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Aptamer-antibody sandwich lateral flow test for rapid visual detection of tetrodotoxin in pufferfish

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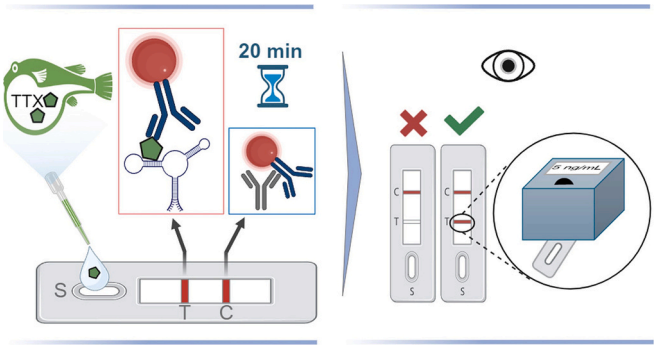
HIGHLIGHTS

- Presence of tetrodotoxin in mediterranean coastal areas increases food poisoning risk
- First antibody-aptamer sandwich-type lateral flow test for tetrodotoxin detection
- 0.3 ng/mL visual limit of detection validated with contaminated pufferfish samples
- Agreement of the LFA results with more costly analytical methodologies
- Stable test devices after more than four years of refrigerated storage

GRAPHICAL ABSTRACT



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ABSTRACT

Tetrodotoxin (TTX) is a highly potent marine toxin which can cause severe poisoning following consumption of contaminated fish and seafood. Thus, a sensitive, reliable and simple test is required for rapid screening of samples and prevention of intoxication. Herein, we translated a previously reported microtiter plate hybrid aptamer-antibody assay into a rapid lateral flow assay (LFA) test. The test relies on an aptamer immobilized on the membrane and an antibody conjugated with gold nanoparticles to provide a visual result when TTX is present in the sample. The optimized test is simple (one-step), rapid (<20 min), highly sensitive (visual limit of detection of 0.3 ng/mL TTX in buffer corresponding to 0.78 mg TTX/kg tissue), specific, reproducible and with long storage life. It was validated by analyzing contaminated pufferfish tissue extracts and it successfully detected TTX below the current limits set by official bodies. The analysis performed with this device in combination with a simple LFA reader for quantification was in excellent agreement with other established methods, further demonstrating the value of this test as a simple, low-cost and reliable analytical tool to ensure food safety, protect

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human health, and broaden the knowledge on the correlation between biological parameters and environmental data.

1. Introduction

Tetrodotoxin (TTX) is a highly potent alkaloid marine toxin that accumulates in pufferfish and other marine species. It blocks sodium voltage-gated channels, thus impairing nerve function, and can cause paralysis, respiratory failure, and even death (Zhao et al., 2018; Katikou et al., 2022; Reverté et al., 2023). Although TTX was assumed to occur only in tropical waters, in the last two decades it has emerged in temperate areas around the world, including the Mediterranean Sea. Since 2003, the toxic pufferfish species *Lagocephalus sceleratus* has been increasingly recorded in Mediterranean coasts, due to the Lessepsian migration through the Suez Canal, reaching not only eastern Mediterranean countries, such as Israel, Lebanon, Turkey, Cyprus and Greece, but also central Mediterranean countries (Bentur et al., 2008; Katikou et al., 2022). In 2007, the first human TTX-poisoning in Europe was reported, attributed to the consumption of a trumpet shell *Charonia lampas lampas* originating from the south of Portugal but purchased in Spain (Rodriguez et al., 2008). Since then, TTX has also been increasingly found in bivalve mollusks of European countries including the United Kingdom, Greece, Netherlands, Portugal, Spain, Italy and France (Katikou et al., 2022).

