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Novel PCR assay for the identification of two transmissible cancers in *Cerastoderma edule*

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ABSTRACT

Transmissible cancers are a rare phenomenon in which cancer cells are passed between individuals, leading to the development of neoplasia in the host organism. Transmissible cancers have been identified in three independent clonal lineages in mammals and eight different clonal lineages in bivalves. This study focused on the development of a multiplex PCR assay for the detection of two types of bivalve transmissible neoplasias (BTN) of cockles *Cerastoderma edule* (CedBTN1 and CedBTN2). The diagnostic sensitivity (Se) and specificity (Sp) of the new PCR of hemolymph samples were assessed using a maximum likelihood estimation in the 88 samples compared to diagnoses obtained by histology and confirmed with genomic analyses. The results showed a Se of 100 % and a Sp of 98.1 % for CedBTN1, and a Se and Sp of 100 % for CedBTN2. The analytical Sp and Se were tested using DNA extractions from infected and non-infected *C. edule* and other bivalves affected by BTN. The assay demonstrated high analytical sensitivity and specificity, detecting down to 0.4 % of circulating CedBTN1 cells at a DNA concentration of 0.05 ng/μL in a hemolymph sample of a cockle with 85.7 % of CedBTN1 cells. The multiplex PCR assay was type-specific for CedBTN and capable of detecting both lineages simultaneously.

Overall, this multiplex PCR assay is a reliable tool for the detection of transmissible cancers in cockles which will facilitate the diagnosis and monitoring of the disease.

1. Introduction

Cancer normally arises from the accumulation of mutations and the uncontrolled proliferation and invasive capabilities of an organism's own cells. Cancer cells can spread from the primary tumor to other tissues, inducing metastases; however, their lifespan is linked to the lifespan of the organism affected by cancer. Nevertheless, there are cancers that can spread between individuals via the transfer of living cancer cells, and these can persist for longer than the organisms that suffer from them, potentially existing for up to 8500 years (Baez-Ortega et al., 2019). These cancers are called clonally transmissible cancers. Such transmissible cancers occurring in wildlife have been identified in dogs (Murgia et al., 2006; Baez-Ortega et al., 2019), Tasmanian devils (Pearse and Swift, 2006; Pye et al., 2016) and recently in marine bivalves' species.

Transmissible cancers in bivalves are a type of disseminated

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neoplasia, a cancer that spreads through the hemolymph to all organs of the individual. The first BTN cancer was described in softshell, *Mya arenaria* analyzing SNPs of mitochondrial genes (cytochrome *c* oxidase subunit I and cytochrome *b*) and confirmed with microsatellites (Metzger et al., 2015). In recent years up to 8 different BTN have already been confirmed (Metzger et al., 2015; Metzger et al., 2016; Garcia-Souto et al., 2022; Michnoswska et al., 2022). This number is probably underestimated since more than 25 different bivalve species affected by disseminated neoplasia have been diagnosed (Díaz, 2015).

BTNs have been observed in only one species, such as the BTN of *Mya arenaria* on the Atlantic coast of North America (Metzger et al., 2015), common edible cockle, *Cerastoderma edule* on the Atlantic coast of Spain (Metzger et al., 2016) and Baltic clam, *Macoma balthica* from Poland (Michnowska et al., 2022). However, BTNs have also been found affecting several species of the same genus. A BTN that arose in a bay mussel, *Mytilus trossulus* has been found infecting individuals of the family Mytilidae worldwide including *M. trossulus*, blue mussels *M. edulis*, Chilean mussels *M. chilensis* and Mediterranean mussels *M. galloprovincialis* (Yonemitsu et al., 2019; Hammel et al., 2022; Skazina et al., 2021; Hammel et al., 2024). BTN have even been found to originate from a species not currently affected by that disease, while affecting another species; as is the case of the BTN described in golden carpet clam, *Polititapes aureus* (Carballal et al., 2013) from the Atlantic coast of Spain originated in pullet carpet clam, *Venerupis corrugata* (Metzger et al., 2016) and the BTN detected in warty venus, *Venus verrucosa* on the Atlantic and Mediterranean coast of Spain spawned from striped venus, *Chamelea gallina* (Garcia-Souto et al., 2022). Furthermore, BTNs have been able to arise more than once in the same species. Two different lineages (MtrBTN1 and MtrBTN2) have been discovered in *M. trossulus* (Metzger et al., 2016). The first MtrBTN1 seems to exclusively affect *M. trossulus*; however, the latter MtrBTN2 affects different species of the same genus. The geographical distribution of these two lineages is also differentiated. While MtrBTN2 has a broader distribution, including coasts of the South Pacific Ocean (Argentina, Chile), North Atlantic Ocean (France, Netherlands), Mediterranean Sea (Croatia), Sea of Okhotsk and Sea of Japan (East Sea); MtrBTN1 is restricted to the North Pacific Ocean (British Columbia) and Sea of Okhotsk, with both lineages overlapping only in Sea of Okhotsk (Metzger et al., 2016; Yonemitsu et al. 2019; Skazina et al., 2021; Hammel et al., 2022; Skazina et al., 2023). Two different BTN sublineages with a common origin have been detected in *Mya arenaria* associated with different Atlantic geographic areas (Canada and United States of America) (Hart et al., 2023). Additionally, two BTN lineages (CedBTN1 and CedBTN2) have been detected infecting *C. edule* along the Atlantic coast of Europe. Phylogenetic analyses of somatic and germinal structural variants from whole-genomes of CedBTN1 and CedBTN2 have detected two origins (Bruzos et al., 2023) as suggested by the study of microsatellites and the mitochondrial cytochrome *c* oxidase subunit I gene (Metzger et al. 2015). Analysis of the mitogenome has revealed nine events of mitochondrial capture by CedBTN from its hosts: up to six captures have been detected in CedBTN1 and three captures in CedBTN2 (Bruzos et al., 2023). CedBTN1 and CedBTN2 share common characteristics of disseminated neoplasia, large cells with a round or oval shape and a high nucleus:cytoplasm ratio with prominent nucleoli and a high frequency of mitotic figures (Carballal et al., 2015). The copy number distributions observed in neoplastic cells of both lineages typically exhibited a modal CN of 4.0, implying ancestral tetraploidy (Bruzos et al., 2023). They are observed in the connective tissue of multiple organs and in vessels and sinuses of the circulatory system (Carballal et al., 2015). However, both lineages differ in cell size and cell interaction. The cells of CedBTN1 neoplasia are larger than those of CedBTN2, and the cells of CedBTN1 are isolated, while the cells of CedBTN2 are clustered and compressed together (Carballal et al., 2001; Bruzos et al., 2023). The distribution of CedBTN1 is the south of the Atlantic coast of Europe (Portugal, Spain, France, Netherlands, United Kingdom and Ireland) while the detection of CedBTN2 is limited to the Galician, and Welsh coasts (Poder and Auffret, 1986; Twomey and Mulcahy, 1988; Díaz et al., 2016; Montaudouin et al., 2021; Bruzos et al., 2023). A differential spatial distribution of each clone was identified, with both coexisting in sandy areas of Galicia, exhibiting variations in prevalence. Specifically, when both lineages coexist within the same population, CedBTN1 shows prevalence ranging from 1.7 % to 9.6 %, while CedBTN2 ranges from 0.4 % to 2.9 % (Bruzos et al., 2023).

