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# Detection and distribution of ostreid herpesvirus 1 in experimentally infected Pacific oyster spat

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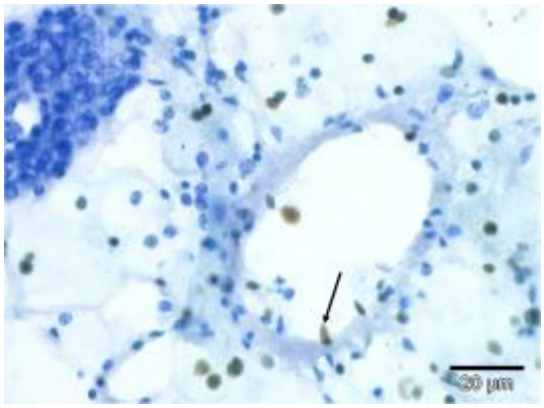
**Abstract :**

High mortality rates are reported in spat and larvae of Pacific oyster *Crassostrea gigas* and associated with ostreid herpesvirus 1 (OsHV-1) detection in France. Although the viral infection has been experimentally reproduced in oyster larvae and spat, little knowledge is currently available concerning the viral entry and its distribution in organs and tissues. This study compares OsHV-1 DNA and RNA detection and localization in experimentally infected oysters using two virus doses: a low dose that did not induce any mortality and a high dose inducing high mortality. Real time PCR demonstrated significant differences in terms of viral DNA amounts between the two virus doses. RNA transcripts were detected in oysters receiving the highest dose of viral suspension whereas no transcript was observed in oysters injected with the low dose. This study also allowed observing kinetics of viral DNA and RNA detection in different tissues of oyster spat. Finally, viral detection was significantly different in function of tissues ($p < 0.005$), time ($p < 0.005$) with an interaction between tissues and time ($p < 0.005$) for each probe.

**Graphical abstract**



**Highlights**

► Viral DNA/RNA localization were studied in oysters by ISH using two virus doses. ► Viral replication was not detected in oysters receiving the lowest virus dose. ► Virus was mainly observed in mantle, gills and heart by ISH using the highest dose. ► Viral detection was significantly different in function of tissues and time.

**Keywords** : *Crassostrea gigas* ; In situ hybridization ; Ostreid herpesvirus 1 ; Viral DNA ; Viral RNA

## Introduction

In France, ostreid herpesvirus 1 (OsHV-1) detection is associated with high mortality rates in Pacific oyster *Crassostrea gigas* spat (Garcia et al., 2011; Renault et al., 1994). OsHV-1 is a double strand DNA virus which belongs to Malacoherpesvirus family (Davison et al., 2005). Studies have been achieved based on the development of molecular diagnosis assays including PCR and *in situ* hybridization in order to detect and localize OsHV-1 in *C. gigas* (Burge and Friedman, 2012; Pepin et al., 2008; Barbosa-Solomieu et al., 2004;). Recently, an *in situ* hybridization (ISH) technique using Digoxigenin (Dig)-labeled RNA probes was developed in order to provide information concerning the expression of three OsHV-1 genes in *C. gigas* tissues 26 hours post infection (hpi) (Corbeil et al., 2015). ISH is a suited method for DNA and RNA detection in virus infected cells and it has been applied to viral disease diagnosis in aquatic organisms (Huang et al., 2004; Kleeman and Adlard, 2000; Walton et al., 1999). Although OsHV-1 has been investigated for the past decade, several aspects of pathogenesis still need to be clarified such as the viral entry, sites where viral replication occurs initially and distribution of virus in persistent or acute infection. Clarification of these aspects could provide a better understanding about OsHV-1 infection and why some oysters do not develop the infection and do not demonstrate mortality. Arzul and co-workers (2002) have reported the detection of viral DNA and proteins in asymptomatic *C. gigas* collected in the field and suggested that the virus is able to persist in host after primary infection without inducing mortality. More recently, Segarra and collaborators (2014b) reported the detection of viral DNA and RNA in infected oysters with or without associated mortality. Nevertheless, OsHV-1 latency has not been characterized by latency-associated transcripts (LATs) unlike vertebrate herpesviruses (Jones, 2003). A

60 protocol based on intramuscular injection of OsHV-1 suspension was recently
61 developed (Schikorski et al., 2011) that allowed reproduction of the virus in
62 experimental conditions in Pacific oyster spat. This approach appears as a valuable
63 tool to better understand interactions between the virus and its host.

64 The major aim of this study was to localize virus DNA and RNA in tissues of *C. gigas*
65 during an experimental infection in Pacific oyster spat. This study also investigated
66 the OsHV-1 detection (DNA and RNA) in animals injected with high or low infection
67 levels. For this purpose, two doses of a viral suspension were tested: a high dose (H)
68 with associated mortality and a low dose (L) without mortality. The infection system
69 used was based on intra-muscular injection of a viral suspension to obtain a
70 synchronous infection among individuals. Collections at several time points and using
71 *in situ* DNA and RNA hybridization and the kinetics of OsHV-1 replication were
72 explored. Tissue distribution of viral DNA and RNA on histological sections might
73 help to localize early replication sites and understand the replication kinetics.

74

## 75 Material and methods

76

### 77 Animals and experimental infection using two viral doses

78 Pacific oysters *Crassostrea gigas* were produced during summer 2012 at the Ifremer
79 hatchery in La Tremblade (Charente Maritime, France). Eighty spat oysters (9 month
80 old, 3 cm in length) were “anesthetized” before injection of the viral suspension
81 (Schikorski et al., 2011). One hundred µL of a low dose of OsHV-1 (µVar, (Segarra et
82 al., 2010)) at $10^1$ copies of viral DNA/µL (L dose, $10^3$ viral DNA copies per oyster) or

a high dose of OsHV-1 at $10^6$ copies of viral DNA/µL (H dose, $10^8$ viral DNA copies per oyster) were injected into the adductor muscle in the hemolymphatic sinus of 40 oysters per conditions using a 1-mL syringe. The low dose was selected to avoid mortality based on preliminary tests (data not shown) whereas the high dose was chosen to induce mortality. Oysters were then placed in three aquaria per dose (10 oysters per tank) containing 5 L of filtered seawater at 22°C for the sampling. Oyster mortality was monitored daily during 96 hours post infection (hpi) and survival was defined for the two viral doses (L and H) in two other tanks (10 oysters per tank and per condition).

**Total DNA extraction and real time PCR analysis**

Samples were collected 2, 6, 10, 14 and 28 hpi for both viral doses. At each time of collection, 2 oysters were sampled from each tank (6 oysters per time per condition). A piece of mantle was sampled from each individual. DNA extraction was then performed with QiAamp tissue mini kit® (QIAgen) according to the manufacturer's protocol. Elution was performed in 100 µL of AE buffer provided in the kit. The detection and quantification of OsHV-1 DNA was carried out using real-time PCR (Pepin et al., 2008). The amplifications were performed using Mx3000P real-time PCR thermocycler (Agilent) to the following conditions: 1 cycle at 95°C for 3 min, 40 cycles of amplification at 95°C for 5 s, 60°C for 20 s. DNA was diluted at 5 ng/µL. Five µL of diluted DNA were added to the reaction mix composed of 10 µL of Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent), 2 µL of each primer OsHVDPFor (Forward) 5'-ATTGATGATGTGGATAATCTGTG-3' and OsHVDPRev (Reverse) 5'-GGTAAATACCATTGGTCTTGTTCC-3' (Webb et al., 2007) at the final concentration

of 500 nM each and 1 µL of distilled water. Assays included a standard curve and a negative control.