A regulatory limit of 2 mg TTX equivalents (equiv.)/kg pufferfish has been established in Japan where pufferfish is traditionally consumed. In Europe, products deriving from fish of the Tetraodontidae family (including different pufferfish species) cannot be sold, and the European Food Safety Authority (EFSA) has concluded that an amount lower than 44 μg TTX equiv./kg shellfish meat is not anticipated to pose a risk to human health (Katikou et al., 2022; Reverté et al., 2023). As TTX is thermostable, cooking does not destroy or eliminate it and intoxication cases following ingestion of contaminated food have been reported globally (Reverté et al., 2023). Considering that doses of 4–42 μg TTX per kg body weight can cause acute poisoning in humans and that no antidote is available (Knutsen et al., 2017), intoxications can be prevented mainly by limiting exposure to contaminated food. Therefore, sensitive, accurate and specific assays or tests that can be performed rapidly and in a user-friendly manner are required for the early detection of TTX.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is currently the most common and powerful technique for the detection and quantification of TTX and its analogues (Bane et al., 2014; Katikou and Vlamis, 2017; Turner et al., 2023). However, the complexity of operation, matrix interferences requiring extensive sample preparation, long analysis time, low throughput and high costs, limit the wide practical application of this technique. Antibody-based assays are alternative tools which can provide accurate and sensitive quantification of TTX in samples at reduced cost and analysis time compared to LC-MS/MS (Bane et al., 2014; Reverté et al., 2015; Reverté et al., 2017a; Reverté et al., 2017b; Reverté et al., 2018; Katikou and Vlamis, 2017; Knutsen et al., 2017; Campàs et al., 2020; Leonardo et al., 2019; Reverté et al., 2023; Turner et al., 2023). However, these multi-step assays are still dependent on equipment and trained professionals. Other methods used for TTX analysis include the mouse bioassay, cell-based assays (CBA), and receptor binding assays among others (Katikou and Vlamis, 2017; Alkassar et al., 2023; Alkassar et al., 2024). Whilst these assays are widely exploited, several hurdles including cost, analysis time and complexity limit their application for in field screening.

Lateral flow assays (LFA) on the other hand can provide rapid and sensitive detection exploiting simple, user-friendly, and low-cost devices (Quesada-González and Merkoçi, 2015; Huang et al., 2021; Bordbar et al., 2021; Mills et al., 2022). These devices are typically based on two biomolecule probes for target capture and detection: one of the probes is immobilized on the membrane, usually nitrocellulose, while the other one is conjugated with a reporter (e.g., gold nanoparticles) and deposited on the conjugate pad. The membrane is laminated with the sample, conjugate and absorbent pad on an adhesive backing card and cased within a plastic cassette. As the liquid sample containing the target analyte is applied to the device, it flows through the strip by capillary force. The target is recognized by the probe-reporter conjugate and the complex formed is detected by the probe at the test line where signal is generated (signal-on). A competitive format is adopted when only one probe is available and, in this case, the presence of target in the sample is demonstrated by a signal decrease at the test line (signal-off). This is the format employed most often for small molecules since their size hinders the binding of two probes simultaneously (Kaiser et al., 2018; Wang et al., 2018). With regards to TTX, only a handful of LFAs have been reported to date. All of them employ a competitive format based on antibody conjugate with gold nanoparticles (AuNPs) or gold nanoflowers (AuNFs) in combination with a TTX-protein conjugate at the test line. The presence of TTX in the sample is indicated by a signal decrease at the test line visible by naked eye (Zhou et al., 2010; Thattiyaphong et al., 2013; Ling et al., 2015; Li et al., 2020; Huang et al., 2023; Sun et al., 2023). There are two reports describing competitive fluorescent LFAs based on fluorescence quenching and signal generation in the presence of the target (Shen et al., 2017a; Shen et al., 2017b) with this signal-on format allowing easier interpretation of results. To date, there are only four commercially available rapid tests for TTX (Table S1), and these are all based on competitive formats. In summary, while the development of all these tests is a step forward towards achieving rapid and facile detection of TTX, competitive immunoassays are more difficult to optimize, and the preparation of toxin-conjugates and result interpretation can also be challenging. Sandwich assays are more robust, with all reagents in excess, and due to this have longer shelf-lives, and are often more sensitive and specific.

Aptamers are single-stranded, synthetic oligonucleotides whose target biorecognition properties have rendered them popular for the detection of virtually any type of target (Arshavsky-Graham et al., 2021; Liu et al., 2021). Their increased stability, easier chemical synthesis, reproducibility and lower cost compared to antibodies have encouraged their use in bioanalysis and particularly for small molecule detection (Wu et al., 2018; Yu et al., 2021; Jing et al., 2022; Vijitvarasan et al., 2022; Zhao et al., 2023; Chen et al., 2024).