The diagnosis of disseminated neoplasia in cockles has traditionally been made by microscopic observation of hemolymph smears, histological sections, and ploidy analysis by flow cytometry (Poder and Auffret 1986; Twomey and Mulcahy, 1988; Grand et al., 2010; Díaz et al., 2010; 2013). However, confirmation of transmissible cancer can only be done by sequencing, procedures that are time-consuming. Due to the contagious nature of these cancers (Collins and Mulcahy, 2003; Diaz et al., 2017) and the fact that they have been associated with massive mortality (Montaudouin et al., 2021), specific, rapid, sensitive, and easy-to-use molecular diagnostic techniques are necessary for early detection, monitoring and prevention of the propagation to non-affected areas. The main goal of this work was to design and evaluate type specific primers for the transmissible cancers lineages of cockles and optimize a diagnostic PCR for their rapid and easy detection.

2. Material and methods

2.1. Sample collection and diagnosis

Within the framework of Scuba Cancers project (grant agreement ID: 716290), a biobank was created with 6854 samples of common cockles *Cerastoderma edule* collected along the Atlantic coast from Morocco to Russia (Morocco, Portugal, Spain, France, Germany, Netherlands, Denmark, Ireland, United Kingdom, Norway, and Russia) from 2017 to 2021 (Bruzos et al., 2023). Species confirmation was conducted through amplification of the internal transcribed spacer region (Freire et al., 2011). In cases where the results were inconclusive, a panel of single nucleotide polymorphisms (SNPs) was analyzed for further validation (Maroso et al., 2019).

All collected bivalves were examined through microscopic observation of hemolymph smears and histological sections to diagnose cases of disseminated neoplasia. Except for the samples from Ireland, which were diagnosed solely by histology.

A total of 115 samples of C. edule were used for retrospective molecular diagnosis of cancer: 52 were unaffected, while 63 had different intensities of cancer, as diagnosed through cytology of hemolymph and histology (56 of the detected cancers exhibited morphological characteristics of CedBTN1, while 7 of the cancers corresponded to CedBTN2). In 36 of these cancers, whole genome genetic variations were analyzed, confirming their assignment: 31 CedBTN1 and 5 CedBTN2 (Bruzos et al., 2023, Suppl. Info. 1).

In addition, samples of one lagoon cockle, *C. glaucum* (Poland), four *V. verrucosa* (Spain) and, two *P. aureus* (Spain) diagnosed with cancer were tested by the molecular assay (Suppl. Info. 1). The *C. glaucum* was diagnosed with transmissible cancer through genetic markers (unpublished data), the samples of *V. verrucosa* were sourced from the study conducted by Garcia-Souto et al. (2022), where they were diagnosed with transmissible cancer and, *P. aureus* samples were collected from the location where the transmissible cancer of the species was discovered (Metzger et al., 2016).

The hemolymph was extracted from the posterior adductor muscle with a 2 ml syringe and a 23G needle. For cytological observation of circulating cells, 50 µl of hemolymph was diluted in 150 µl Alsever antiplatelet solution (0.11 M glucose, 37 mM sodium citrate, 11 mM EDTA and 0.38 M NaCl). This solution was used to carry out the smear using a cytochamber and Universal 320R centrifuge (7 min, 130xg, 4 °C) (Hettich Lab Technology™) and it was stained with the Hemacolor® kit (Sigma-Aldrich). The percentage of circulating neoplastic cells was determined by counting the neoplastic cells present in 500 cells within

hemolymph monolayers.

A 5 mm cockle section containing all the organs was fixed in Davidson's solution (10 % glycerin, 20 % Formaldehyde 36–40 %, 30 % Ethanol, 30 % filtered seawater, 10 % acetic acid) for 24 h at 4 °C and stored in 70 % ethanol. The tissues were processed using histological techniques and sections were stained with Harris' haematoxylin and eosin.

