Received: 1 February 2021 | Accepted: 26 March 2021

DOI: 10.1111/jfd.13375

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Comparison of diagnostic methods for *Tetracapsuloides bryosalmonae* detection in salmonid fish

Veronika Seidlova[1,2] | Eva Syrova[1,3] | Hana Minarova[1,3] | Jan Zukal[4] | Vojtech Balaz[1] | Monika Nemcova[1,2] | Ivana Papezikova[1,2] | Jiri Pikula[1,2] | Heike Schmidt-Posthaus[5] | Jan Mares[2] | Miroslava Palikova[1,2]

[1]Department of Ecology and Diseases of Zoo Animals, Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic

[2]Department of Zoology, Fisheries, Hydrobiology and Apiculture, Mendel University in Brno, Brno, Czech Republic

[3]Department of Infectious Diseases and Preventive Medicine, Veterinary Research Institute, Brno, Czech Republic

[4]Institute of Vertebrate Biology, Czech Academy of Sciences, Brno, Czech Republic

[5]Department of Pathobiology, Centre for Fish and Wildlife Health, University of Bern, Bern, Switzerland

Correspondence
Veronika Seidlova, Department of Ecology and Diseases of Zoo Animals, Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic.
Email: seidlovav@vfu.cz

Funding information
This study was funded by the project PROFISH CZ.02.1.01/0.0/0.0/16_019/0000869. The project is financed by the European Regional Development Fund in the operational programme VVV MŠMT.

Abstract

Diagnostic accuracy of pathogen detection depends upon the selection of suitable tests. Problems can arise when the selected diagnostic test gives false-positive or false-negative results, which can affect control measures, with consequences for the population health. The aim of this study was to compare sensitivity of different diagnostic methods IHC, PCR and qPCR detecting *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease in salmonid fish and as a consequence differences in disease prevalence. We analysed tissue from 388 salmonid specimens sampled from a recirculating system and rivers in the Czech Republic. Overall prevalence of *T. bryosalmonae* was extremely high at 92.0%, based on positive results of at least one of the above-mentioned screening methods. IHC resulted in a much lower detection rate (30.2%) than both PCR methods (qPCR32: 65.4%, PCR: 81.9%). While qPCR32 produced a good match with IHC (60.8%), all other methods differed significantly ($p < .001$) in the proportion of samples determined positive. Both PCR methods showed similar sensitivity, though specificity (i.e., the proportion of non-diseased fish classified correctly) differed significantly ($p < .05$). Sample preservation method significantly ($p < .05$) influenced the results of PCR, with a much lower DNA yield extracted from paraffin-embedded samples. Use of different methods that differ in diagnostic sensitivity and specificity resulted in random and systematic diagnosis errors, illustrating the importance of interpreting the results of each method carefully.

KEYWORDS

diagnostic sensitivity, diagnostic specificity, immunohistochemistry, polymerase chain reaction, prevalence, proliferative kidney disease

1 | INTRODUCTION

Some infectious diseases manifest when fish are exposed to a pathogen supported by favouring environmental conditions (Kopp et al., 2018). *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea), an endoparasitic infectious agent causing proliferative kidney disease (PKD) (Pojezdal et al., 2020), can cause high mortality in fish populations at permissive water temperature (Clifton-Hadley et al., 1986; Hedrick et al., 1993; Okamura et al., 2001; Syrová et al., 2020), in both, farmed and wild salmonid species, in Europe and North America

(Hedrick et al., 1993; Henderson & Okamura, 2004). The parasite's lifecycle involves two hosts, bryozoans and salmonid fish infected via spores in the water (Longshaw et al., 1999). The fish get infected through the skin and gills (Grabner & El-Matbouli, 2010; Hedrick et al., 1993; Longshaw et al., 2002). After initial infection, the parasite is distributed through the blood system and invades the inner organs (Clifton-Hadley, Richards, et al., 1986; Longshaw et al., 2002), with the kidney as the main target organ (Tops et al., 2006). *T. bryosalmonae* forms both extrasporogonic and sporogonic developmental stages, with the extrasporogonic stage lasting for 2–3 weeks and the sporogonic stage settles in the lumen of the fish kidney tubules (Chilmonczyk et al., 2002; Ferguson & Needham, 1978). Some infected salmonids can also excrete spores into the water via urine, infecting bryozoan (Feist et al., 2001; Hedrick et al., 1993; Morris et al., 2002; Sterud et al., 2007).

Clinical outbreaks, generally associated with mortality, occur in summer and early autumn when water temperatures increase. At temperature below 10°C, fish may get infested without disease development (Gay et al., 2001); when temperature surpasses 15°C, however, infestation results in parasite proliferation and disease (Bettge et al., 2009; Ferguson, 1981; Ferguson & Ball, 1979; Gay et al., 2001; Hedrick et al., 1993). It is assumed that fish, unlike mammals, are able to regenerate renal tissue through nephron neogenesis and, as such, can survive the clinical phase of PKD (Bettge, Segner, et al., 2009; Bettge et al., 2009; Schmidt-Posthaus et al., 2012).