We recently selected high affinity aptamers against TTX and demonstrated that the unique cage-like structure of TTX facilitated the formation of an antibody-TTX-aptamer complex enabling the detection of TTX with a sandwich assay (Shkembi et al., 2021). Here, we report the implementation of the developed sandwich assay in an LFA format for the detection of TTX in pufferfish. The test is based on an α-TTX IgG@AuNPs conjugate, with the TTX aptamer immobilized on the membrane (Fig. 1 and Supplementary Information Fig. S1). In the presence of TTX in the sample, a sandwich is formed at the test line between the antibody, TTX and the aptamer, and signal is generated. The test was optimized and characterized in terms of sensitivity, reproducibility, specificity, and stability. Finally, the optimized LFA was applied to the detection of TTX in extracts from contaminated pufferfish and compared to other techniques such as CBA and LC-MS/MS. To the best of our knowledge, this is the first example of a sandwich, signal-on rapid LFA test for TTX detection, as well as the first one to use an aptamer, and furthermore in a hybrid aptamer-antibody sandwich format.

2. Materials and methods

2.1. Material and apparatus

Tetrodotoxin (TTX) (98.8 % purity) was obtained from Latoxan (France), and 1 mg/mL TTX standard solutions were prepared in 0.1 M sodium acetate buffer pH 4.8. Saxitoxin (SXT) and domoic acid (DA) were obtained from the National Research Council of Canada (NRC, Halifax, Canada). Okadaic acid potassium salt (OA) from *Prorocentrum concavum*, brevetoxin (PbTx-2) from *Ptychodiscus brevis*, goat α-mouse IgG antibody, skimmed milk powder, Empigen BB and streptavidin-HRP (SA-HRP) were from Merck (Spain). Mouse monoclonal α-TTX IgG antibody (CABT-L3089, CD Creative Diagnostics) was purchased from Deltaclon S.L. (Spain). The Pacific ciguatoxin (CTX1B) standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, St Lucia, Australia). The D3 TTX-binding aptamer (Shkembi et al., 2021) with a 5′-biotin was synthesized by Biomers.net (Germany). Neutravidin (NeuA), phosphate buffered saline tablets (PBS; 10 mM phosphate buffer pH 7.4, 137 mM NaCl, 2.7 mM KCl), assay buffer (PBS with 1.5 mM $MgCl_2$), PBST (PBS with 0.05 % (*v/v*) Tween-20), carbonate-bicarbonate buffer (0.2 M, pH 9.4), bovine serum albumin (BSA) and sucrose were provided by Fisher Scientific (Spain). Gold nanoparticle colloid suspension (AuNPs; 40 nm diameter) at optical density (OD) 1 in water was from BBI Solutions (United Kingdom). Nitrocellulose (NC) membranes (grades FF80HP, FF120HP and FF170HP), Whatman CF4 sample pads and CF7 wicking pads were purchased from Cytiva (Spain). Fiberglass sample/conjugate pads (grades 8951 and 8964) were from Ahlström (Finland), backing cards from DCN (USA) and the cassettes from TV Plastics (India). All other reagents were provided by Fisher Scientific (Spain) and Scharlau (Spain). Ultra-pure double deionized water (18.2 MΩ.cm) was used for all experiments. The Varian Cary 100 Bio UV–Visible spectrophotometer, the JEOL JEM-1011 transmission electron microscope (TEM) operated at 100 K, the Malvern Zetasizer Nano ZS, the Molecular Devices SPECTRAmax 340PC-384 microplate spectrophotometer and the Branson ultrasonic water bath M2800-E were used for the preparation and characterization of the gold nanoparticle conjugate. The ClaremontBio Automated Lateral Flow Reagent Dispenser (ALFRD) set at 6 V coupled with the Chemyx Fusion Classic 200 automated syringe pumping system set at 0.25 mL/min were utilized for reagent dispensing on the nitrocellulose membranes and the AUTOKUN HGS220 guillotine cutter for cutting the strips. The ChemBio Diagnostics CubePlus lateral flow reader (Fig. S2) was used to measure the intensity of the lines of the LFA tests.