Tissue and hemolymph leftovers were frozen at −80 °C for posterior use in molecular techniques.

Neoplastic cells were recognized following the descriptions previously reported for these species (Carballal et al., 2001, Carballal et al., 2013; Garcia-Souto et al., 2022). The diagnosis of disseminated neoplasia, based on the proportion of circulating neoplastic cells of hemolymph (Díaz et al., 2010) and the abundance, distribution, and progression of the neoplastic cells through the cockle tissues (Díaz et al., 2016); was classified into 4 stages,: unaffected (stage N0), low (stage N1 < 15 % of neoplastic cells circulate in the hemolymph and infiltrate solid tissues in small numbers), moderate (stage N2 distinguished by 15–75 % of neoplastic cells in the hemolymph and presence of small infiltration foci in one or more organs), and high severity (stage N3, >75 % of neoplastic cells in the hemolymph and massive tissue infiltration).

2.2. Genomic DNA extraction

DNA extractions from the hemolymph, digestive gland, mantle and foot from bivalves were individually performed using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's protocol. DNA quality and quantity were checked in a Nanodrop One spectrophotometer (Thermo Scientific).

2.3. Computational design of neoplasia specific primers

2.3.1. Marker variant selection

Within the framework of Scuba Cancers project, ten CedBTNs samples (7 CedBTN1 from France, Portugal, and Spain and 3 CedBTN2 from Spain and United Kingdom) were selected based on their high tumor purity levels (≥97 % of neoplastic cells in hemolymph) to form a 'golden set' (Bruzos et al., 2023). Cancer genomes were sequenced from hemolymph cells and tumor variants were identified as described in Bruzos et al. (2023). Illumina paired-end DNA sequencing reads were aligned to the common cockle reference genome assembly (GenBank: GCA_947846245.1) using BWA v0.7.17, with default BWA-MEM settings. The aligned reads were sorted and indexed using samtools (v1.9), and duplicate reads were marked using biobambam2 (v2.0.87). A computational search was conducted to identify genomic variants that could potentially serve as specific markers for each of the two target cancer lineages, CedBTN1 and CedBTN2, using the sample genomes from the 'golden set'.

The marker search prioritized structural variants (SVs) due to their established link with cancer, significant impact, minimal homoplasy, and distinctive molecular profile. Deletions were selected over other SVs (such as translocations, inversions, duplications) because they represent fewer complex events, yet appropriate for reliable allelic discrimination via PCR-based genotyping. Deletions were identified by using three alternative computational algorithms. The first one, Delly v0.7.9 (Rausch et al., 2012), discovers the events through the integration of discordant read-pair and split-read evidence. The second one, Lumpy v0.2.13 (Layer et al., 2014) combines discordant read-pair and split-read analysis with read-depth evaluation. The third one, Manta v1.6.0 (Chen et al., 2016), relies on discordant read-pair and split-read analysis followed by target assembly breakpoint validation.

Structural variant calls from each algorithm were integrated using Svimmer (v0.1; github.com/DecodeGenetics/svimmer). Deletions with precise (base-level) breakpoints identified by all the three methods were chosen, to select a high-confident set of structural variants. The selected deletions were genotyped on 10 tumors genomes from the 'golden set' using Graphtyper v2.0 (Eggertsson et al., 2019), which assesses alternative structural variant haplotypes after encoding them into directed acyclic graphs. After encoding the presence of each variant on a custom binary matrix, all the non-relevant events meeting the following criteria were discarded: a) deletions without VCF PASS filter category b) deletions with undetermined genotype on any tumour genome c) deletions identified on available unaffected *C. edule* genomes, likely representing germline variation, and d) deletions outside genic regions. Finally, those deletion events specific to either of the two target lineages (CedBTN1 or CedBTN2), shared by all the available cancer genomes of the 'golden set' from that lineage, likely representing early clonal somatic events, were considered as marker candidates. Among the marker candidates, it was decided to prioritize variants of moderate size (deletions between 1 and 2 Kb), for validation purposes.

Finally, after manual bam file inspection of the top candidate deletions with Integrative Genomics Viewer (IGV) software, two marker deletions were selected: CedBTN1: *STARD9* deletion (chr1:46638024–46639236; size = 1.2 Kb) and CedBTN2: *BIN1* deletion (chr6:23657409–23659027; size = 1.6 Kb) (Fig. 1).

2.3.2. Amplification design

Primer design was performed using the Primer3 software (Untergasser et al., 2012) following a variant size discriminative strategy. Source DNA sequence consisted of 1000 bp from the *C. edule* reference genome flanking each deletion (500 bp upstream and 500 bp downstream region).

After masking local repetitive sequences, optimal primer pairs were retrieved, considering product size ranges between 150 and 500 bp. Optimal primer size was set on 20 nucleotides (accepted range 18–24 nucleotides), optimal primer melting temperature was set on 59 °C (accepted range 57–62 °C) and optimal primer GC % was set on 59 % (accepted range 30–70 %). The best two primer pair combinations for both selected regions.

2.3.3. In silico PCR

In silico PCR evaluation with the software Ipcress (exonerate package v2.4.0, Slater and Birney, 2005) confirmed the specificity of the amplification strategy, rendering a single amplification product per experiment. The analysis was done with *C. edule* reference genome sequence (Bruzos et al., 2023), considering potential amplification products smaller than 1 Mb. Thus, two primer pairs, each specific for a CedBTN lineage, that allow discriminating both based on the size of the amplified DNA product were selected (CedBTN1-F/R (404pb) and CedBTN2-F/R (160pb)) (Table 1).