A number of different molecular and immunological tools are commonly used for detection of *T. bryosalmonae* and/or PKD. For example, routine histopathology with haematoxylin and eosin staining can be used to detect the sporogonic and extrasporogonic stages of the parasite within the kidney (Klontz and Chacko 1983), while the lectin-based procedure is a more specific staining method to localize and identify *T. bryosalmonae* specifically (Castagnaro et al., 1991; Hedrick et al., 1992). More recently, immunohistochemistry (IHC) using a monoclonal antibody and the polymerase chain reaction (PCR) have been developed. While PCR testing methods have been developed, the majority of primers used amplify non-specific sequences and/or achieve inconsistent amplification efficiency (Morris et al., 2002). At present, the 5F/6R PKX sensitive primer set for amplification of a 435-bp segment from the SSU-rDNA gene of *T. bryosalmonae* is most commonly used (Kent et al., 1998). It is also possible to detect parasite DNA using quantitative PCR (qPCR) based on sequence-specific DNA, as described by Bettge, Wahli et al. (2009).

Multiple aspects may influence the diagnostic accuracy of pathogen detection, including the pathogen abundance and infection load, which will differ between environmental conditions and/or fish species, as well as the sample preservation used and extracted DNA concentration. As data on assay specificity and sensitivity are of high practical importance, the aim of this study was to compare the results of three methods presently used for detection of *T. bryosalmonae* in kidney samples, that is IHC, conventional PCR and qPCR, using two different cycle determinations. We hypothesize that during microscopy, a random selection of optic fields increases the number of false-negative results. We further hypothesize that both PCR methods show higher sensitivity compared to microscopy resulting in higher disease prevalence. Likewise, false-positive results using PCR methods may occur due to possible misinterpretation of signals detected at higher numbers of PCR cycles.

2 | MATERIAL AND METHODS

Between 2015 and 2017, a total of 388 specimens of two different salmonid species (brown trout *Salmo trutta* m. *fario* and rainbow trout *Oncorhynchus mykiss*) were obtained from different sites in the Czech Republic, 124 fish from the recirculating aquaculture facility and 264 fish from eight rivers (for more details, see Table 1). Full necropsy was performed immediately after euthanasia of the animal, and samples of caudal kidney were fixed in 10% formalin for histopathology and immunohistochemistry and 70% ethanol for PCR and qPCR diagnosis. All parts of the experiment were performed in accordance with EU Directive 2010/63/EU on animal experimentation.

2.1 | Immunohistochemistry

Caudal kidney samples (n = 212) from brown trout were fixed in 10% buffered formalin and then embedded in paraffin, sectioned and stained using mouse monoclonal anti-*T. bryosalmonae* antibodies (AquaMAb-P01, Aquatic Diagnostics), followed by biotin-conjugated goat anti-mouse IgG, based on the protocol of Adams et al. (1992). *T. bryosalmonae* structures were visualized with the aid of streptavidin-HRP staining (Merck KGaA), followed by AEC (3-amino-9-ethylcarbazole) staining (Dako Chemicals) (Bettge, Segner, et al., 2009). Ten microscopic fields (200 × magnification) per slide were randomly selected, and the mean number of parasites per field was counted for all kidneys examined.

2.2 | DNA extraction, PCR and qPCR assays

DNA was extracted from the all kidney samples (n = 388) of suspected *T. bryosalmonae* infection using either the NucleoSpin® Tissue kit for samples stored in 70% ethanol or the NucleoSpin®DNA FFPE kit for paraffin-embedded samples (both kits produced by Macherey-Nagel GmbH & Co. KG). Around 20 mg of infected kidney tissue was sampled in each case, and DNA was extracted following the protocol of the manufacturer. Specific primers PKX-5F: 5-CCT

TABLE 1 Fish samples (kidney tissue) used in the study

Habitat	n	Sample preservation	n
Aquaculture	124	e	124
Rivers	264	e	113
		p	151
Total			388

Abbreviation: e, ethanol; p, paraffin-embedded.

ATT CAA TTG AGT AGG AGA-3 and PKX-6R: 5-GGA CCT TAC TCG TTT CCG ACC-3 (according to Kent et al., 1998) were used for PCR amplification of the 435-bp segment from the SSU-rDNA gene of *T. bryosalmonae*. PCR amplification was performed using a reaction volume of 25 μl containing water 6.5 μl, Q5® High-Fidelity Master Mix (New England BioLab® Inc.) 12.5 μl and forward primer (10 μM) 2 μl, reverse primer (10 μM) 2 μl and 2 μl of extracted DNA. The amplification conditions consisted of an initial denaturation at 98°C for 3 s, followed by 40 cycles of 20 s at 98°C, 45 s at 55°C, 50 s at 72°C and a final extension of 72°C for 2 min. The PCR products were analysed by 1.5% agarose gel electrophoresis stained with DNA Stain G (SERVA) and visualized on a UV transilluminator. PCR product molecular weight was determined using the DNA molecular weight standard, 100 bp DNA Ladder. PCR products (435 bp) were commercially sequenced using Sanger sequencing. Sequences similar to each gene sequence were identified in GenBank using MegaBLAST. We also used qPCR detection as a further comparison method. The primers and probe for 18S rDNA were performed as described previously in Bettge, Wahli, et al., (2009). Forward and reverse primers were designed to amplify a 73-base pair region PKDtaqf1: 5′-GCG AGA TTT GTT GCA TTT AAA AAG-3′ and PKDtaqr1 5′-GCA CAT GCA GTG TCC AAT CG-3′. The internal probe was labelled at the 5′end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA) (probePKD: 5′-CAA AAT TGT GGA ACC GTC CGA CTA CGA-3′). qPCR amplification was performed using a reaction volume of 20 μl containing water 4.2 μl, LightCycler® 480 Master (Roche) 10 μl, forward primer 300 nM, reverse primer 300 nM, probe 200 nM and 5 μl extracted DNA. The amplification conditions consisted of an initial denaturation at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. All samples were analysed in duplicate and each real-time PCR run included a negative control (molecular grade water only) and a positive control (*T. bryosalmonae* DNA checked by Sanger sequencing). Paired tissue samples of 77 *Salmo trutta* specimens preserved by both methods (paraffin embedding after formalin fixation and ethanol preservation) were used for the comparison of PCR results.

