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Temperature, pH and bile dependent *in vitro* cultivation of *Hexamita salmonis* from rainbow trout *Oncorhynchus mykiss* intestine

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ABSTRACT: The intestinal diplomonadid flagellate *Hexamita salmonis* was isolated from the intestinal contents of freshwater reared rainbow trout *Oncorhynchus mykiss* in Denmark and maintained in axenic cultures in the laboratory. A cell culture medium (MEM) or MEM supplemented with a bile solution (Keister's modified TYI-S-33) and penicillin, gentamicin and nystatin (mycostatin) was used as culture substrate. Temperature, pH and bile requirements for laboratory maintenance were investigated. Optimum temperature for *H. salmonis* population increase was determined to be 10°C. Satisfactory although decreased growth was found at 5°C. At 15°C and 20°C flagellate cells survived but did not exhibit significant population growth. In the pH range from 4 to 10 parasite survival was seen from 5.5 to 9.0, although optimum pH conditions for population increase were pH 7.5 to 8.0. Bile in low concentrations (30 to 960 mg l^{-1}) enhanced flagellate propagation slightly. This study on the basic requirements explains the occurrence of the flagellate in rainbow trout in less warm seasons and biotopes as well as particular microhabitats in the host.

KEY WORDS: *Hexamita salmonis* · Parasite · Protozoa Diplomonadida · *In vitro* cultivation Rainbow trout · *Oncorhynchus mykiss* · MEM · TYI-S-33

INTRODUCTION

The intestinal diplomonadid flagellate *Hexamita salmonis* Moore, 1923, has been recorded from a range of salmonid hosts (Lom & Dyková 1992, Woo & Poynton 1995) and is often found associated with pathogenic conditions in hatchery fish (Moore 1923, Davis 1956, Ferguson 1979). The pathogenicity of this protozoan has been doubted by some authors and experiments have been performed to elucidate this point (Uzmann et al. 1965). However, a crucial point in this dispute is to determine the basic growth requirement of *H. salmonis* itself, information which for the most part is lacking. This will also contribute to an understanding of the parasite's site selection in the host and the seasonal variation in occurrence (Rosengarten 1985, Uldal & Buchmann unpubl.). A valuable approach in this regard is *in vitro* cultivation of the organism in question. Following development of culture methods for the related genus *Giardia* (Karapetyan 1962, Meyer 1976, Keister 1983), a wide range of isolates have been characterized biochemically and biologically. This has demonstrated differing strain characteristics (Thompson et al. 1994). Similar variations are likely to be found in *H. salmonis*. The first cultivation of a *H. salmonis* strain using human cord serum in a culture medium was achieved by Uzmann & Hayduk (1963) and subsequently an evaluation of the pathogenicity of particular isolate was performed (Uzmann et al. 1965). In the present paper we present a new easy culture technique for *H. salmonis*, the isolation and cultivation of a Danish strain of this protozoan species and the subsequent determination of temperature, pH and bile requirements for this particular parasite strain.

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MATERIALS AND METHODS

Parasite isolation. A total of 8 specimens of *Oncorhynchus mykiss* fry (4 to 5 cm total body length) heavily infected with *Hexamita salmonis* and kept at 7.8°C were dissected. The entire intestine including the pyloric region with content was removed from each fish and placed in a 250 ml screw cap glass culture flask containing 145 ml culture medium. The culture flask with medium and 8 intestines were then incubated at 7.8°C for 28 d. Thereafter the parasite culture was subcultivated by transferring the cell culture suspension to screw cap glass tubes (dilution 1:3) or screw cap glass culture flasks with new medium, which were incubated for 5 d at 7.8°C. From this stage further subcultivations could be conducted by transferring the parasite cell culture to new medium (dilution 1:5). Addition of nitrogen gas was not used.

Culture medium. The medium was composed of Eagle's Minimum Essential Medium (MEM) (Sigma Cat. No. M 0643) supplemented with 10% foetal bovine serum (Gibco BRL Cat. No. 10106-078), inactivated at 56°C for 30 min, and 4% Keister's modified bile-supplemented TYI-S-33 medium (Keister 1983). For isolation of parasites pH was adjusted to 7.5. Inhibition of bacterial and fungal growth was achieved by adding the antibiotics penicillin (2000 U ml^{-1}), gentamicin (50 µg ml^{-1}) and nystatin (mycostatin) (100 U ml^{-1}).

This medium but without the addition of bile-supplemented TYI-S-33 was used when studying the influence of bile addition on the growth of *Hexamita salmonis* populations.

Parasite cell counting. To determine the concentration of *Hexamita salmonis* cells in culture flasks the content was thoroughly stirred and aliquots of 1 to 15 µl were placed on slides under a 12 × 12 mm coverslip and counted using a light microscope at a magnification of 100 to 250×. Only motile cells were included and each determination included 3 to 5 subsamples. The concentration of cells ml^{-1} was then calculated.