2.4. Multiplex PCR assay for the diagnosis of neoplasia

The optimal annealing temperature was determined individually for each primer pair and verified for multiplex PCR using a temperature gradient (52, 54, 58, 60, 62, and 64 °C). Incubation times were optimized without compromising the results, starting from those suggested in the technical sheet of the JumpStart™ Taq DNA polymerase (Sigma-Aldrich). Amplifications were carried out in a Proflex Thermal Cycler (ThermoFisher Scientific) in volume of 25 µl containing 2 µl of genomic DNA (100 ng), 400 µM of dNTPs (ThermoFisher Scientific), 0.4 µM each specific primer CedBTN1-F/R and CedBTN2-F/R, 0.2 U μl^{-1} JumpStart™ Taq DNA polymerase (Sigma-Aldrich) and PCR buffer with $MgCl_2$ at 1x concentration (Sigma-Aldrich). A positive control for each CedBTN type and a negative control (no DNA) were used.

Cycling conditions were initial denaturation at 94 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 65 °C for 2 min, with a final extension of 65 °C for 10 min. PCR products were evaluated by electrophoresis in 2 % agarose gels stained with SYBR™ Safe (Thermo Fisher Scientific).

Two PCR products from each type of cancer were sequenced to verify that the amplification had been as designed. PCR products were then

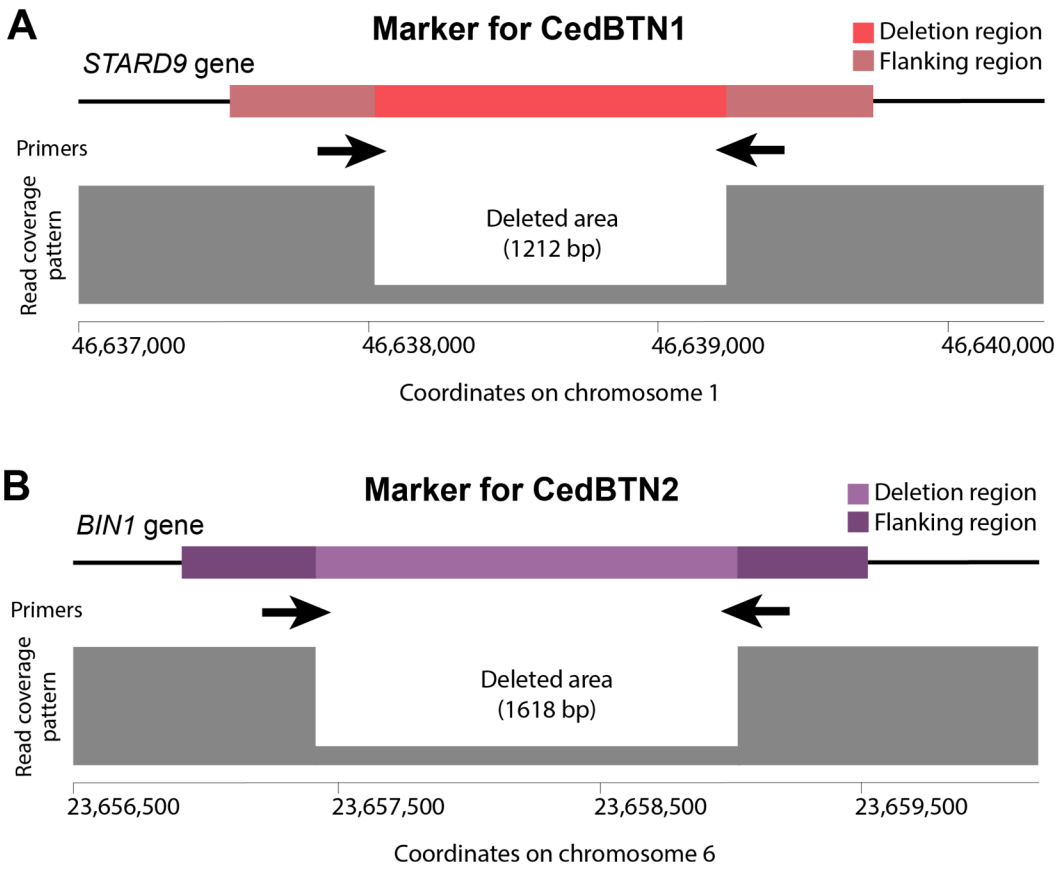

Fig. 1. Schematic representation of the area from the genes used as markers for cockle transmissible cancers (CedBTNs), annealing region of primers and read coverage pattern from a representative sample affected by the cancer **(A)** CedBTN1 lineage, 1212 bp deletion in the STARD9 gene, **(B)** CedBTN2 lineage, 1618 bp deletion in the BIN1 gene on chromosome 6.

Table 1
Sequences of the primers designed in this study for the detection of the transmissible cancer lineages CedBTN1 and CedBTN2, in the cockle species *Cerastoderma edule*, along with the size of the amplified sequence. F: forward, R: reverse.

Primer	Sequence (5́- 3́)	Amplicon
CedBTN1-F	TTATGTGGTGCTAGGTGGGGAT	404pb
CedBTN1-R	GGAGAAATGCAAGACACCAGTAAG	
CedBTN2-F	TCACTGAACCTTTGAATAGCTCA	160pb
CedBTN2-R	ACCTTTGCTCATCTCAAGACA	

purified with ExoSAP-IT (Thermo Fisher Scientific) and sequenced in both directions with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), following manufacturer's protocol. The sequences were analysed on SeqStudio Genetic Analyzer (Thermo Fisher Scientific). Sequences were aligned with NCBI pairwise blast tool to confirm the sequence identity between the amplicons and the target *C. edule* deleted genomic regions.