2.3 | Statistical analysis

Prevalence of *T. bryosalmonae* was calculated for each data set as the percentage of positive samples from the total number of samples analysed. Differences between sample preservation methods were tested on 61 selected kidney samples (only brown trout from rivers caught during December 2017) using the chi-square test. Normal distribution of DNA concentration and number of PCR cycles was tested using the Kolmogorov–Smirnov and Shapiro–Wilk tests, respectively. Differences in DNA concentration obtained from samples preserved by different methods were compared using the *t* test. The accuracy of each screening method as regards the detection of positives, sensitivity and specificity were tested using the difference test between proportions. The McNemar chi-square test was used for comparing matches between particular screening methods in order to control for potential false-positive or false-negative results. This test is applicable in situations where frequencies in the 2 × 2 table represent dependent samples. As the number of PCR cycles was not normally distributed, and transformations did not improve normality of this variable, statistical analysis was conducted using the non-parametric Mann–Whitney *U* test. The number of stained parasites counted on each section was used as a measure of infection severity. The sensitivity of test was calculated as the number of diseased that are correctly classified, divided by all diseased individuals and the specificity as the number of non-diseased correctly classified divided by all non-diseased individuals. For analysis, was used software Statistica for Windows®13.2 (StatSoft, Inc.).

3 | RESULTS

The two methods of sample preservation used (formalin vs. ethanol fixation of kidney specimens) significantly influenced the results of PCR tests. In all three cases (PCR, qPCR32 and qPCR36 [32 and 36 referring to the number of amplification cycles]), ethanol-preserved samples showed a higher prevalence of *T. bryosalmonae* infestations compared to formalin-fixed, paraffin-embedded samples. In addition, formalin-fixed samples revealed much lower DNA concentrations ($t = 16.95$, $p < .001$; Figure 1). As such, only ethanol-preserved samples were used for subsequent statistical analysis comparing the different diagnostic methods.

The overall prevalence of *T. bryosalmonae* infection, based on combining positive findings from all screening methods, was extremely high at 92.0% (218 of 237 ethanol-preserved samples). IHC provided lowest detection rate (30.2%) and the proportion of positive samples using IHC differed significantly from detection rate by all PCR methods (difference test, $p < .001$). The results of the different PCR methods also differed significantly, with qPCR32 showing a much lower *T. bryosalmonae* prevalence (65.4%) compared to the

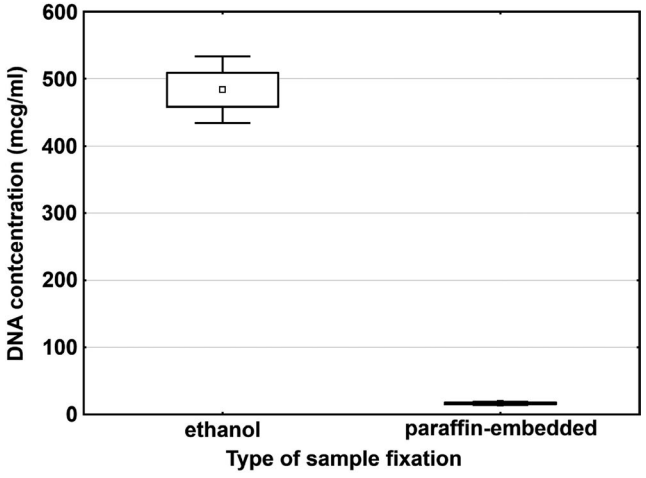

FIGURE 1 Difference in DNA yield extracted from ethanol-preserved (e) and paraffin-embedded samples (p). Explanations: Square = mean, box = ±SE, whiskers = ±1.96*SE

other methods (Table 2). To address this further, we analysed the match between results of different screening methods in greater detail (Figure 2) to control for potential false-positive or false-negative results (Figure 2). While we observed significant differences between all PCR methods (Table 3), despite a relatively high match score (>71.7%), IHC displayed similar positivity (1/1) to qPCR32 (McNemar $\chi^2 = 0.50$; $p = .481$). Any mismatches consisted mainly of IHC-negative and qPCR-positive combinations (0/1; Figure 3), which showed a significantly higher number of amplification cycles ($Z = -6.11$; $p < .001$) than cases of matched positivity (1/1; Figure 3). Four exceptional cases showing the opposite pattern (1/0) occurred at a low infection severity, that is between 0.1 and 3.5 parasites per section.