2.2. Preparation of the α-TTX IgG@AuNPs conjugate

The minimum concentration of the IgG antibody required for conjugation with the AuNPs to achieve colloidal stability was determined by titration using a gold aggregation test as described in the Supplementary Information. For the final preparation of the conjugate, the AuNPs were incubated with the chosen concentration of the α-TTX IgG for 30 min at 22 °C under tilt rotation, and BSA was then added to a final concentration of 1 % (*w/v*) for blocking. After another incubation for 30 min under the same conditions, the conjugate was washed three times by resuspending with conjugate buffer (5 mM sodium borate buffer pH 9, 1 % (*w/v*) BSA, 10 % (*w/v*) sucrose) and purification by centrifugation for 30 min at 14000 rpm and 10 °C. The final conjugate was resuspended with the same conjugate buffer and stored at 4 °C until further use. For characterization, UV–Vis spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential were used with particle suspensions prepared in water.

2.3. Construction of the LFA tests

A schematic illustration of the test strips is shown in Fig. 1a. They were prepared by assembling (in the given order) the NC membrane, the wicking (absorbent) pad, the conjugate pad containing the α-TTX IgG@AuNP conjugate and the sample pad on an adhesive backing card with a 3 mm overlap between each part. For the construction of the test (TL) and control (CL) lines on the NC membrane, NeuA (0.3 mg/mL in H_2O) and goat α-mouse IgG (1.2 mg/mL in H_2O) were automatically dispensed, with a 7 mm distance between them and the membrane was then dried for 2 h at 37 °C. The biotinylated D3 TTX aptamer (38 μM inH_2O) was dispensed on the previously dispensed NeuA at the TL and incubated for 1 h at 4 °C (Ali et al., 2020). The NC membranes were blocked with membrane blocking solution (10 mM carbonate-bicarbonate buffer pH 9.4, 5 % (*w/v*) skimmed milk powder, 0.5 % (*v/v*) Empigen BB) for 30 min under mild shaking at 22 °C, washed twice