Determination of temperature optimum. A parasite cell culture (5000 cells ml^{-1}) was established and duplicates of 88 ml cell culture in 250 ml glass flasks were placed in thermostat-regulated incubators at 5, 10, 15 and 20°C without light. Parasite cell concentrations in individual culture flasks were determined at intervals of a few days for 18 d. The flagellates survived well the transfer from 7.8°C to this range of temperatures.

Determination of pH optimum. Parasite cell cultures (150 ml, 10000 cells ml^{-1}) were established in 250 ml flasks and pH was adjusted gradually over 48 h from 7.5 to the appropriate final incubation pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 or 10.0. The cultures were incubated at 10°C in a thermostat-regulated incubator and parasite cell concentrations were determined at intervals of a few days for the following 14 d.

Determination of bile requirements. A total of 15 cell cultures (100 ml, 20000 cells ml^{-1}) were established. Two cultures did not contain bile, 2 contained 4% Keister's medium, and the remaining 11 flasks contained bile (bovine Sigma B-8381) in increasing concentrations from 30 to 29120 mg l^{-1}).

RESULTS

Temperature optimum

At both 5 and 10°C the cell cultures multiplied rapidly. After inoculation a lag phase of a few days was observed. This was then replaced by an exponential growth phase and finally a maximum was seen, followed by a more or less slight decrease (Fig. 1).

The population increase of *Hexamita salmonis* was pronounced and fastest at 10°C. Within 10 d the duplicate cell cultures reached a maximum of 250000 and 290000 cells ml^{-1}, respectively. At 5°C the growth curve was less steep and the maximum cell concentrations (180000 and 215000 cells ml^{-1}) were achieved only after 15 d.

At both 15 and 20°C an initial decline in parasite count was replaced after 10 d by a steady low-count parasite population (less than 8000 and 2000 cells ml^{-1}, respectively).

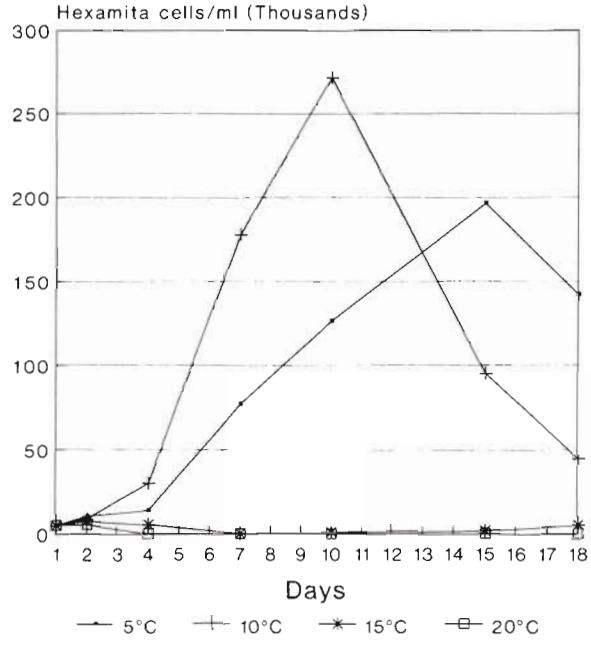

Fig. 1 Temperature dependent, *in vitro* population increase of *Hexamita salmonis* in MEM with 4% Keister's medium and 10% foetal calf serum (pH 7.5) (means of duplicate groups)

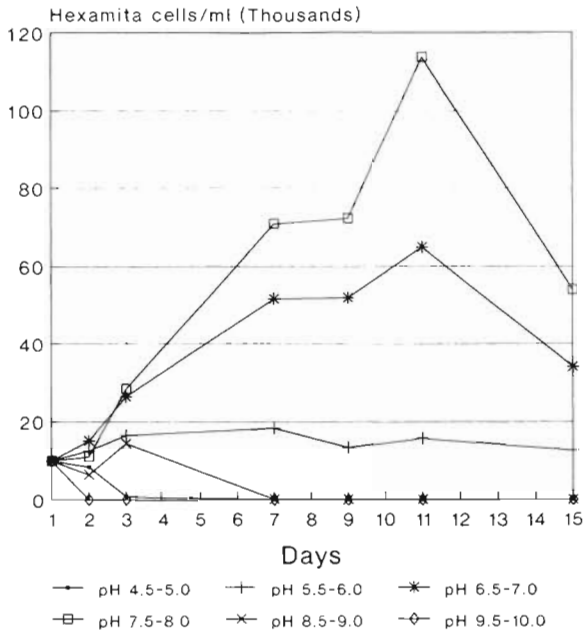

Fig. 2. The pH dependent, *in vitro* population increase of *Hexamita salmonis* in MEM with 4% Keister's medium and 10% foetal calf serum (temperature 10°C) (means of 2 groups in each interval)

pH optimum

The fastest growth rates were observed at pH 7.5 and 8.0, at which cell counts above 110000 cells ml^{-1} were seen (Fig. 2). Above this pH (pH 8.5 and 9.0) cell counts decreased rapidly to below 100 cells ml^{-1}. At lower pH values (pH 6.0 and 5.5) maximum counts did not exceed 27000 and 10000 cells ml^{-1}, respectively (Fig. 2). The lowest pH values were unsuitable for *Hexamita salmonis*. No survival of cells was observed after 3 d at pH 4.5 and 5.0, and at pH 4.0 all cells were killed within 24 h. This was also observed at pH 9.5 and 10.0.