2.5. Analytical specificity and sensitivity of the PCR assays

The specificity of the primers against type of CedBTN was evaluated using (1) hemolymph DNA from cockles previously diagnosed with high and moderate severity of neoplasia, (39 cockles), (2) 52 unaffected cockles and (3) from other bivalves highly affected by disseminated neoplasia: one *C. glaucum,* two *P. aureus* and four *V. verrucosa* (Suppl. Info. 1). In addition, it was evaluated whether multiplex PCR could detect a co-infection of both types of neoplasia in the same sample. Samples were created by combining varying proportions of hemolymph DNA from two cockles, FRCE17/701 highly affected by CedBTN1 and UGCE17/2401 highly affected by CedBTN2 (CedBTN1:CedBTN2 ratio; 100:0, 75:25, 50:50, 25:75, 0:100).

The analytical sensitivity of the multiplex PCR was evaluated with eleven-fold serial dilutions at 50 % of DNA extraction of hemolymph of cockles infected with each type of neoplasia with high severity. DNA concentration of the dilutions was measured by Qubit fluorometric quantification with 1xdsDNA high sensitivity assay kit (Thermo Fisher Scientific).

To evaluate whether host DNA interferes in PCR reactions, two assays were performed. Firstly, hemolymph DNA from 23 CedBTN1 cockles with different percentages of circulating neoplastic cells (100 % to 0.4 %). There were no samples available to perform it with CedBTN2. Secondly, tissue DNA (gill, digestive, mantle and foot) from three CedBTN1 cockles of each severity; three CedBTN2 cockles of moderate severity and two of high severity (Suppl. Info. 1).

2.6. Diagnostic specificity and sensitivity

The specificity and sensitivity of the diagnostic PCR for CeBTN1 and CeBTN2 have been estimated using a maximum likelihood method using TAG V.2.0 program (Pouillot et al., 2002). The algorithm includes 2 assumptions: (i) diagnostic tests are independent, and ii) test diagnostic values are considered to have constant sensibility and specificity when applied to different populations. The diagnosis obtained from the 88*C. edule* samples through the PCR developed in this study has been compared with the previous diagnosis by histology and confirmed CedBTN through genomic analyses in Bruzos et al. (2023). Based in the genetic population structure of the species *C. edule* (Vera et al., 2022; Bruzos et al., 2023), results have been analyzed for two groups: (1)

cockles originating from Spain, France, and Portugal populations; (2) cockles originating from Germany, Denmark, Netherlands, Ireland, Norway, and the United Kingdom populations.

3. Results

3.1. Analytical specificity multiplex PCR

The analytical specificity of multiplex PCR for the detection of transmissible cancers was confirmed using DNA from *C. edule* infected with high and moderate severity of CedBTN1 or CedBTN2 obtaining products of the expected size of 404 bp or 160 bp, respectively (Fig. 2, Suppl. Info. 2). CedBTN1 was detected in the hemolymph of 33 cockles from Spain, France, Portugal, and Ireland. CedBTN2 was detected in the hemolymph of 8 cockles from Spain, United Kingdom, Ireland, and Portugal (Fig. 2). All PCRs confirmed the previous diagnosis made by histological sections of tissue and/or monolayer smears of hemolymph. In one case (PACE17/497), which had been diagnosed with only CedBTN1 lineage, both lineages were detected through this multiplex PCR (Suppl. Info. 2). The sequence of the PCR-amplification products matched the selected deletion from CedBTN1 and CedBTN2.

The hemolymph of 51 non-infected cockles from 15 different origins from 9 countries (Portugal, Spain, France, Germany, Netherlands, Denmark, Ireland, United Kingdom, Norway) did not show the bands assigned to the types of BTN. However, 1 sample from Ireland (ICCE19/365) that had not been detected as neoplastic by microscopic methods tested positive for CedBTN1 via multiplex PCR (Suppl. Info. 2). Additionally, 69 % of non-infected cockles successfully amplified a sequence of approximately 1.7 Kb corresponding to the genes without the deletion, which is used for the specific diagnosis of the types of neoplasia. Only samples from Denmark and Germany failed to amplify this sequence, along with 4 samples from the United Kingdom (4 out of 12) and 1 from the Netherlands (1 out of 4) (Suppl. Info. 2).

This PCR multiplex assay did not amplify the DNA of other bivalves with cancer, such as *Polititapes aureus*, *Venus verrucosa*, and *Cerastoderma glaucum* (Suppl. Info. 3).

3.2. Analytical sensitivity multiplex PCR

The analytical sensitivity of the multiplex PCR for CedBTN1 and CedBTN2 was tested using hemolymph DNA serial dilutions of cockles heavily affected by the neoplasia. The multiplex PCR successfully detected CedBTN1 at concentrations as low as 0.016 ng of cockle gDNA per µL and CedBTN2 at concentrations as low as 0.059 ng of cockle gDNA per µL (Fig. 3).

It was evaluated whether host cells could interfere with the diagnosis from cockle hemolymph with different percentages of CedBTN1 cells and by analyzing different tissues of affected cockles. The multiplex PCR was able to diagnose all hemolymph samples analyzed, detecting up to 0.4 % of circulating CedBTN1 cells. The sequence without the deletion (1.6–1.7 Kb) was amplified in all samples where the proportion of normal hemocytes was greater than 76.2 % (Fig. 4). Moreover, both CedBTN lineages were detected by PCR with 100 % accuracy in gill and digestive tissues across all disease severities. However, detection was not observed in the feet, and only in 66.7 % of mantle samples for low severity of CedBTN1. When analyzing *C. edule* DNA templates artificially coinfected with CedBTN1 and CedBTN2, amplification sequences for both were observed across all ratio variations tested. No selective amplification of a specific lineage was evident, indicating that both lineages can be diagnosed simultaneously (Suppl. Info. 4).