In terms of total match, qPCR32 was best matched with IHC (60.8%) and displayed high sensitivity (93.8%) and better specificity (46.6%) than the standard PCR method (25.7%; difference test, $p < .001$).

4 | DISCUSSION

In Europe and North America, both farmed and wild salmonid fishes are under pathogen pressure of *T. bryosalmonae*, the causative agent of PKD, one of the most economically important fish diseases (Clifton-Hadley et al., 1986; Hedrick et al., 1993). Until the late 1990s, the organism causing PKD was unknown and the disease considered untreatable (Canning et al., 2000; Kent & Hedrick, 1985). Still, up to date, no treatment has been approved. Understandably, reliable diagnostic methods are required to help control and prevent PKD. However, the various diagnostic methods presently available are known to provide differing disease prevalence results (Nowak et al., 2006; Rüssmann et al., 2001; Suresh & Smith, 2004; Whyte et al., 2002). From a practical point of view, therefore, it is important to ascertain the error rates of the methods used.

Though prevalence of PKD can reach 100% in parts of Europe (Lewisch et al., 2018; Palikova et al., 2017; Vasemägi et al., 2017;

TABLE 2 Prevalence of *Tetracapsuloides bryosalmonae* based on positivity in different screening methods

	Positive	Tested	Prevalence	Difference test—two-sided	
IHC	64	212	30.2%	IHC vs. PCR	$p < .001$
PCR	194	237	81.9%	IHC vs. qPCR32 cycles	$p < .001$
qPCR32 cycles	155	237	65.4%	PCR vs. qPCR32 cycles	$p < .001$
qPCR36 cycles	210	237	88.6%	PCR vs. qPCR36 cycles	$p = .040$

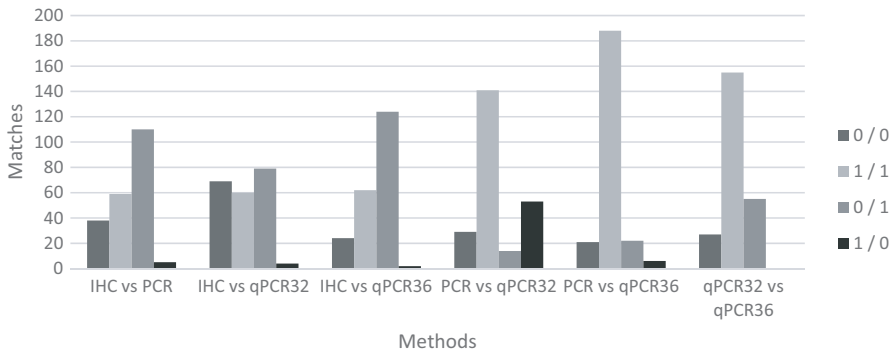

FIGURE 2 Detailed match between particular results of all screening methods. Explanations: 0—negative result, 1—positive result

TABLE 3 General match between the results of screening methods

	IHC vs. PCR	IHC vs. qPCR32	IHC vs. qPCR36	PCR vs. qPCR32	PCR vs. qPCR36	qPCR32 vs. qPCR36
Total matched results	97	129	86	170	209	182
%	45.8%	60.8%	40.6%	71.7%	88.2%	76.8%
Number of tested samples	212	212	212	237	237	237
McNemar chi-square test	4.12 $p = .042$	0.5 $p = .481$	15.92 $p < .001$	72.48 $p < .001$	131.85 $p < .001$	86.62 $p < .001$

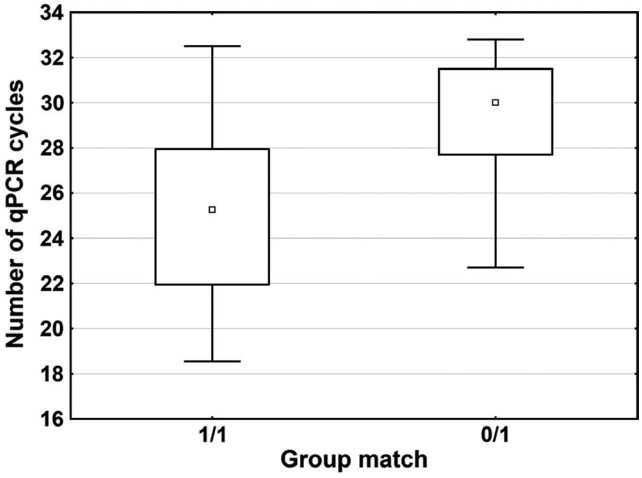

FIGURE 3 The number of amplification cycles applied in the cases of matched positivity (1/1 cases) and mismatched cases with IHC-negative and qPCR-positive combination (0/1 cases). Explanations: Square = median, box = 25%–75%, whiskers = Minimum–Maximum