### *In situ* hybridization (ISH)

ISH was performed adapting a protocol previously described by Renault and Lipart (Renault and Lipart, 1998). In the present study, several ISH approaches were performed: ISH using a DNA probe and ISH using two different RNA probes described by Corbeil et al. (2015). C2/C6 (C2: CTTTTTACCATGAAGATACCCACC and C6: GTGCACGGCTTACCATTTTT), ORF 7 (ORF 7-1: GGCATTCACCCCTGACTCTA and ORF 7-2: CTGGAAGATGGGTTTCTCCA) and ORF 87 (ORF 87-1: GGTGGCCACACAAGAACAAT and ORF 87-2: GGTGGCAGGCACATCTATCT) probes (sense SP6 and anti-sense T7) were synthetized by incorporation of Digoxigenin-11-dUTP (Roche) as described by Corbeil et al. (2015). The C2/C6 probe was used to detect viral DNA. ORF 7 contains motifs V and VI characteristic of SF2 helicases and ORF 87 encodes BIR protein. These two viral genes were selected to search for virus RNAs as they have been previously detected in experimentally infected spat (Segarra et al., 2014a) and could be potentially involved in early stages of the viral cycle.

Oysters were fixed for 48 h in Davison fixative (22 % formaldehyde, 33 % ethanol 95, 12 % glycerol, 33 % of 0.2 µm filtered seawater and 10 % acetic acid). After embedding in paraffin wax, formalin-fixed tissues were sectioned (7 µm) and cross sections were collected onto silane-coated slides (Sigma). Histological cross sections were hybridized with 125 µL of hybridization buffer (4X SSC, 50 % deionised formamide, 10 % dextran sulfate, 1X Denhardt's solution, 250 µg/mL yeast t-RNA).

The DNA probe was used at the final concentration of 2.5 ng/µL in hybridization buffer, RNA probes were used at 250 ng/mL. Sections and DNA probe (no RNA) were denatured in hybridization buffer at 95°C for 5 min. Histological section and RNA probes were not denatured before hybridization. Hybridization was carried out at 42°C overnight in a humid chamber. After washing in 1X SSC with additional 0.2 % BSA for 10 min, sections were incubated in 1X PBS containing 6 % milk protein (Regilait®) for 30 min at room temperature. Linked probes were detected with anti-Digoxigenin-POD, Fab' fragments from sheep (Roche) diluted 1:50 in 1X PBS during 1 hour at room temperature. After five washes in 1X PBS, the revelation of the antibody was performed with 3′-Diaminobenzidine tetrahydrochloride TAB (Sigma) in the dark at room temperature (10 min). The reaction was stopped with distilled water. Sections were stained for 20 s in Unna blue and dehydrated, cleared with xylene and mounted for microscopy observation. DNA *in situ* hybridization was also carried out on non-denatured histological sections in order to control for the absence of single stranded DNA. With RNA probes, this step was omitted as target RNAs and RNA probes are single strand. Moreover, for each targeted RNA, both sense and anti-sense probes were tested on histological sections in order to confirm hybridization specificity.

**Statistical analysis**

Kaplan-Meier (Kaplan and Meier, 1958) survival curves were used to compare survival between infected oysters with the low (L) and the high (H) viral suspension doses. One way analysis of variation (ANOVA) was used to compare quantity of viral DNA (log-transformed) from low and high viral suspension doses. Statistical analyses

were performed using Minitab 16.2.1 statistical software. An ordinal logistic regression model was built to describe the relationship between the viral detection, the time and tissues for each probe. Then, contrast statement (Nichols, 1997) was performed to compare the viral detection level between tissues. A contrast is a linear combination of variables whose coefficients add up to zero, allowing comparison of different tissues. Analyses were carried out using the Statistical Package for the Social Sciences (SPSS, v.23). Results were declared statistically significant at the two-sided 5 % (ie, $p< 0.05$).

## Results

### Oyster survival and viral DNA detection by real-time PCR

Oyster mortality was daily monitored and Kaplan–Meier survival curves were generated for the low (L) and the high (H) viral suspension doses. No mortality was occrured for the L group during the monitoring period. Oyster survival injected with the dose H was 30 % at 48 hpi, 90 % at 72 hpi and 100 % at 96 hpi (Figure 1). The mean viral DNA amounts increased gradually during the infection for both doses, L and H (Figure 2). Nevertheless, viral DNA amounts were significantly different (ANOVA, $p<0.05$) between the doses. OsHV-1 DNA amounts ranged from 0.5 to 5.73 x $10^1$ and 1.2 x $10^2$ to 7.7 x $10^5$ viral DNA copies per ng of total DNA between 2 and 28 hpi for doses L and H, respectively (Figure 2). The viral amounts plateaued after 12 hpi for the L group (Figure 2).

### Viral DNA and RNA detection using *in situ* hybridization

178 In order to observe the tissue distribution of OsHV-1, viral DNA and RNA were
179 detected by ISH during the experimental infection. No viral DNA and RNA signals
180 were detected during the study for animals receiving the dose L (Figure 3).

181 The intensity of hybridization signal for the dose H varied among time, tissues and
182 probes (Table 1). Ordinal logistic regression test was performed for each probe
183 (DNA, RNA ORF 7, RNA ORF 87) and viral detection was significantly different in
184 function of tissues ($p<0.005$), time ($p<0.005$) with an interaction between tissues and
185 time ($p<0.005$). Then, the viral detection level between tissues was compared using
186 contrast test.

187 Positive hybridization with the DNA probe was observed in connective tissues of
188 mantle and digestive gland at 6 hpi (Table 1). DNA signals were mainly observed in
189 mantle (Figure 4a), gills and heart (Figure 4b). No significant difference (contrast test)
190 was observed between the heart and hemolymph sinus (Figure 4c) and mantle, and
191 between the heart and gills (Table 2). Nevertheless, significant differences were
192 reported between heart and other tissues (e.g adductor muscle ($p<0$), digestive gland
193 ($p<0.001$), and gonad ($p<0$)) during infection (Table 2). Statistically significant
194 differences were found also between gills and gonad ($p<0$), gills and adductor muscle
195 ($p<0$), gills and digestive gland ($p<0.012$), and gills and mantle ($p<0.002$) (Table 2).
196 Gonad, adductor muscle and digestive gland showed lower DNA detection than the
197 mantle, gills and heart. No significant difference was observed between gonad
198 connective tissue (Figure 4d), adductor muscle and the digestive gland (Table 2).