3.3. Diagnostic specificity and sensitivity

Based on the analyses via PCR compared with the diagnosis by histology and confirmed with genomic studies (Table 2, Suppl. Info. 5), the diagnostic specificity estimated by the maximum likelihood method for lineage CedBTN1 was 0.9808 with a 95 % confidence interval of (0.9973, 8.8758), and for lineage CedBTN2 it was 1.0000, indicating that no false positives were detected. The sensitivity for both lineages was 1.0000, indicating that the diagnostic PCR accurately detected all true positive cases.

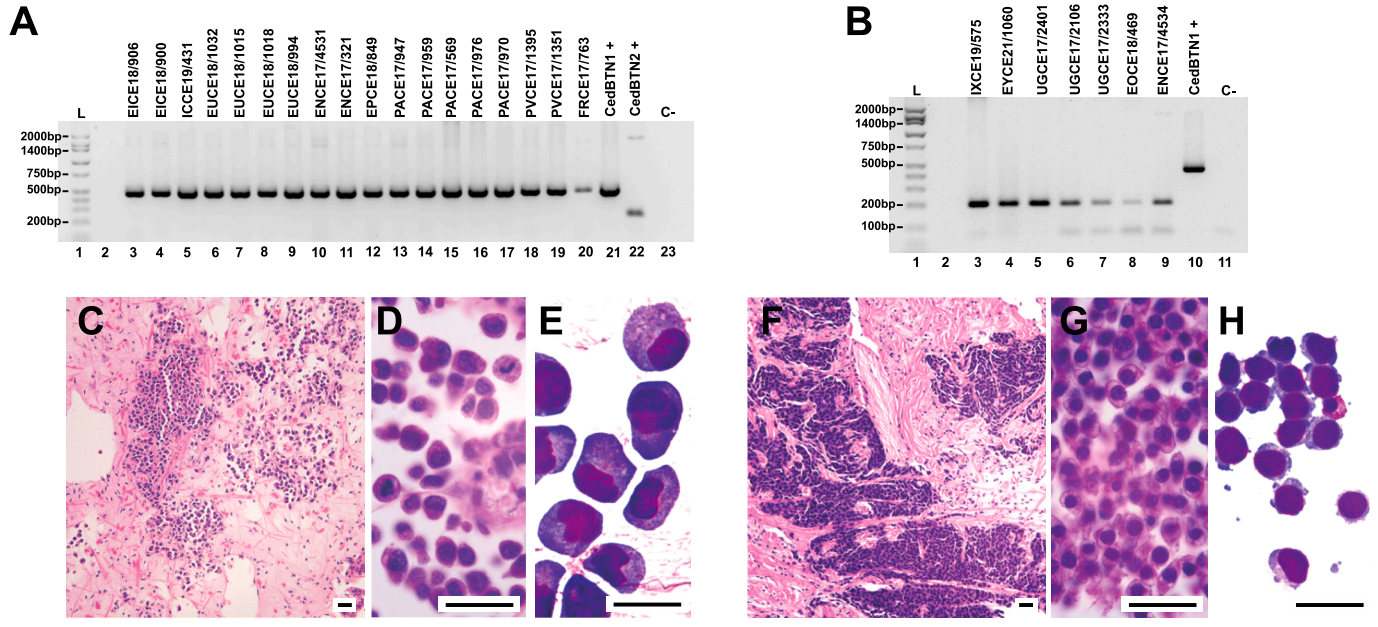

Fig. 2. Electrophoresis gel showing multiplex PCR amplification products of cockle transmissible cancers (CedBTNs) from different populations: infected by **(A)** CedBTN1 linage and **(B)** CedBTN2 linage. Lanes: A 1 and B 1 ladder; A: 21, 22 and B 10 positive controls; A 23 and B 11 negative controls. The microphotographs illustrate the morphology of CedBTN cells: **(C-E)** CedBTN1 linage and **(F-H)** CedBTN2 linage. C & F depict infiltration of CedBTN into connective tissue. D and G detailed view of CedBTN infiltration into tissue. E and H detail of CedBTN in hemolymph. C, D, F, and G Histological sections stained with Hematoxylin and Eosin. E and H hemolymph smears stained by Hemacolor. Scale bar 20 µm.