Syrová et al., 2020), mortality rates vary considerably. For example, while mortality generally ranges around 30% in the Czech Republic, it may reach up to 85% under conditions of stress or secondary infection and/or higher water temperatures (Bettge, Segner, et al., 2009; Okamura et al., 2001). In the present study, prevalence of *T. bryosalmonae* based on the positivity of at least one of our screening methods was as high as 92%. While *T. bryosalmonae* was detected both in aquaculture and in rivers by PCR, IHC indicated a much lower prevalence (30.2%) than both PCR methods (qualitative PCR = 81.9%, qPCR32 = 65.4%), that is the proportion of positive results differed significantly between IHC and both PCR methods but the results obtained by the PCR methods did not differ. Comparison was possible because of measuring paired kidney samples that originated from the same fish. It is known that PCR is a more sensitive method than IHC, and this is shown in our study by some tissues being PCR positive but IHC negative. Abd-Elfattah et al. (2014) or Skovgaard and Buchmann (2012) reported that not all PCR-positive samples were confirmed by IHC. Bettge, Segner, et al. (2009) recorded a correlation between PCR methods and IHC; however, only a poor correlation was observed when fish were either strongly or weakly affected by the parasites (in terms of DNA yield). In such cases, examination may be complicated by parasite distribution being clustered in the target organ, thereby increasing the probability of false-negative results. Though we used specific primers for PCR, false-positive or false-negative results can be an essential problem for data interpretation (see Morris et al., 2002). To address this, we implemented qPCR using variable regions of the conserved 18S rDNA (according to Bettge, Segner, et al., 2009) to ensure higher sensitivity. Morris et al. (2002) tested all commonly used 18S rDNA primers for qualitative PCR but found that only primers 3F/4R and 5F/6R were specific. According to Kent et al. (1998), qualitative PCR determination of 5F/6R primer sensitivity at DNA equivalent is 8.29 parasite cells g^{-1} of tissue and 0.00829 parasite cells per reaction, which allows detection of sporogonic stages and the preclinical levels of extrasporogonic stages. In general, we found good correlation between PCR methods (PCR vs. qPCR 32 cycles; 65.4%, $p < .001$).

Surprisingly, IHC confirmed a significantly lower prevalence than both PCR methods in the present study, contrary to the results of Bettge, Wahli, et al., (2009), with the best match overall being with qPCR32 (60.8%), having almost 94% of specificity but only 46% sensitivity. The number of parasites visible on tissue sections generally increases during the course of infection (Bettge, Segner, et al., 2009; Hedrick et al., 1993); nevertheless, in most cases, molecular methods are able to confirm the causative agent in fish showing neither mortality nor enlarged kidneys. Our samples from recirculating aquaculture were taken, when PKD was manifest (pathological findings) but samples from rivers were taken outside the optimal period of PKD screening (lesions and clinical disease were minimal); thus, it can be assumed that IHC is able to confirm the presence of the causative agent even when it is present in low quantities, despite its lower sensitivity. In this case, the high prevalence detected by the PCR methods was due to the methods' higher sensitivity (>92%).

Ethanol- and formalin-fixed tissues are both invaluable resources for molecular studies of pathogens (Sengüven et al., 2014); molecular studies are highly dependent on the quality and quantity of nucleic acids extracted from the tissue. Our results showed that the sample preservation method used can influence the detection probability of the PKD pathogen. Obtaining high-quality DNA from paraffin-embedded tissues can be a difficult task as formalin damages tissue nucleic acids, which subsequently results in extensive DNA fragmentation. Further, extracted DNA often contains remnants of substances that inhibit the PCR amplification reaction. Consequently, unlike ethanol fixation, which permits extraction of large amounts of high molecular weight DNA (Bramwell and Burns, 1988; Ribeiro et al., 2004), FFPE (formalin-fixed paraffin-embedded), formalin fixation only allows DNA amplifications of up to 300 bp (base pair), and very often amplimers of up to just 100 bp are obtained (Bonin et al., 2003). The lower DNA yield obtained from formalin-fixed samples had a significant effect on PCR results in our study, with PCR (sequence size 330 bp) confirming a significantly different detection rate when samples were fixed using either ethanol or formalin, despite the paired origin of samples from the same fish. However, there was no such difference observed when using qPCR (amplified sequence size 76 bp).

It has previously been shown that transmission of parasitic spores to the fish host via bryozoans is dependent on water temperature, fish migration, hydrodynamic dispersal of spores and/or bryozoan statoblasts (Bettge, Segner, et al., 2009; Bettge, Wahli, et al., 2009; Clifton-Hadley, Bucke, et al., 1986; Hedrick et al., 1993). Water temperature, in particular, has been shown to influence the proliferation of *T. bryosalmonae* in both invertebrate (Tops et al., 2006) and fish (Bettge, Wahli, et al., 2009) hosts, suggesting seasonal and habitat-related variation in parasite DNA quantity, and thus disease detection probability, when using different screening methods (IHC or PCR).

At our study localities, fish positive for *T. bryosalmonae* could be recognized by all three screening methods, with prevalence ranging from 6.7% to 100%. Despite IHC showing a much lower detection

probability than qPCR, the results did not differ significantly between locality type when ethanol-preserved samples only were used.