199 Differences in viral RNA detection were reported depending on collection time and
200 tissues. No RNA detection was observed in gonadal connective tissues using ORF 7
201 and ORF 87 (Table 1). Positive signals were detected at 14 hpi in mantle, gill and

heart using ORF 7 RNA probe (Table 1). RNA signals detected in the mantle (Figure 5) and heart were significantly differents of other tissues (Table 2).

First transcripts were detected only at 28 hpi with the ORF 87 RNA probe (Table 1). No significant difference was found between mantle, gills, heart and adductor muscle using the ORF 87 RNA probe (Table 2). Nevertheless, a significant difference was found between mantle and the digestive gland ($p<0.007$), mantle and gonad ($p<0.012$), heart and digestive gland ($p<0.022$), heart and gonad ($p<0.032$), gills and gonad ($p<0.047$) and digestive gland and gills ($p<0.031$) (Table 2). No discrete signals were present when ORF 7 and ORF 87 sense probe (identical sequence as virus mRNAs) were reacted with infected oyster tissues.

## Discussion

Significant differences in terms of mortality rate and viral DNA detection were observed between the two viral doses. Although no mortality was reported in oysters receiving the lowest dose, all oysters injected with the highest dose had died by 96 hpi. Viral replication appeared less important for the dose L than the dose H based on real-time PCR. Some authors (Oden et al., 2011; Sauvage et al., 2009) previously reported that viral DNA copies up to $10^4$ found in naturally infected oysters could be interpreted as evidence of an expressed viral infection leading to mass mortality. In another study, Renault et al. (2014) noted that up to $10^4$ copies of viral DNA per mg of wet tissues were only detected a few days before mass mortality occurred in the field.

Viral DNA detection was positive by real-time PCR whereas viral RNA and DNA detection were negative on histological sections by ISH for Pacific oyster spat infected with the lowest dose (L). These results could be partly explained through the higher sensitivity of real-time PCR in comparison with ISH (Biesaga et al., 2012). Viral DNA and proteins have been previously reported in adult oysters in absence of mortality (Arzul et al., 2002). Although no mortality was observed 96 hpi after injection of the lowest dose of viral suspension, the increasing level of viral DNA detected by real-time PCR during the course of the experiment suggested that OsHV-1 replicated in oysters receiving this dose. Chaves et al. (2011) showed that an adequate infectious dose is critical in reproducing the clinical infection of avian influenza A virus (H7N1) in chickens. These authors concluded that chickens exposed to lower doses can be infected and shed virus representing a risk for the dissemination of the viral agent (Chaves et al., 2011). Moreover, these results are in accordance with previous ones reporting an increase of OsHV-1 DNA detection and low mortality rates in oysters belonging to a low susceptible family (Segarra et al., 2014b). Although they are infected, some Pacific oyster appear to be able to manage the viral replication and to recover (He et al., 2015). The results reported in the present study suggested that injecting a viral suspension containing a low number of OsHV-1 DNA copies could be an useful tool to better understand the infection processes and how oysters are able to manage the viral infection. Recently Paul-Pont et al. (2015), demonstrated that a sufficient initial dose of viral particles is needed to induce high mortalities in Pacific oysters.

In this study, results showed viral RNA detection in connective tissue of different organs including mantle and gills. These organs were previously detected positive for viral DNA in naturally infected spat and adult oysters (Arzul et al., 2002). Mantle and

gills appeared as target organs (table 2). These results were consistent with previous epidemiological studies (Arzul et al., 2002; Pepin et al., 2008; Sauvage et al., 2009). Viral RNA was detected at 14 hpi principally in mantle and heart, by ISH using the ORF 7 probe. This result is in agreement with a previous study that detected viral transcripts before 26 hpi based on real-time PCR (Segarra et al., 2014a). However, the ORF 87 probe did not allow detection of viral transcripts on histological section before 28 hpi. Differences in probe sensitivity could explain such differences. The adductor muscle was detected positive at the final collection (28 hpi) even though the infection was performed by injection of the viral suspension in the hemolymphatic sinus of this organ. This result suggested that the adductor muscle is not an early site of viral replication. Miller et al. (2005) showed that the dissemination of Simian Immunodeficiency Virus infection to systemic lymphoid tissues occurred within 1–3 days of vaginal inoculation, although virus production at this site was established later.

The heart also appeared to be an organ of interest in terms of viral replication. Viral DNA and RNA were detected as early as 10 hpi and 14 hpi in this organ, respectively. The heart of *Crassostrea gigas* was previously described as infected by OsHV-1 (Arzul et al., 2002; Lipart and Renault, 2002). Results from the present study suggested that this organ could be a site of early replication of the virus and may play a key role in virus spread in the entire oyster body through hemolymph. Schikorski et al. (2011) reported viral DNA detection by real-time PCR in circulating hemocytes collected during an experimental infection of Pacific oyster spat. They suggested that the virus might penetrate rapidly in the hemolymphatic system, an open circulatory system in oysters (Schikorski et al., 2011). Moreover, labeled cells interpreted as circulating hemocytes were also detected in the root of the aorta in the present study.

The injection of viral suspension in the hemolympathic sinus of adductor muscle might allow the virus to reach rapidly the heart via hemolymph. Li et al. (1994) showed leukocytes are primary targets of the causative agent of Marek's disease, an *Alphaherpesvirus*. Moreover, leukocytes have been recognized as sites of latency in bovine herpesvirus type 1 (Mweene et al., 1996), in the rabbit model of herpes simplex virus type 1 (Seto et al., 1997) and in equid herpesvirus-1 (Edington et al., 1994; Welch et al., 1992). In this context, another study focus on the heart should be investigated in order to know if this organ could be considered as a first site of viral replication after the entry of the viral. Viral replication in heart cells might be a source of viral particle release. Infectious particles might be then transported to other organs including the adductor muscle and tissues by hemolymph.

No viral RNA detection was observed in gonad from oysters receiving the highest viral dose (H) suggesting there was not viral replication in this organ. Nevertheless, viral DNA was detected in the gonad of oysters. Viral DNA and proteins have been previously detected in gonads of adult oysters (Arzul et al., 2002).

To conclude, this study allowed localizing OsHV-1 DNA and RNA in several organs. Viral DNA and viral replication were mainly observed in mantle, gills and heart in oysters receiving the highest viral dose (H). However, we did not observed viral RNA by ISH in animals infected with the lowest viral dose (L). A real-time PCR based study should be performed in different organs to detect OsHV-1 DNA and RNA in order to further define target organs and tissues in oysters.

**Competing interests**: This work was supported by Ifremer (Institut Français pour l'Exploitation de la Mer). The authors declare that they have no competing interests.