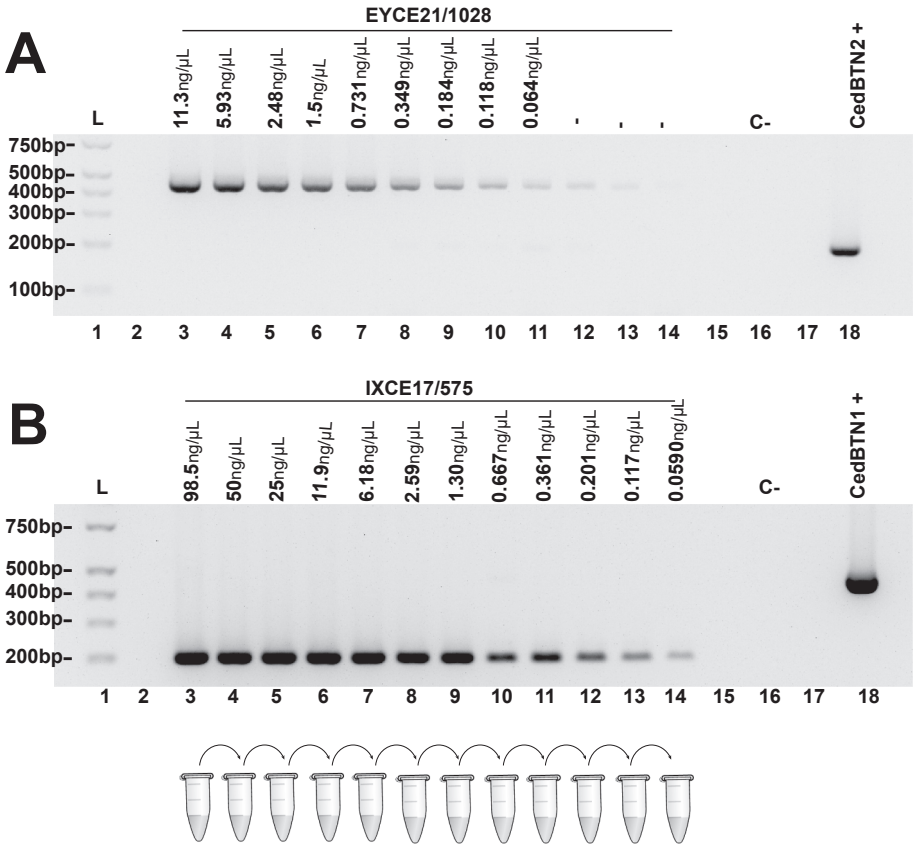

Fig. 3. Electrophoresis gel showing multiplex PCR amplification products of cockle transmissible cancers (CedBTNs) in assays performed to evaluate the analytical sensitivity using DNA dilutions (assessed concentration per qubit) from (A) EYCE21/1028 cockle infected by the CedBTN1 lineage and (B) in IXCE 17/575 cockle infected by the CedBTN2 lineage. Lanes: 1 ladder; 18 positive controls; 16 negative controls. Lanes: 1 ladder; 18 positive controls; 16 negative controls.

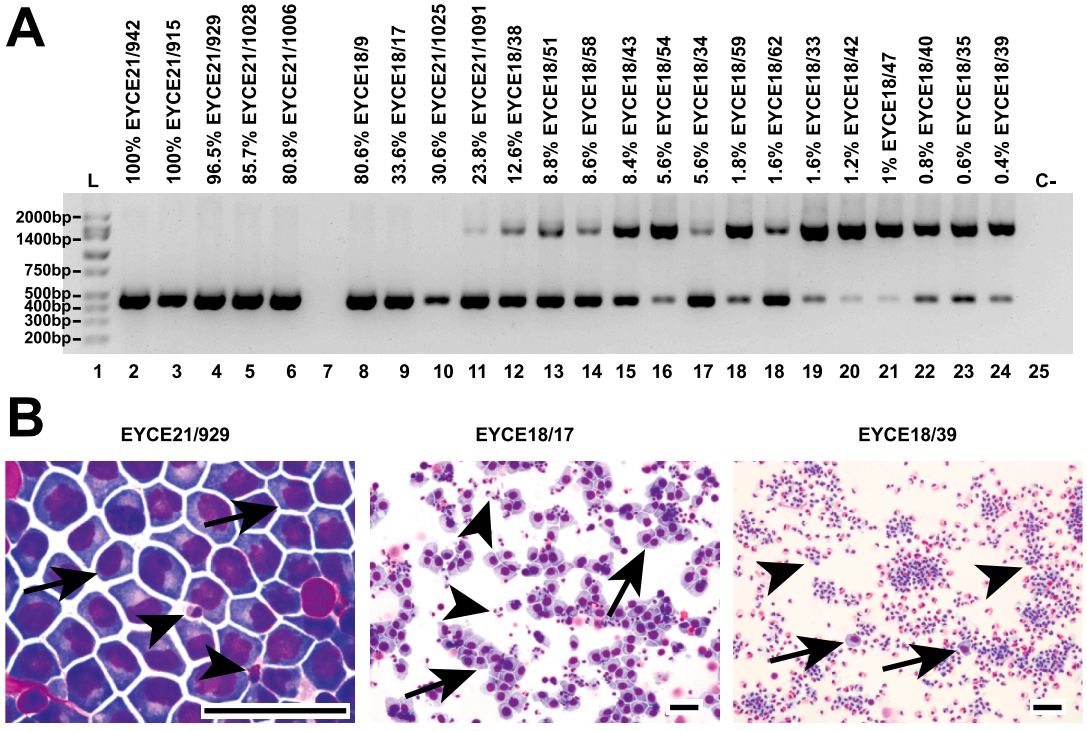

Fig. 4. **(A)** Electrophoresis gel displays the amplification products of multiplex PCR for cockle transmissible cancers (CedBTNs) in assays conducted to evaluate the analytical sensitivity using hemolymph DNA from cockles of the same population but with varying percentages of circulating BTN. Lanes: 1 ladder; 25 negative control. **(B)** Microphotographs of monolayer smears displaying hemolymph samples with varying percentages of CedBTN cells. Head arrow hemocytes and arrow CedBTN cells. Stained with Hemacolor. Scale bar 50 µm.

Table 2
Number of cockle samples in which each transmissible cancer lineage (CedBTN1 and CedBTN2) was detected by histology or multiplex PCR.

n = 115	Histology examination	Multiplex PCR
unaffected	52	51
CedBTN1	56	56
CedBTN2	7	7
CedBTN1 + CedBTN2	0	1

4. Discussion

The development of rapid, cost-effective, and user-friendly molecular diagnostic techniques for detecting mollusk diseases in marine environments is crucial. Several molecular diagnostic methods have been developed for detecting various pathologies in bivalves (Carrasco et al., 2017; Ríos et al., 2020). Some of these techniques can even detect multiple pathogens simultaneously (Canier et al., 2020), which is highly advantageous for disease monitoring and management.