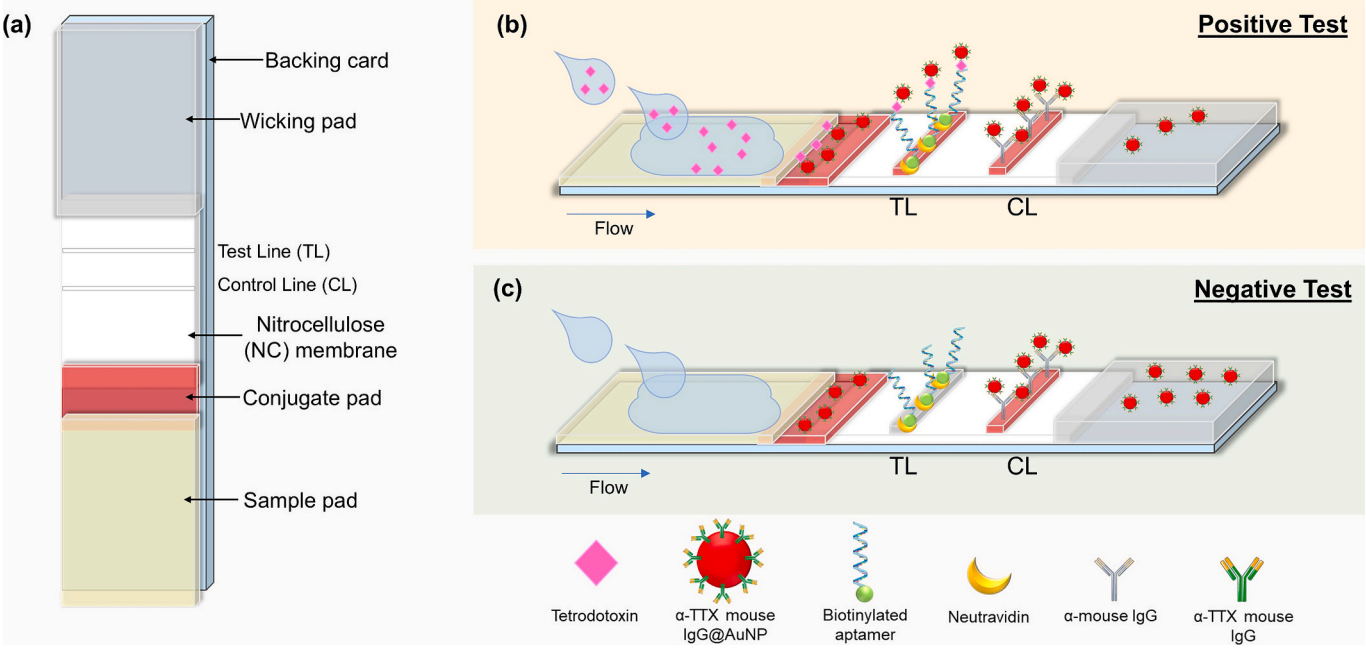


Fig. 1. Schematic illustration of the TTX LFA tests. (a) Design of the strips and representation of the detection process (b) in the presence and (c) absence of TTX.

with PBS for 10 min and finally dried for 2 h at 37 °C. For the conjugate pad preparation, this was first briefly soaked in pre-treatment solution (5 mM sodium borate buffer pH 8.8, 1 % (*w/v*) BSA, 0.05 % (*v/v*) Tween 20), followed by drying for 1 h at 37 °C. The α-TTX IgG@AuNPs conjugate suspension (OD 5–10 in conjugate buffer) was then manually dispensed onto the pre-treated conjugate pad with a micropipette ensuring uniform spreading and dried for 1 h at 37 °C. Following assembly of the material in the order described, the strips were cut at 4 mm, inserted into the plastic housing cassettes and stored in vacuum-sealed bags with desiccants at 4 °C.

2.4. Optimization of the LFA tests

Several parameters were optimized to enhance the performance of the LFA tests. For the immobilization of the aptamer on the NC membrane, two strategies were evaluated: direct dispensing of a pre-incubated mixture of NeuA (0.3 mg/mL) with the biotinylated aptamer (10–75 μM) or stepwise immobilization of the NeuA followed by the aptamer. For the latter approach, NeuA was first spotted (0.5 μL of 0.3 mg/mL in PBS) at the test line of the NC membrane, dried for 2 h at 37 °C, followed by spotting the aptamer (0.5 μL of 10–75 μM in PBS) on the previously dispensed NeuA and incubation for 2 h at 4 °C. After immobilization, the membranes were blocked and dried. The strips were dipped in 20 μL of solution containing the α-TTX IgG@AuNPs (OD 5) and TTX in assay buffer and let to run for approximately 30 min. Other parameters optimized were the type of NC membrane (FF80HP, FF120HP or FF170HP), the amount of the α-TTX IgG@AuNPs conjugate used per test (OD 5, 7 or 10) and the type of sample pad (Ahlström 8951 or 8964 fiberglass or Cytiva CF4 cellulose). Different concentrations of TTX were used when evaluating the different material (0–5 ng/mL).

2.5. Sensitivity and reproducibility

To evaluate the sensitivity and reproducibility of the test, a series of TTX standard solutions were analyzed with the tests on different days. TTX was prepared at 40 ng/mL in assay buffer and then serially diluted two-fold down to 0.04 ng/mL using the same buffer. Subsequently, 150 μL of each solution were added to the sample window of the tests and let to run for 20 min. Blank samples containing only assay buffer were analyzed in parallel. The cassettes were imaged with a smartphone, or with the Cube lateral flow reader to measure the intensity of the test lines. To construct the calibration curve, the intensity of the test lines was plotted against the logarithm of the concentration of TTX and the data were fitted into a four-parameter sigmoidal curve (4PL) using GraphPad Prism 8. The limit of detection (LOD) was interpolated from the curve as bottom$+3SE_{bottom}$, where bottom is the bottom of the fitted curve and SE its standard error. Duplicate measurements were acquired in three independent experiments on different days to evaluate the reproducibility of the tests.

2.6. Specificity of the LFA test

The specificity was studied by analyzing samples containing TTX or other marine toxins and recording the signals of the test lines. TTX was tested at 1 ng/mL, while saxitoxin (STX), domoic acid (DA), okadaic acid (OA) and brevetoxin (PbTx-2) were used at 20 ng/mL and Pacific ciguatoxin (CTX1B) at 20 pg/mL, individually or in combination with TTX. Each sample (150 μL in assay buffer) was applied to the sample window of the cassette and after 20 min, the cassettes were imaged and the test line intensities were recorded using the Cube LFA reader. Each sample was analyzed in duplicate with two independent experiments. The test line intensities were normalized to the signals obtained for TTX alone.