**Acknowledgement**

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Table 1: Detection of ostreid herpesvirus 1 in highly infected Pacific oyster spat by real-time PCR and ISH according the organ analysed at 2 hpi, 6 hpi, 10 hpi, 14 hpi and 28 hpi "+++": indicates high intensity signal; "++": medium intensity signal; "+": low intensity signal; "-": no signal observed. /: tissue not observed on the histological section. [a]: number of viral DNA copies/ng of total DNA (based on a sample of the mantle). nd: no data.

| Time (hpi) | Oysters | qPCR [a] | *In situ* hybridization | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DNA (C2C6) | | | | | | RNA (ORF 7) | | | | | | RNA (ORF 87) | | | | | |
| | | | Mantle | Gills | Digestive gland | Adductor muscle | Heart | Gonad | Mantle | Gills | Digestive gland | Adductor muscle | Heart | Gonad | Mantle | Gills | Digestive gland | Adductor muscle | Heart | Gonad |
| 2 | 1 | $3,16.10^2$ | - | - | - | - | / | / | - | - | - | - | / | / | - | - | - | - | / | / |
| | 2 | $3,37.10^1$ | - | - | - | - | / | / | - | - | - | - | / | / | - | - | - | - | / | / |
| | 3 | $4,73.10^1$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 4 | $2.79.10^2$ | - | - | - | - | / | / | - | - | - | - | / | / | - | - | - | - | / | / |
| | 5 | $6,08.10^3$ | - | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / |
| | 6 | $3,59.10^1$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| 6 | 7 | $2,10.10^4$ | - | - | + | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 8 | $9,03.10^3$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 9 | $8,90.10^3$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 10 | $3,72.10^3$ | + | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 11 | $5,08.10^3$ | - | - | - | - | / | / | - | - | - | - | / | / | - | - | - | - | / | / |
| | 12 | $1,45.10^3$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| 10 | 13 | $1,30.10^5$ | + | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 14 | $3,02.10^5$ | ++ | + | - | - | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 15 | $6,07.10^4$ | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | 16 | $4,02.10^5$ | +++ | + | + | - | / | + | - | - | - | - | / | - | - | - | - | - | / | - |
| | 17 | $1,24.10^5$ | - | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| | 18 | $1,97.10^5$ | ++ | - | - | - | / | - | - | - | - | - | / | - | - | - | - | - | / | - |
| 14 | 19 | $2,79.10^5$ | +++ | ++ | + | - | / | / | + | - | - | - | / | / | - | - | - | - | / | / |
| | 20 | $7,34.10^5$ | +++ | ++ | ++ | - | +++ | - | + | - | - | - | +++ | - | - | - | - | - | - | - |
| | 21 | $5,39.10^5$ | ++ | + | + | - | ++ | / | ++ | - | - | - | ++ | / | - | - | - | - | - | / |
| | 22 | $3,11.10^5$ | ++ | + | - | - | / | / | - | - | - | - | / | / | - | - | - | - | / | / |
| | 23 | $6,48.10^5$ | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | 24 | $1,13.10^5$ | +++ | ++ | - | - | ++ | - | ++ | + | - | - | +++ | - | - | - | - | - | - | - |
| 28 | 25 | $4,95.10^5$ | +++ | ++ | + | ++ | ++ | / | ++ | ++ | - | - | ++ | / | ++ | + | - | + | ++ | / |
| | 26 | $7,77.10^5$ | ++ | +++ | - | + | ++ | ++ | +++ | ++ | + | ++ | - | - | - | - | - | - | - | - |
| | 27 | $1,22.10^6$ | +++ | ++ | ++ | ++ | / | + | +++ | +++ | ++ | ++ | / | - | ++ | ++ | ++ | ++ | / | - |
| | 28 | nd | +++ | +++ | + | ++ | / | + | +++ | ++ | - | ++ | / | - | ++ | ++ | - | - | / | - |
| | 29 | $1,06.10^6$ | +++ | +++ | ++ | ++ | +++ | + | +++ | ++ | + | ++ | +++ | - | +++ | ++ | - | ++ | +++ | - |
| | 30 | $3,15.10^5$ | +++ | +++ | ++ | ++ | / | + | +++ | +++ | ++ | - | / | - | ++ | ++ | - | - | / | - |
| Number of individual positives | | | 16/28 | 13/28 | 11/28 | 6/28 | 8/9 | 6/19 | 10/28 | 7/28 | 4/28 | 4/28 | 5/9 | 0/19 | 5/28 | 5/28 | 1/28 | 3/28 | 2/9 | 0/19 |
| Positives % | | | 57 | 46 | 39 | 18 | 89 | 32 | 36 | 25 | 14 | 14 | 55 | 0 | 18 | 18 | 4 | 11 | 22 | 0 |

320 Table 2: Contrast statement's test between tissues of *Crassostrea gigas* for each
321 probe (DNA, RNA ORF 7 and RNA ORF 87). The infection level of ostreid
322 herpesvirus 1 detected by ISH was compared in different tissues (adductor muscle,
323 mantle, digestive gland, gonad, heart and gills). Significant differences were noted in
324 bold (*p<0.05*).

| | *p value* | | |
|---|---|---|---|
| Contrast | DNA | RNA (ORF 7) | RNA (ORF 87) |
| C1 Adductor muscle/ Mantle | ***0*** | ***0*** | 0.062 |
| C2 Digestive gland/ Mantle | ***0*** | ***0*** | ***0.007*** |
| C3 Gills/ Mantle | ***0.002*** | ***0.032*** | 0.415 |
| C4 Gonad/ Mantle | ***0*** | ***0*** | ***0.012*** |
| C5 Heart/ Mantle | 0.234 | 0.407 | 0.94 |
| C6 Adductor muscle/ Heart | ***0*** | ***0.001*** | 0.139 |
| C7 Digestive gland/ Heart | ***0.001*** | ***0*** | ***0.022*** |
| C8 Gills/ Heart | 0.163 | ***0.016*** | 0.553 |
| C9 Gonad/ Heart | ***0*** | ***0*** | ***0.032*** |
| C10 Adductor muscle/ Gonad | 0.668 | 0.073 | 0.273 |
| C11 Digestive gland/ Gonad | 0.087 | 0.177 | 0.902 |
| C12 Gills/ Gonad | ***0*** | ***0.002*** | ***0.047*** |
| C13 Adductor muscle/ Gills | ***0*** | 0.058 | 0.241 |
| C14 Digestive gland/ Gills | ***0.012*** | ***0.017*** | ***0.031*** |
| C15 Adductor muscle/ Digestive gland | 0.141 | 0.55 | 0.208 |

325

326 Figure 1: Survival of *Crassostrea gigas* spat oysters during an experimental infection
327 with two OsHV-1 doses. H: High dose ($10^6$ copies of viral DNA/µL) and L: Low dose
328 ($10^1$ copies of viral DNA/µL). n=10 oysters/condition.

329

330 Figure 2: Viral DNA detection curves by real time quantitative PCR in spat oysters.
331 Average n=6 per condition. Error bars represent standard error of the mean (SEM).

332

333 Figure 3: Absence of viral detection by *in situ* hybridization for lowly infected oysters
334 (L dose) at 26 hpi using viral DNA probe in mantle connective tissue.