5 | CONCLUSIONS

Based on our results, provisional use of formalin-fixed samples appears to be inappropriate for PCR analysis of *T. bryosalmonae* prevalence. Instead, qPCR32 (ethanol-preserved samples) would appear to be the best PCR screening method for *T. bryosalmonae* and should be used as standard for analysis of the different factors influencing prevalence of *T. bryosalmonae* in salmonid fishes. Our study illustrates the importance of careful interpretation of results based on different screening and sample preservation methods before undertaking follow-up control actions.

ACKNOWLEDGEMENTS

We thank Kevin Roche for his correction and improvement of the English text.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

VS and MP designed the study. JZ performed the statistical analysis. VS, ES, HM, MP, VB, MN, IP, H S-P and JM collected field data and processed the samples. VS and JP produced the first draft of the manuscript. All authors contributed substantially to revising the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This experiment was performed in accordance with EU Directive 2010/63/EU on animal experimentation and was approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (accreditation by the Ministry of Agriculture of the Czech Republic no. 28414/2009-17210).

DATA AVAILABILITY STATEMENT

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ORCID

Ivana Papezikova https://orcid.org/0000-0003-1396-9497
Heike Schmidt-Posthaus https://orcid.org/0000-0003-2628-4454
Miroslava Palikova https://orcid.org/0000-0002-5482-7172

REFERENCES

Abd-Elfattah, A., Kumar, G., Soliman, H., & El-Matbouli, M. (2014). Persistence of *Tetracapsuloides bryosalmonae* (Myxozoa) in chronically infected brown trout *Salmo trutta*. *Diseases of Aquatic Organisms*, *111*(1), 41–49. https://doi.org/10.3354/dao02768

Adams, A., Richards, R. H., & de Mateo, M. M. (1992). Development of monoclonal antibodies to PK 'X', the causative agent of proliferative kidney disease. *Journal of Fish Diseases*, *15*(6), 515–521. https://doi.org/10.1111/j.1365-2761.1992.tb00683.x

Bettge, K., Segner, H., Burki, R., Schmidt-Posthaus, H., & Wahli, T. (2009). Proliferative kidney disease (PKD) of rainbow trout: Temperature- and time-related changes of *Tetracapsuloides bryosalmonae* DNA in the kidney. *Parasitology*, *136*(6), 615–625.

Bettge, K., Wahli, T., Segner, H., & Schmidt-Posthaus, H. (2009). Proliferative kidney disease in rainbow trout: Time-and temperature-related renal pathology and parasite distribution. *Diseases of Aquatic Organisms*, *83*(1), 67–76. https://doi.org/10.3354/dao01989

Bonin, S., Petrera, F., Niccolini, B., & Stanta, G. (2003). PCR analysis in archival postmortem tissues. *Molecular Pathology*, *56*(3), 184. https://doi.org/10.1136/mp.56.3.184

Bramwell, N. H., & Burns, B. F. (1988). The effects of fixative type and fixation time on the quantity and quality of extractable DNA for hybridization studies on lymphoid tissue. *Experimental Hematology*, *16*(8), 730–732.

Canning, E. U., Curry, A., Feist, S. W., Longshaw, M., & Okamura, B. (2000). A new class and order of myxozoans to accommodate parasites of bryozoans with ultrastructural observations on *Tetracapsula bryosalmonae* (PKX organism). *Journal of Eukaryotic Microbiology*, *47*(5), 456–468. https://doi.org/10.1111/j.1550-7408.2000.tb00075.x

Castagnaro, M., Marin, M., Ghittino, C., & Hedrick, R. P. (1991). Lectin histochemistry and ultrastructure of rainbow trout *Oncorhynchus mykiss* kidneys affected by proliferative kidney disease. *Diseases of Aquatic Organisms*, *10*(3), 173–183. https://doi.org/10.3354/dao010173

Chilmonczyk, S., Monge, D., & De Kinkelin, P. (2002). Proliferative kidney disease: Cellular aspects of the rainbow trout, *Oncorhynchus mykiss* (Walbaum), response to parasitic infection. *Journal of Fish Diseases*, *25*(4), 217–226. https://doi.org/10.1046/j.1365-2761.2002.00362.x

Clifton-Hadley, R. S., Bucke, D., & Richards, R. H. (1986). Economic importance of proliferative kidney disease of salmonid fish in England and Wales. *Veterinary Record*, *119*(12), 305–306. https://doi.org/10.1136/vr.119.12.305

Clifton-Hadley, R. S., Richards, R. H., & Bucke, D. (1986). Proliferative kidney disease (PKD) in rainbow trout *Salmo gairdneri*: Further observations on the effects of water temperature. *Aquaculture*, *55*(3), 165–171. https://doi.org/10.1016/0044-8486(86)90112-2

Feist, S. W., Longshaw, M., Canning, E. U., & Okamura, B. (2001). Induction of proliferative kidney disease (PKD) in rainbow trout *Oncorhynchus mykiss* via the bryozoan *Fredericella sultana* infected with *Tetracapsula bryosalmonae*. *Diseases of Aquatic Organisms*, *45*(1), 61–68. https://doi.org/10.3354/dao045061