2.7. Stability of the LFA test

The LFA tests were prepared as described earlier, packaged in vacuum-sealed plastic bags with desiccants and stored at either 4 °C or 37 °C for up to 6 weeks to perform an Arrhenius accelerated thermal stability study. A TTX standard (1 ng/mL in assay buffer) and a blank control (only assay buffer) were analyzed with the LFA tests stored at the two temperatures at different time intervals in duplicate and imaged with a smartphone. The test line intensities were estimated and used to predict the long-term storage of the tests under refrigerated conditions (Laboria et al., 2011).

2.8. Pufferfish samples

Extracts of three *L. sceleratus* pufferfish captured on Crete coasts of the Libyan Sea in 2019 (PF1, PF2, PF3) were obtained from a previous study (Alkassar et al., 2023). For the preparation of the extracts, the fish were dissected, and the different tissues (gonads, intestine, liver, muscle, and skin) were retrieved. TTX was extracted from each of these tissues using 0.1 % (*v/v*) acetic acid. Extracts from a *Lagocephalus lagocephalus* TTX-free pufferfish were used as controls. All the extracts (200 mg tissue/mL) were stored at −20 °C until use.

2.9. Analysis of the pufferfish extracts with the LFA tests

The pufferfish tissue extracts were analyzed in triplicate with an Enzyme Linked Aptamer Assay (ELAA) using microtiter plates (SI). For LFA, different sample dilution factors, based on the ELAA results, were considered to ensure correct quantification regardless of the TTX content in each tissue. Specifically, 1 μL of each extract was serially diluted with assay buffer to achieve final dilution factors of 500 to 5000. Subsequently, 150 μL of diluted sample was applied to the sample window of the cassette and let to run for 20 min. The intensity of the test lines was measured with the Cube LFA reader. The concentration of TTX in each sample was calculated after interpolation of the intensity of the test line into a TTX calibration curve constructed in parallel. All samples and standards were analyzed in duplicate.

3. Results and discussion

3.1. Design of the LFA test

In our previous work we reported the selection of novel TTX aptamers and combined them with an α-TTX IgG antibody to develop a sandwich assay to detect TTX in contaminated pufferfish extracts using microtiter plates (Shkembi et al., 2021). Herein, we demonstrated the implementation of this system into an LFA test with a classical sandwich-type design (Fig. 1a and Fig. S1). The aptamer with a 5′-biotin modification was immobilized on the NC membrane through affinity interactions with NeuA to construct the test line, while the α-TTX mouse IgG antibody was adsorbed on gold nanoparticles and dried on a conjugate pad. For the control line, an α-mouse IgG antibody was dispensed. For analysis, the sample is diluted with assay buffer and added to the sample window of the device. As it flows along the strip by capillary forces, it rehydrates the conjugate pad containing the α-TTX IgG@AuNPs conjugate, which in turn binds TTX present in the sample. The IgG/TTX complex migrates towards the test line where it is captured by the aptamer. Excess gold conjugate is captured at the control line by the α-mouse IgG, thus confirming the correct performance of the test, while remaining liquid flows towards the wicking (absorbent) pad. The presence of TTX in the sample is indicated by the development of red colour at the test and control lines (Fig. 1b), while only the control line is visible when there is no TTX in the sample (Fig. 1c and Fig. S1).