335

336 Figures 4. Detection of viral DNA by *in situ* hybridization (ISH) in experimentally
337 infected spat 14 hpi (H dose). Positive results are characterized by brown/black
338 precipitates (arrows). Fig. 4a: positive cells in mantle connective tissue. Fig. 4b:
339 positive muscular cells in the heart ventricle. Fig. 4c: positive cells (hemocytes) in a
340 hemolymph sinus. Fig 4d: positive cell in gonad connective tissue.

341

342 Figures 5. *In situ* hybridization probe ORF 7 (complementary to viral mRNA) binding
343 specifically (discrete labelling of cells: arrows) to OsHV-1 mRNA gene 7 (gene
344 encoding an SF2 helicase) in mantle connective tissue at 28 hpi (H dose).

345

346

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Figure 1



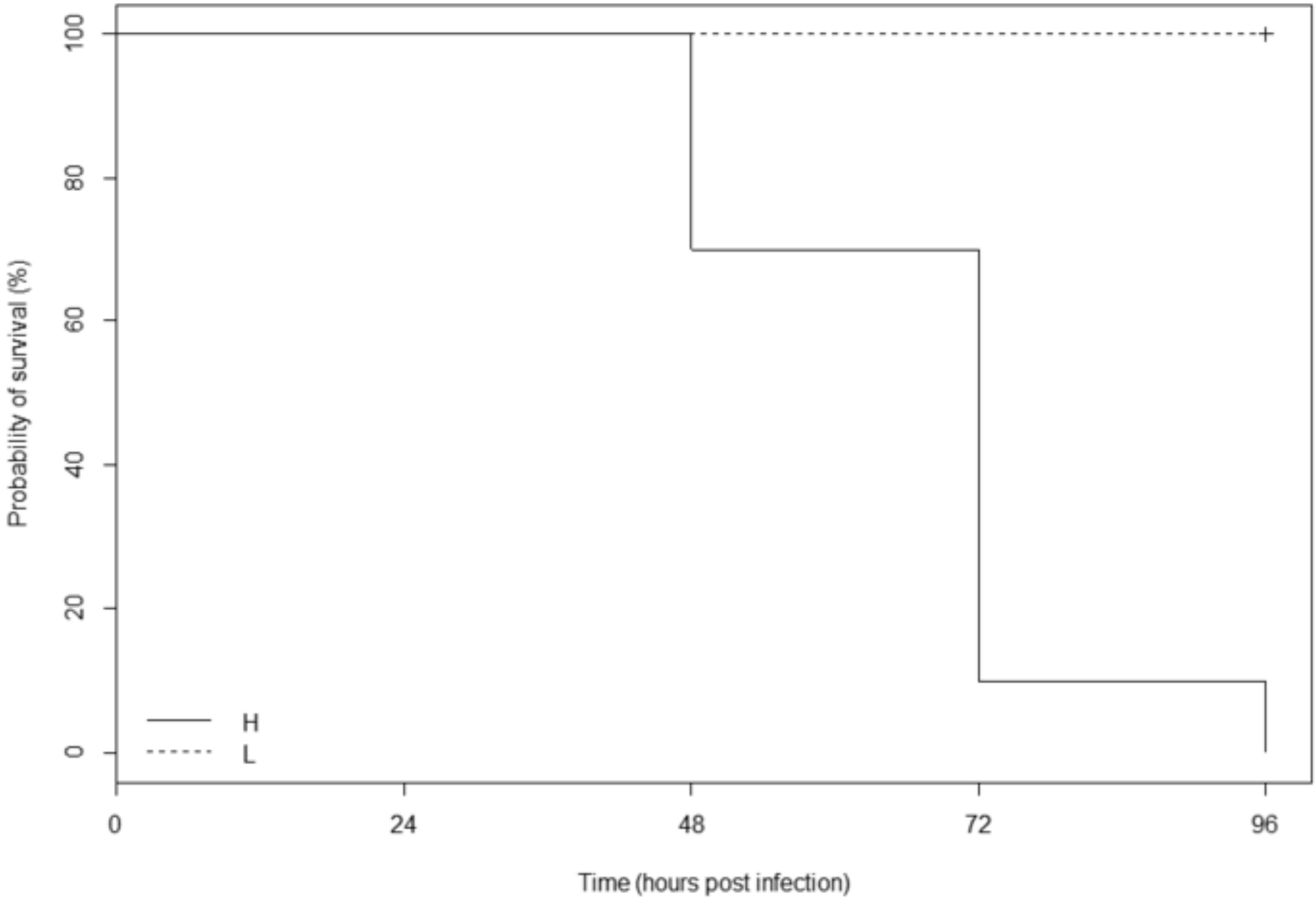

Figure 2



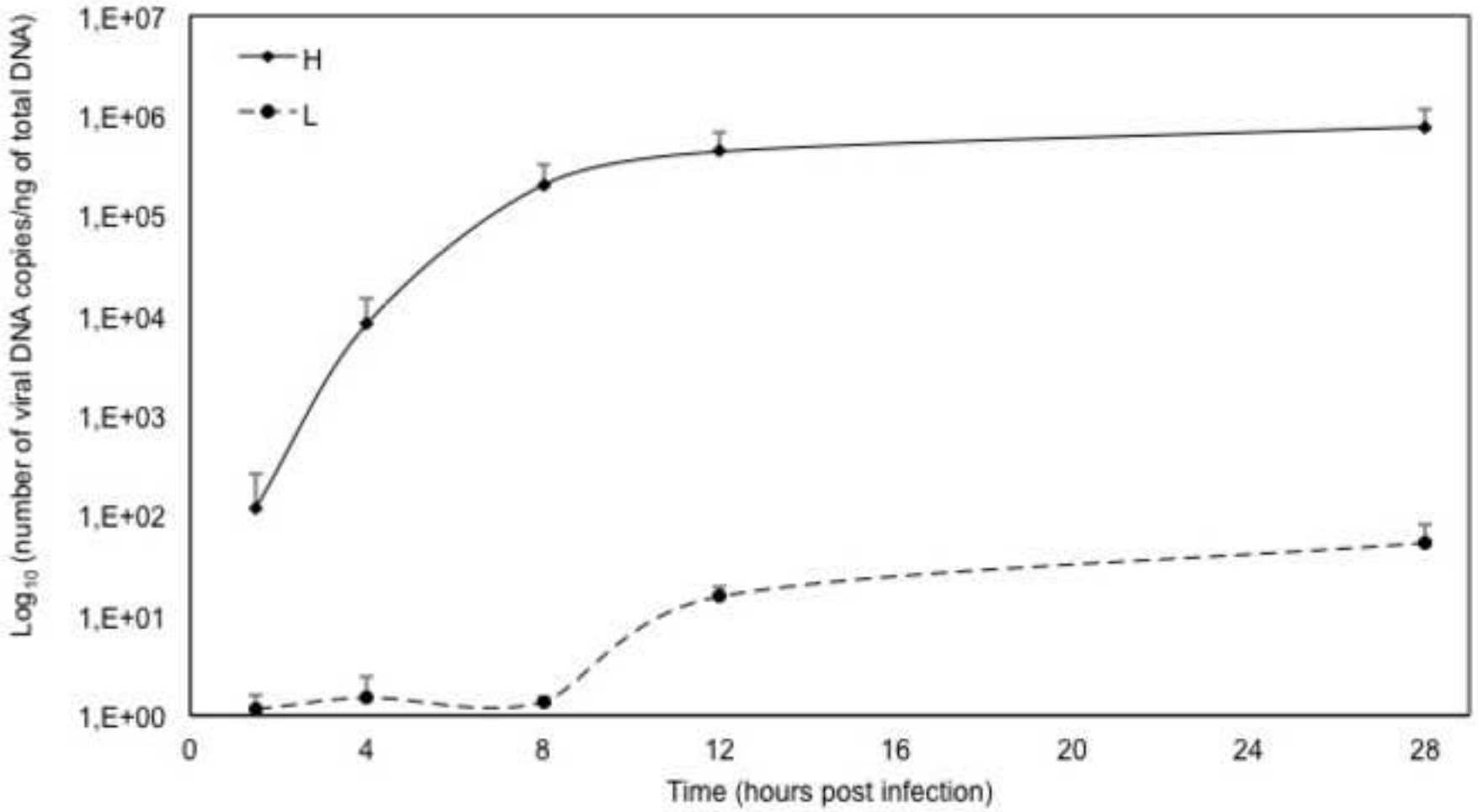