Ferguson, H. W. (1981). The effects of water temperature on the development of proliferative kidney disease in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases*, *4*(2), 175–177. https://doi.org/10.1111/j.1365-2761.1981.tb01122.x

Ferguson, H. W., & Ball, H. J. (1979). Epidemiological aspects of proliferative kidney disease amongst rainbow trout *Salmo gairdneri* Richardson in Northern Ireland. *Journal of Fish Diseases*, *2*(3), 219–225. https://doi.org/10.1111/j.1365-2761.1979.tb00161.x

Ferguson, H. W., & Needham, E. A. (1978). Proliferative kidney disease in rainbow trout *Salmo gairdneri* Richardson. *Journal of Fish Diseases*, *1*(1), 91–108. https://doi.org/10.1111/j.1365-2761.1978.tb00008.x

Gay, M., Okamura, B., & De Kinkelin, P. (2001). Evidence that infectious stages of *Tetracapsula bryosalmonae* for rainbow trout *Oncorhynchus mykiss* are present throughout the year. *Diseases of Aquatic Organisms*, *46*(1), 31–40. https://doi.org/10.3354/dao046031

Grabner, D. S., & El-Matbouli, M. (2010). *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) portal of entry into the fish host. *Diseases of Aquatic Organisms*, *90*(3), 197–206. https://doi.org/10.3354/dao02236

Hedrick, R. P., MacConnell, E., & De Kinkelin, P. (1993). Proliferative kidney disease of salmonid fish. *Annual Review of Fish Diseases*, *3*, 277–290. https://doi.org/10.1016/0959-8030(93)90039-E

Hedrick, R. P., Marin, M., Castagnaro, M., Monge, D., & De Kinkelin, P. (1992). Rapid lectin-based staining procedure for the detection of the myxosporean causing proliferative kidney disease in salmonid fish. *Diseases of Aquatic Organisms*, *13*(2), 129–132. https://doi.org/10.3354/dao013129

Henderson, M., & Okamura, B. (2004). The phylogeography of salmonid proliferative kidney disease in Europe and North America. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *271*(1549), 1729–1736. https://doi.org/10.1098/rspb.2004.2677

Kent, M. L., & Hedrick, R. P. (1985). PKX, the causative agent of proliferative kidney disease (PKD) in Pacific salmonid fishes and its affinities with the myxozoa 1. *The Journal of Protozoology*, *32*(2), 254–260. https://doi.org/10.1111/j.1550-7408.1985.tb03047.x

Kent, M. L., Khattra, J., Hervio, D. M. L., & Devlin, R. H. (1998). Ribosomal DNA sequence analysis of isolates of the PKX myxosporean and their relationship to members of the genus Sphaerospora. *Journal of Aquatic and Animal Health*, *10*, 12–21. https://doi.org/10.1577/1548-8667(1998)010%3C0012:RDSAOI%3E2.0.CO;2

Klontz, G. W., & Chacko, A. J. (1983). Methods to detect the organism causing proliferative kidney disease in salmonids. *Bulletin of the European Association of Fish Pathologists*, *3*, 33–36.

Kopp, R., Palíková, M., Papežíková, I., Mareš, J., Navrátil, S., Pikula, J., & Pohanka, M. (2018). Oxidative stress response of rainbow trout (*Oncorhynchus mykiss*) to multiple stressors. *Acta Veterinaria Brno*, *87*(1), 55–64. https://doi.org/10.2754/avb201887010055

Lewisch, E., Unfer, G., Pinter, K., Bechter, T., & El-Matbouli, M. (2018). Distribution and prevalence of *T. bryosalmonae* in Austria: A first survey of trout from rivers with a shrinking population. *Journal of Fish Diseases*, *41*(10), 1549–1557.

Longshaw, M., Feist, S. W., Canning, E. U., & Okamura, B. (1999). First identification of PKX in bryozoans from the United Kingdom-molecular evidence. *Bulletin-European Association of Fish Pathologists*, *19*, 146–148.

Longshaw, M., Le Deuff, R. M., Harris, A. F., & Feist, S. W. (2002). Development of proliferative kidney disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following short-term exposure to *Tetracapsula bryosalmonae* infected bryozoans. *Journal of Fish Diseases*, *25*(8), 443–449. https://doi.org/10.1046/j.1365-2761.2002.00353.x

Morris, D. C., Morris, D. J., & Adams, A. (2002). Molecular evidence of release of *Tetracapsula bryosalmonae*, the causative organism of proliferative kidney disease from infected salmonids into the environment. *Journal of Fish Diseases*, *25*(8), 501–504. https://doi.org/10.1046/j.1365-2761.2002.00352.x

Nowak, B., Mueffling, T. V., Caspari, K., & Hartung, J. (2006). Validation of a method for the detection of virulent *Yersinia enterocolitica* and their distribution in slaughter pigs from conventional and alternative housing systems. *Veterinary Microbiology*, *117*(2–4), 219–228. https://doi.org/10.1016/j.vetmic.2006.06.002

Okamura, B., Anderson, C. L., Longshaw, M., Feist, S. W., & Canning, E. U. (2001). Patterns of occurrence and 18S rDNA sequence variation of PKX (*Tetracapsula bryosalmonae*), the causative agent of salmonid proliferative kidney disease. *Journal of Parasitology*, *87*(2), 379–385.