3.2. Preparation and characterization of the α-TTX IgG@AuNPs conjugate

The α-TTX IgG antibody was conjugated to 40 nm AuNPs by passive adsorption. Prior to conjugate preparation, the amount of antibody and pH conditions required for assuring conjugate stability were determined through a gold aggregation test, and the minimum antibody concentration providing colloidal stability was 6 μg/mL at pH 8 (Fig. S3). For more efficient bioreceptor spacing and considering that the particles would be blocked with BSA (Parolo et al., 2020), the final concentration of the antibody used was 4 μg/mL. The conjugate was characterized by UV–Vis spectroscopy and a characteristic peak at 526 nm was observed for the bare AuNPs (40 nm), while a red-shift to 533 nm was observed after conjugation with the α-TTX IgG, verifying the presence of protein on the surface of the particles, while the peak maintained its width, demonstrating the narrow size distribution of the conjugate (Fig. S4a). Moreover, TEM images revealed spherical and homogenous particles after conjugation (Fig. S4b) with an increased average diameter of 39.1 ± 3.2 nm as compared to the bare AuNPs (37.5 ± 4.0 nm). DLS analysis showed a diameter of 110.6 ± 0.5 nm for the α-TTX IgG@AuNP conjugate and 42.2 ± 0.1 nm for the bare AuNPs (Fig. S4c). The discrepancy in the measurements obtained with the two methods, TEM and DLS, is probably due to the difference in the technique: TEM measures the physical diameter of the particles as compared to the hydrodynamic diameter provided by DLS, which are typically larger (Bhattacharjee, 2016). Finally, the zeta potential was −46.7 ± 1.1 mV for the bare AuNPs and −26.3 ± 2.3 mV for the α-TTX IgG@AuNPs conjugate, demonstrating the stability of the particles before and after conjugation with the antibody (Fig. S4d). On the other hand, the increased zeta potential of the conjugate, which is a measurement of surface charge, attests to the successful immobilization of protein on the surface of the particles which leads to decreased repulsive charges between the particles, also observed in the TEM images. Overall, these studies confirmed the preparation of a stable gold conjugate.

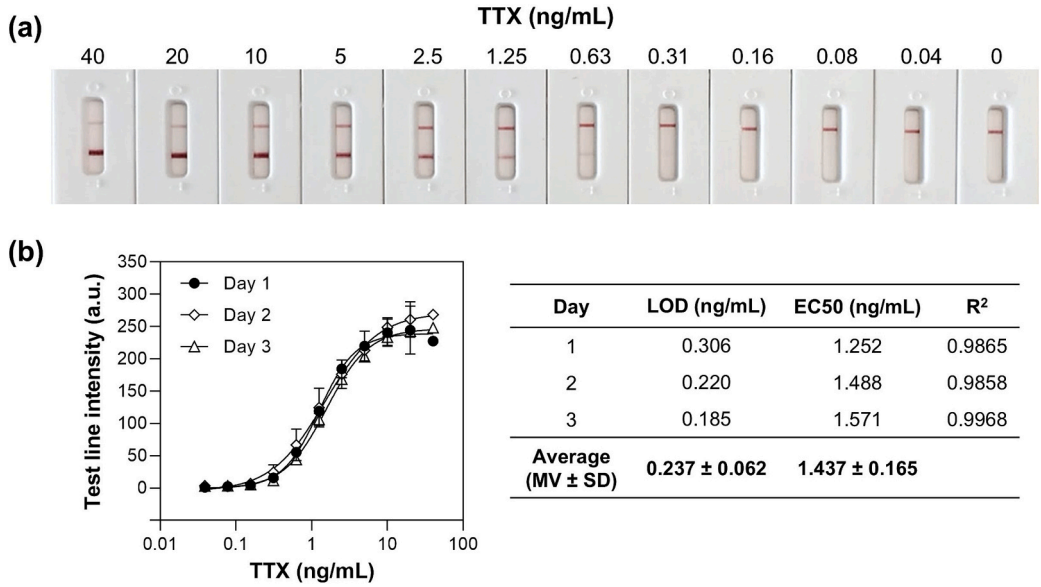
3.3. Optimization of the LFA tests and sample analysis conditions