Figure 3



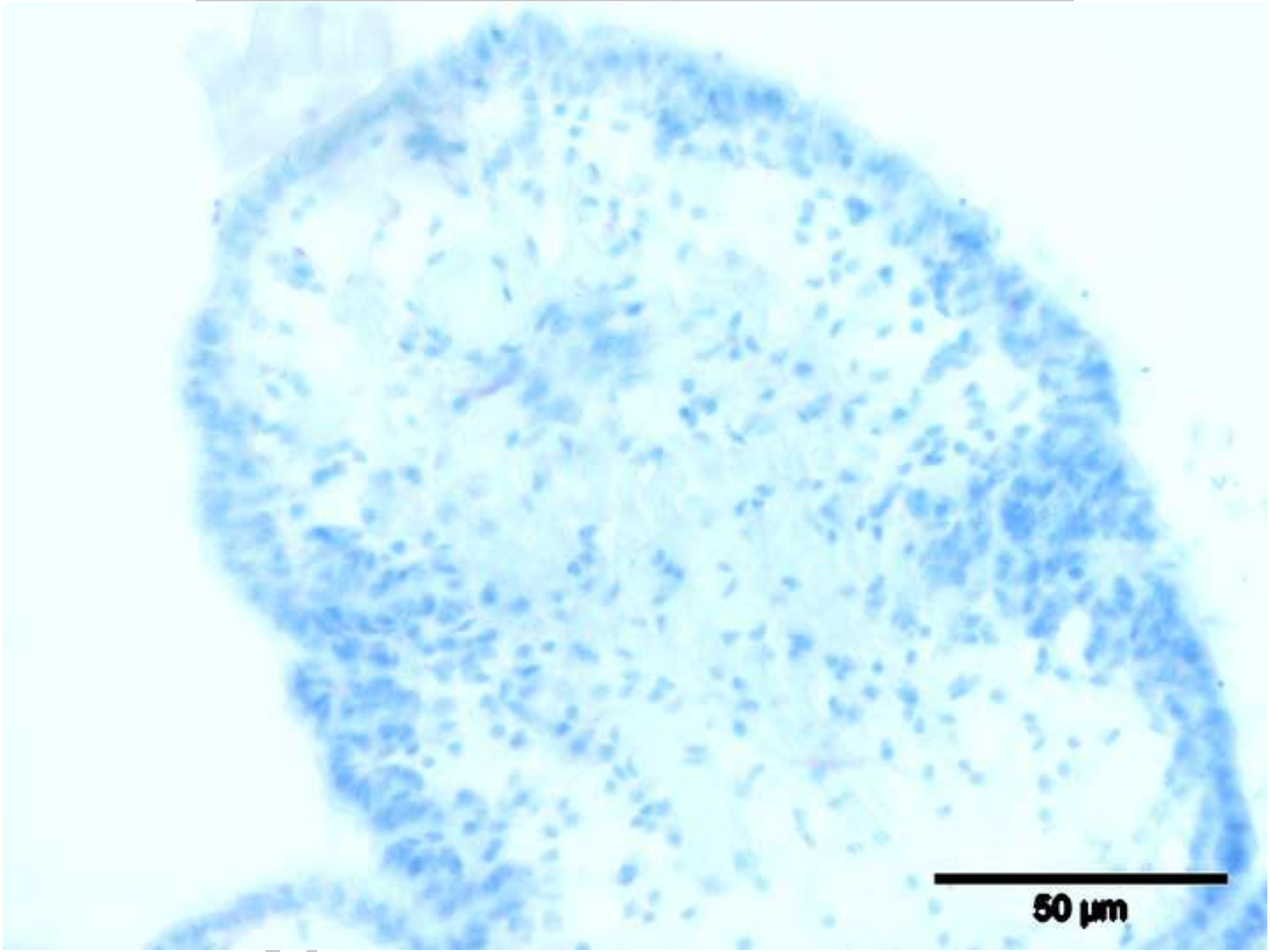

Figure 4a



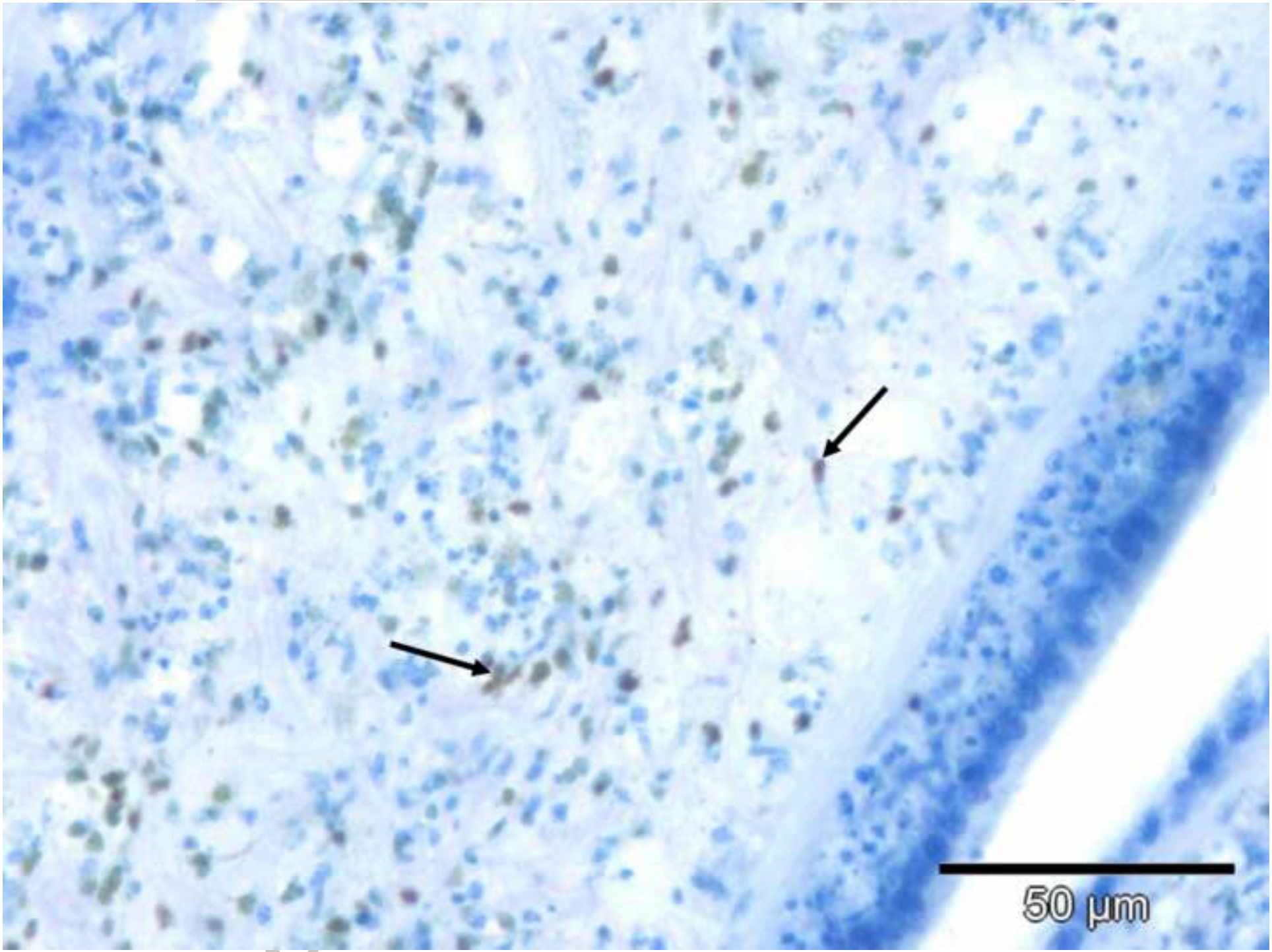

Figure 4b



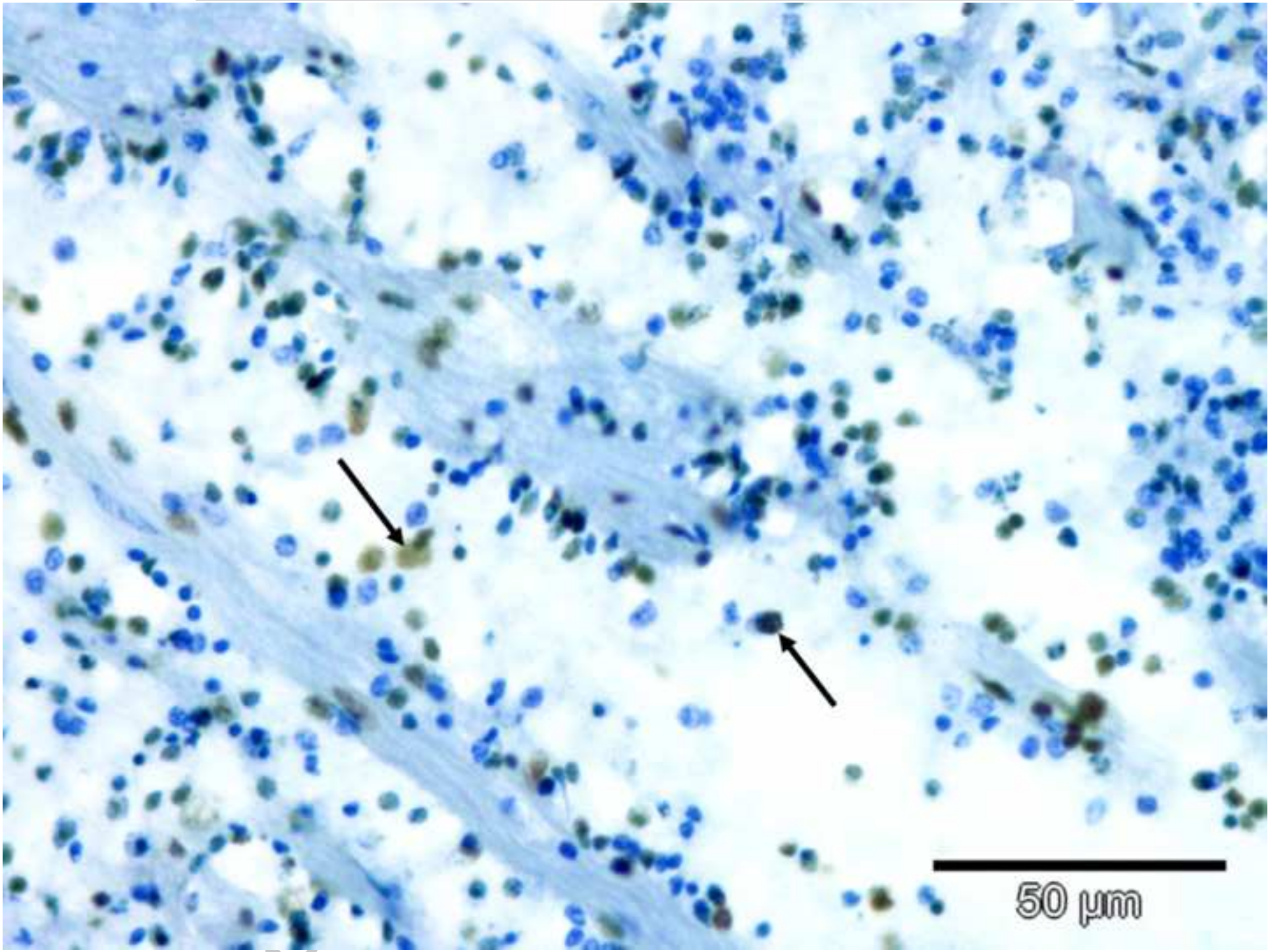

Figure 4c



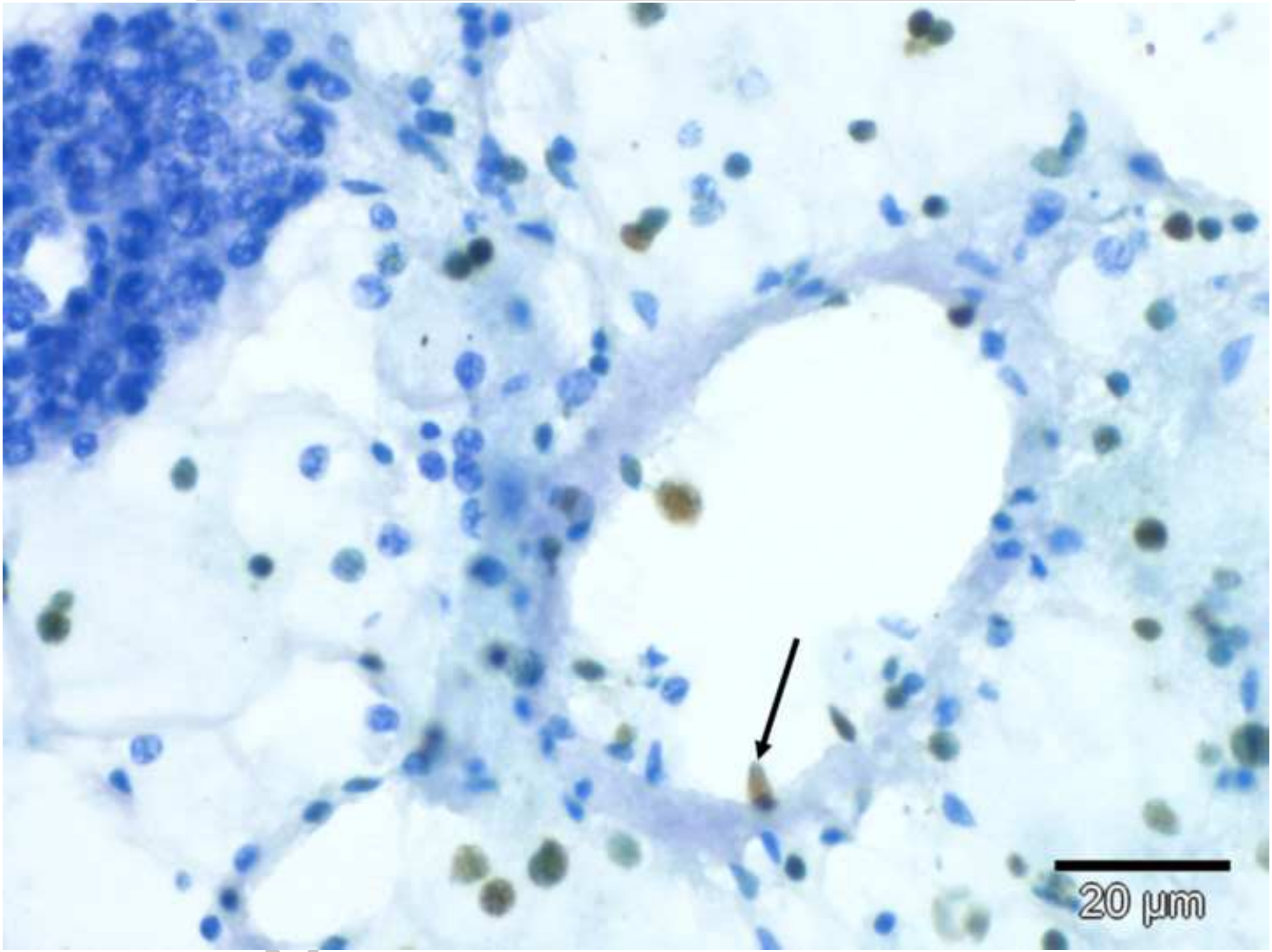

Figure 4d



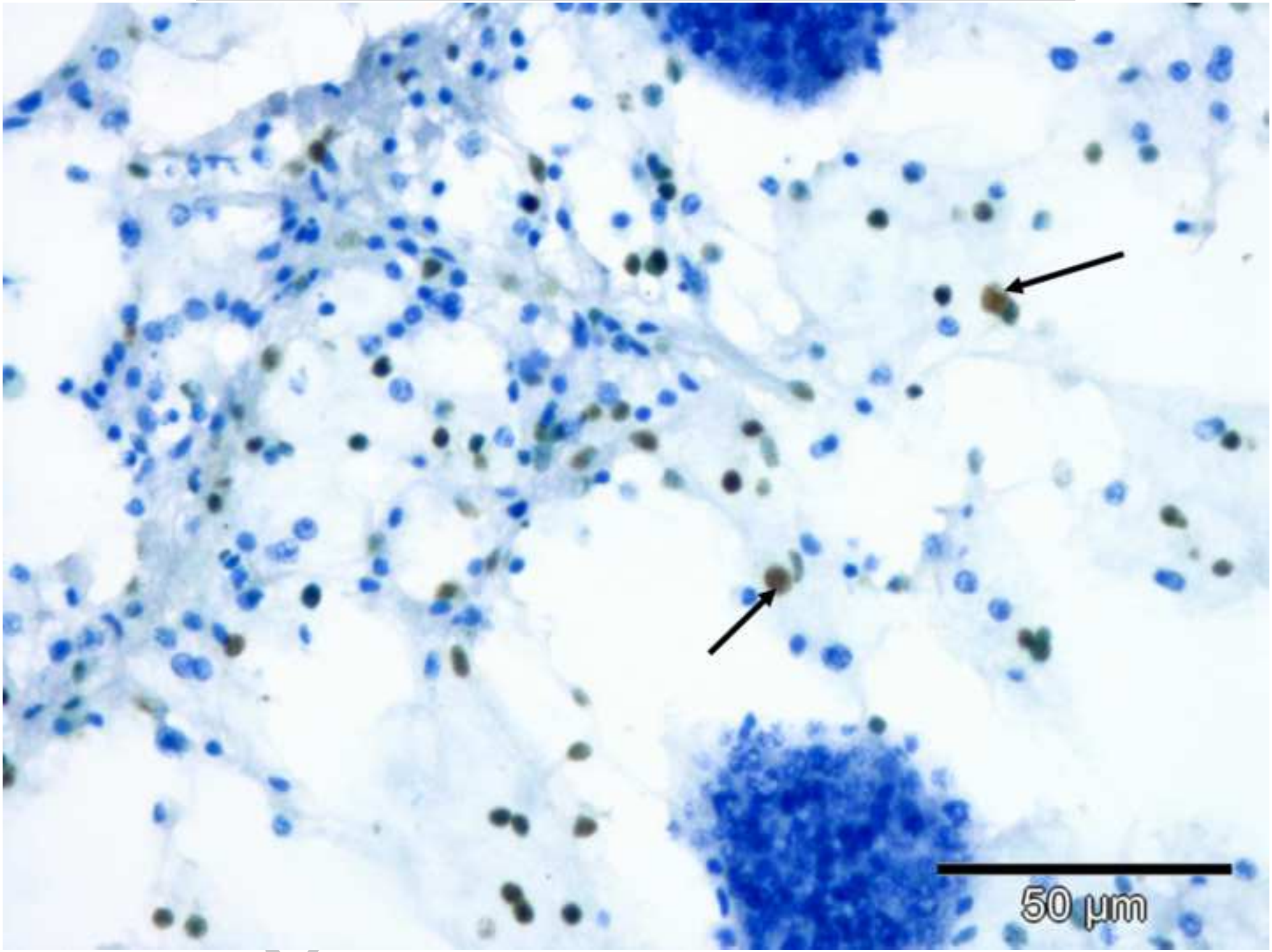

Figure 5