: negative DBDB reaction; +ve: positive DBDB reaction; ND: not determined

| Weeks post-infection | Fish No. 1 | | Fish No. 2 | | Fish No. 3 | | Fish No. 4 | |
|---|---|---|---|---|---|---|---|---|
| | HCT | DBDB | HCT | DBDB | HCT | DBDB | HCT | DBDB |
| 0 | 0 | –ve | 0 | –ve | 0 | –ve | 0 | –ve |
| 1 | 0 | –ve | 0 | –ve | 0 | –ve | 0 | –ve |
| 2 | 0 | –ve | (+) | –ve | (+) | –ve | (+) | –ve |
| 3 | 0 | –ve | 1.35 | +ve | 1.16 | +ve | 1.56 | +ve |
| 4 | (+) | –ve | 3.75 | ND | 2.88 | ND | 3.50 | ND |
| 5 | 0.85 | +ve | 2.00 | ND | 1.00 | ND | 1.00 | ND |
| 6 | 2.65 | +ve | 0.63 | ND | (+) | ND | 0.08 | ND |
| 7 | 0.99 | ND | 0.24 | ND | (+) | ND | 0.90 | ND |
| 8 | 0.45 | +ve | 0.35 | +ve | (+) | –ve | 0.43 | +ve |
| 9 | 0.37 | +ve | 0.78 | +ve | 0 | –ve | 1.40 | +ve |
| 10 | 1.03 | +ve | 2.50 | +ve | 0 | –ve | 0.90 | +ve |
| 11 | 2.05 | +ve | 0.70 | +ve | 0 | –ve | 0.38 | +ve |

The present results indicate that the Cs-V1 probe is *Cryptobia salmositica*-specific, but not strain-specific. It may also be used for diagnosis of *C. salmositica* infection in rainbow trout. The DBDB technique, using dried blood from filter paper, was developed as a field technique to study the epizootiology of salmonid cryptobiosis. The technique can be used to identify the reservoir host(s) of *C. salmositica* and to determine the prevalence of infection in fish. Fish blood can be collected and dried on filter paper in the field and brought back to the laboratory for processing. The technique does not require DNA purification, and the blood sample collection and storage are relatively simple and rapid under field conditions.

The sensitivity of a DNA probe is related to the number of target DNA fragments and the method of labeling the probe (Barker 1994). Using a radioactive $^{32}P$-labelled nuclear DNA probe, trypanosome DNA was detected when there were 10 000 (devoid of blood) or 100 000 (mixed with host blood) trypanosomes per dot (Massamba & Williams 1984, Gibson et al. 1988). In the present study, *Cryptobia salmositica* was detected in less than 0.02 ml of the blood using a non-radioactive DIG-labelled probe when there were approximately 100 000 parasites $ml^{-1}$ blood. The detection using radioactive $^{32}P$-labelled probes is related to the number of copies of the 32P probe bound to the target DNA. However, the non-radioactive DIG-labelled probe is not only related to the number of target DNA fragments, but is also amplified by the antigen and antibody cascade reaction. This amplification increases its sensitivity (Hill et al. 1991). The polymerase chain reaction has been used to further increase the sensitivity of DNA detection (Sturm et al. 1989, Barker 1994), however, it is time-consuming to perform and it requires expensive and highly technical equipment (Barker 1994), which is at present not readily available in many laboratories.

The present study showed that the DBDB technique was less sensitive than the HCT for detection of *Cryptobia salmositica* in fish blood. However the HCT may not be a very practical field technique when large numbers of samples have to be examined in a relatively short time. Also the blood has to be kept cold and examined soon after collection (Woo & Wehnert 1983). In the present study fish blood DNA mixed with the target *C. salmositica* DNA might have reduced the sensitivity of DBDB by covering some of the target DNA or by decreasing the amount of target DNA bound to the

membrane as was suggested earlier for trypanosomes (Massamba & Williams 1984). Nevertheless, the DBDB technique is a reliable and rapid method for the identification of *C. salmositica* although its use as a diagnostic technique may require further studies.

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