Palikova, M., Papezikova, I., Markova, Z., Navratil, S., Mares, J., Mares, L., Vojtek, L., Hyrsl, P., Jelinkova, E., & Schmidt-Posthaus, H. (2017). Proliferative kidney disease in rainbow trout (*Oncorhynchus mykiss*) under intensive breeding conditions: Pathogenesis and haematological and immune parameters. *Veterinary Parasitology*, *238*, 5–16.

Pojezdal, Ľ., Adamek, M., Syrová, E., Steinhagen, D., Minářová, H., Papežíková, I., Seidlová, V., Reschová, S., & Palíková, M. (2020). Health surveillance of wild brown trout (*Salmo trutta fario*) in the Czech Republic revealed a coexistence of proliferative kidney disease and *Piscine Orthoreovirus-3* infection. *Pathogens*, *9*(8), 604.

Ribeiro, C. N. M., Peres, L. C., & Pina-Neto, J. M. (2004). DNA extraction and quantification from touch and scrape preparations obtained from autopsy liver cells. *Brazilian journal of medical and biological research*, *37*(5), 635–642.

Rüssmann, H., Kempf, V. A., Koletzko, S., Heesemann, J., & Autenrieth, I. B. (2001). Comparison of fluorescent in situ hybridization and conventional culturing for detection of *Helicobacter pylori* in gastric biopsy specimens. *Journal of Clinical Microbiology*, *39*(1), 304–308. https://doi.org/10.1128/JCM.39.1.304-308.2001

Schmidt-Posthaus, H., Bettge, K., Forster, U., Segner, H., & Wahli, T. (2012). Kidney pathology and parasite intensity in rainbow trout *Oncorhynchus mykiss* surviving proliferative kidney disease: Time course and influence of temperature. *Diseases of Aquatic Organisms*, *97*(3), 207–218. https://doi.org/10.3354/dao02417

Sengüven, B., Baris, E., Oygur, T., & Berktas, M. (2014). Comparison of methods for the extraction of DNA from formalin-fixed, paraffin-embedded archival tissues. *International Journal of Medical Sciences*, *11*(5), 494. https://doi.org/10.7150/ijms.8842

Skovgaard, A., & Buchmann, K. (2012). *Tetracapsuloides bryosalmonae* and PKD in juvenile wild salmonids in Denmark. *Diseases of Aquatic Organisms*, *101*(1), 33–42. https://doi.org/10.3354/dao02502

Sterud, E., Forseth, T., Ugedal, O., Poppe, T. T., Jørgensen, A., Bruheim, T., Fjeldstad, H.-P., & Mo, T. A. (2007). Severe mortality in wild Atlantic salmon *Salmo salar* due to proliferative kidney disease (PKD) caused by *Tetracapsuloides bryosalmonae* (Myxozoa). *Diseases of Aquatic Organisms*, *77*(3), 191–198.

Suresh, K., & Smith, H. (2004). Comparison of methods for detecting *Blastocystis hominis*. *European Journal of Clinical Microbiology and Infectious Diseases*, *23*(6), 509–511. https://doi.org/10.1007/s10096-004-1123-7

Syrová, E., Palíková, M., Mendel, J., Seidlová, V., Papežíková, I., Schmidt-Posthaus, H., Somerlíková, K., Minářová, H., Mareš, L., Mikulíková, I., Pikula, J., & Mareš, J. (2020). Field study indicating susceptibility differences between salmonid species and their lineages to proliferative kidney disease. *Journal of Fish Diseases*, *43*(10), 1201–1211. https://doi.org/10.1111/jfd.13221

Tops, S., Lockwood, W., & Okamura, B. (2006). Temperature-driven proliferation of *Tetracapsuloides bryosalmonae* in bryozoan hosts portends salmonid declines. *Diseases of Aquatic Organisms*, *70*(3), 227–236. https://doi.org/10.3354/dao070227

Vasemägi, A., Nousiainen, I., Saura, A., Vähä, J. P., Valjus, J., & Huusko, A. (2017). First record of proliferative kidney disease agent *Tetracapsuloides bryosalmonae* in wild brown trout and European grayling in Finland. *Diseases of Aquatic Organisms*, *125*(1), 73–78. https://doi.org/10.3354/dao03126

Whyte, P., Mc Gill, K., Collins, J. D., & Gormley, E. (2002). The prevalence and PCR detection of salmonella contamination in raw poultry. *Veterinary Microbiology*, *89*(1), 53–60. https://doi.org/10.1016/S0378-1135(02)00160-8

How to cite this article: Seidlova V, Syrova E, Minarova H, et al. Comparison of diagnostic methods for *Tetracapsuloides bryosalmonae* detection in salmonid fish. *J Fish Dis*. 2021;44:1147–1153. https://doi.org/10.1111/jfd.13375