However, there is currently no molecular tool available for detecting transmissible cancers in cockles. The nuclear genes STARD9 and BIN1 have been used for the detection of CedBTN lineage-specific deletions: chr1:46638024-46639236del and chr6:23657409-23659027del, respectively. This represents the first development of a tool capable of simultaneously detecting all transmissible cancers identified in this species. Population studies with whole genome data of the species *Cerastoderma edule* from the North Atlantic revealed two distinct populations bordering French Brittany (Vera et al., 2022; Bruzos et al., 2023). Samples from both populations were evaluated to avoid non-specific reactions of the primers. For the detection of molecular markers for CedBTN, the use of the mitochondrial genome was ruled out due to the detection of 9 mitochondrial DNA haplotypes that could be a consequence of host mitochondria captures in the cockle-to-cockle transmission of these neoplastic cells (Bruzos et al., 2023).

There is no universal molecular assay capable of detecting transmissible cancers across all affected species; these tools need to be adapted according to each lineage of each species. Additionally, reducing false positives detection is one of the main challenges of molecular techniques, especially given the lack of gold standard samples for validation. Diagnostic molecular techniques exist for transmissible cancers of Tasmanian devils, mussels, and soft-shell clams. For Tasmanian devils *Sarcophilus harrisii*, a multiplex PCR was designed based on specific structural variations of each lineage, using interchromosomal translocations as markers (DFT1 2/X and DFT2 4/5) (Kwon et al., 2018). For transmissible cancer of soft-shell clams *Mya arenaria* and Mytilidae, qPCR was performed for independent amplifications of specific cancer alleles. Two allele-specific qPCR were designed to amplify integration sites of the LTR-retrotransposon Steamer in *Mya arenaria* (Giersch et al., 2022). Multiple qPCR assays targeting various loci were designed to diagnose the two lineages affecting different Mytilidae species: 5 specific alleles of cancer in mussels were targeted across mitochondrial (mtCR and mtCOI) and nuclear (EF1a, H4, and EF1a-i3) genes (Yonemitsu et al., 2019; Burioli et al., 2021). Recently, the nuclear gene EF1a and the mitochondrial control region MtCR from *M. trossulus* have been multiplexed using probes to optimize the detection of MtrBTN2 in *M. galloprovincialis*, *M. edulis* and hybrids of both species through ddPCR (Hammel et al., 2024).

Transmissible cancers are characterized by the presence of neoplastic cells with the same genotype in different individuals, suggesting the transmission of cancer cells between hosts. This transmission may occur through the release of cancer cells into the marine environment (Burioli et al., 2021; Giersch et al., 2022), emphasizing the need to develop specific molecular techniques for detecting transmissible cancers outside of their host or in hosts of other species. Such techniques would allow for the rapid evaluation of entire populations and the implementation of disease control and prevention measures. Similar approaches have been used to study other pathogens affecting bivalves, such as those described in previous studies (Audemard et al., 2004; Carrasco et al., 2017, 2008; Arzul et al., 2014). In this study, we developed a diagnostic method with high sensitivity (Se 100 %), detecting the two lineages of transmissible cancers of cockles at low DNA concentrations (less than 0.06 ng/μL) and low percentages of circulating cells (0.4 %). The method is also high specific (Sp 98.1 %), detecting only the cockle transmissible cancer lineages and not those of other species that share their habitat. These characteristics enable the detection of transmissible cancers in new geographic locations and further research on potential host-transmitter relationships.

The phenotypic differences in cell size between the two CedBTN lineages (Carballal et al., 2001; Bruzos et al., 2023) could influence a higher diagnosis of CedBTN1 compared to CedBTN2 by microscopical methods, as the size of CedBTN2 is closer to that of hemocytes, making its diagnosis more challenging in the initial stages of the disease. Implanting diagnostic PCR in transmissible cancers of cockles could increase its detection. Furthermore, the coexistence of both mitochondrial lineages in the same habitat and the potential for simultaneous infection of the same individual have been documented (Bruzos et al., 2023), highlighting the importance of molecular diagnostic techniques for detecting coinfection.

Multiplex PCR offers rapid detection of the presence/absence of different CedBTN lineages. However, PCR does not provide information on the intensity, cannot distinguish between dead and active cancer cells, and false-positive results may occur from contaminated samples (Aranguren and Figueras, 2016). The development of techniques for eNA (eDNA and eRNA) detection will be the new challenges for transmissible cancers in the coming years as it has been developed to detect other bivalve diseases in environmental compartments (Mérou et al., 2022; Giersch et al., 2022; Bass et al., 2023). Despite the great advantages offered by molecular diagnostic techniques, these techniques should not replace histological techniques because they offer complementary information on tissue lesions, health status, and possible emerging diseases (Aranguren and Figueras, 2016).

In conclusion, we have designed a fast, cost-effective, specific, sensitive, and easy-to-use PCR based method for the detection of CedBTN. This assay can simultaneously discriminate between the two CedBTN lineages, facilitating disease monitoring, detection of new affected areas, or new species involved in transmission. The proposed PCR assay represents a significant contribution to the field of transmissible cancers, offering a reliable and accessible tool for laboratories worldwide.

CRediT authorship contribution statement

Martín Santamarina: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Alicia L. Bruzos:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Visualization. **Ana Pequeño-Valtierra:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. **Jorge Rodríguez-Castro:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Project administration. **Seila Díaz:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing, Conceptualization. **Jose M. C. Tubio:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing, Project administration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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