Bile requirements

All cell cultures replicated slowly during the first 5 d whereafter cultures containing from 30 to 960 mg bile l^{-1} exhibited superior growth performance compared to either higher or no bile concentration (Fig. 3). The MEM alone supported satisfactory growth of *Hexamita salmonis* but addition of moderate bile amounts or 4% Keister's medium seemed to augment growth moderately. Bile concentrations of 3600 and 14000 mg l^{-1} were associated with a steady decline in cell counts during the 8 d following inoculation. Concentrations above this level (29120 mg l^{-1}) were lethal to the cell cultures within a few hours.

DISCUSSION

A number of field studies have indicated that the *Hexamita salmonis* organisms in salmonids exhibit seasonal occurrence (Moore 1923, Davis 1956) and select particular microhabitats in the host intestine (Moore 1923, Davis 1956, Ferguson 1979, Poynton 1986, Buchmann et al. 1995, Uldal & Buchmann unpubl.). Satisfactory explanations of these observations are, however, not available. The present *in vitro* cultivation method provides us with a tool to elucidate the basic biological characteristics of this diplomonadid flagellate.

Using this method, it was evident that temperature has a crucial influence on *Hexamita salmonis* multiplication. With inferior growth at 15 and 20°C, optimal performance at 10°C and significant growth at 5°C it is evident that this flagellate is adapted to lower temperatures. This can explain the pronounced occurrence of the parasite in winter, spring and early summer (Davis 1956, Buchmann et al. 1995) and its subsequent survival in summer. Comparative temperature experiments were not presented by Uzmann & Hayduk (1963) although they chose to cultivate the organism at 10°C.

Hexamita salmonis is known to occupy restricted microhabitats in the host's intestinal tract (Moore 1923,

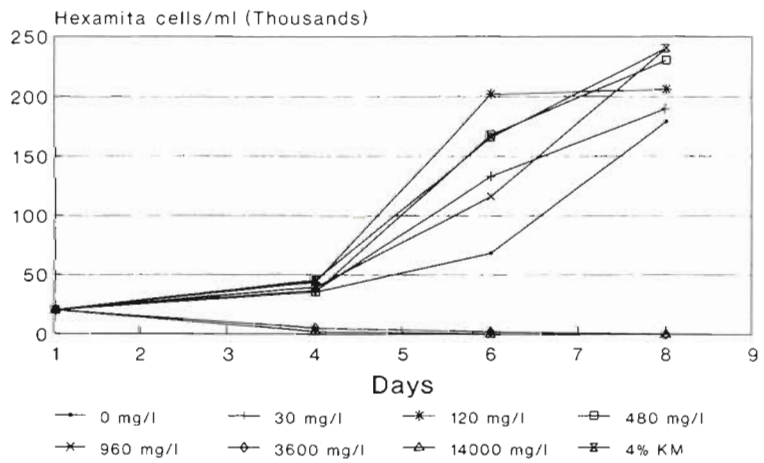

Fig. 3. Bile dependent, *in vitro* population increase of *Hexamita salmonis* in MEM with 10% foetal calf serum. Bile was added in concentrations from 30 to 14000 mg l^{-1} (pH 7.5). The 29120 mg l^{-1} culture died within a few hours. Cultures with no bile addition and with 4% Keister's medium (KM) (means of duplicates) are also shown

Uldal & Buchmann unpubl.). Whether this is due to the parasite's special requirements (Uldal & Buchmann unpubl.) or related to the age of the infection (Moore 1923) is unknown. The present observations on pH and bile dependent growth suggest that *H. salmonis* is well adapted to the anterior part of the intestine. Relatively low pH values (pH 6) (due to the inlet of acidic stomach contents into the anterior intestine and pyloric region) are well tolerated by the organism, although neutral and slightly basic conditions are optimal (7.0 to 8.0). Although the present *in vitro* studies demonstrated that serum-complemented MEM alone will support growth satisfactorily, the addition of low amounts of bile augments flagellate population increase. This could also explain the location of these diplomonadids in the anterior part of the intestine. The TYI-S-33 medium (Keister 1983) alone did not appear to support growth of the *H. salmonis* strains studied (data not shown). Judging from the maximum number of cells recorded in our culture flasks (290000 cells ml^{-1}) it appears that the MEM medium supplemented with Keister's medium is superior to the medium (100000 cells ml^{-1}) used by Uzmann & Hayduk (1963). However, parasite strain differences and incubation method may influence this variation.

During recent years systemic infections of salmonids with *Hexamita salmonis*-like organisms have been reported (Mo et al. 1990, Kent et al. 1992, Poppe et al. 1992). Biological, biochemical and molecular comparative studies using the described *in vitro* technique could contribute significantly to the elucidation of relations and affiliations between intestinal and systemic organisms.

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