With the aim of developing an accurate and robust TTX LFA test, several experimental parameters were optimized. Initially, the immobilization of the aptamer on the NC membrane was studied. Its concentration should be optimized, since excess could lead to non-specific signals or generate steric hindrance, whilst low concentrations could result in rapid saturation of the signal at the test line and consequent decreased sensitivity of the test (Parolo et al., 2020). Therefore, a series of concentrations of the aptamer with a 5′-biotin label were tested in combination with NeuA, to facilitate immobilization through affinity interactions. Two different strategies were evaluated: direct absorption of a NeuA/biotinylated aptamer pre-incubated mixture (Wu et al., 2018) and a two-step approach of first dispensing NeuA on the NC membrane followed by the aptamer as a second layer on top (Ali et al., 2020). The FF120HP membrane was used for this study and the test was performed in dipstick format. The highest specific signals in the presence of TTX were observed when 25 μM of the aptamer were used to immobilize employing the two-step strategy (Fig. S5) and this concentration was used in all following experiments. The type of NC membrane was evaluated next, and the membranes FF80HP, FF120HP and FF170HP with large, medium and small pore size, respectively, were used to prepare the detection strips. Faint and diffused test line bands were observed on the strips prepared with the FF80HP membrane (largest pore size and fastest capillary flow rate), while clear and sharp lines were obtained with the other two membranes (Fig. S6a). The FF170HP membrane was finally chosen because it was hypothesized that the slower capillary flow rate increases the contact time between the antibody-TTX complex and the aptamer at the test line, increasing the sensitivity of the test. Moreover, an OD of 5 for α-TTX IgG@AuNPs conjugate was considered optimum since higher amounts of the conjugate did not improve the sensitivity of the test, and no signal was observed for the blank control (Fig. S6b). Finally, three types of sample pads were compared: two were made of fiberglass (Ahlström grades 8951 and 8964) and one of cellulose (CF4). Both materials are widely exploited in LFAs with the cellulose sample pads having more filtration capacity. The intensity of the test lines in the presence of TTX was similar for all three sample pads, with slightly less intensity observed with the cellulose pad (Fig. S6c). Considering the general characteristics of wicking rate and water absorption, the Ahlström 8964 fiberglass pad was chosen.

3.4. Analytical performance of the LFA test

Test strips were prepared using the optimum conditions and materials described above and were used to evaluate the analytical performance of the assay, by analyzing TTX standard solutions in the range of 0.04–40 ng/mL using serial 1/2 dilutions. A gradual increase in the intensity of the test line is observed for TTX concentrations of ≥0.3 ng/mL and up to 10 ng/mL at which point the signal saturates (Fig. 2a). Based on these results, the visual LOD was defined as approximately 0.3 ng/mL TTX. The sensitivity of the test was evaluated on three different days and after quantifying the intensity of the test lines with the Cube LFA Reader (Fig. S2), sigmoidal calibration curves were constructed with very high reproducibility (Fig. 2b). The calculated LODs were in the range of 0.185–0.306 ng/mL, with an average of 0.237 ± 0.062 ng/mL TTX and an average half-maximal effective concentration (EC50) value of 1.437 ± 0.165 ng/mL TTX. The test achieved a visual LOD of 0.31 ng/mL and a dynamic range of 0.31–10 ng/mL TTX, spanning approximately 1.5 orders of magnitude. The microplate format on which the LFA test was based on exhibited very similar analytical performance (LOD of 0.310 ng/mL and EC50 of 1.1 ng/mL) (Shkembi et al., 2021). Based on the analysis of different TTX standard solutions on different days, both the intra-assay and inter-assay coefficients of variation (CV) were <13 % (Table S2). These results demonstrate that the developed test was not only very sensitive, but it also exhibited high precision and reproducibility. The microplate assay is more precise and accurate than the LFA test based on the lower intra-assay and inter-assay CVs (0.1–12.8 % and 2.8–4.3 %, respectively) (Shkembi et al., 2021) but it does not provide the advantages of speed, simplicity, portability and affordability.

There are very few works in the literature detailing the development of LFAs for TTX (Table S3), and the majority of the tests employ a TTX-BSA conjugate at the test line to compete with TTX in the sample for binding to an α-TTX IgG immobilized on AuNPs (Zhou et al., 2010; Ling et al., 2015; Huang et al., 2023), AuNFs (Thattiyaphong et al., 2013; Li et al., 2020) or coloured latex microspheres (Huang et al., 2023). There is one report in which the same strategy was used but, in this case, TTX was detected simultaneously with OA by combining two gold nanoparticle immunoconjugates and constructing two test lines, one for each toxin (Ling et al., 2019). The visual LODs of all the above-mentioned tests were in the range of 1–40 ng/mL TTX. Higher sensitivity was demonstrated in two other reports where fluorescence detection was employed instead of colorimetric. A competitive signal-off test was demonstrated by Sun et al. who used TTX-BSA at the test line and a TTX IgG antibody conjugated to fluorescent microspheres for detection, reporting a sensitivity of 0.05 ng/mL (Sun et al., 2023). A competitive signal-on LFA was designed using AuNFs (Shen et al., 2017b) conjugated with IgG antibody as quenchers for the fluorescently labelled TTX-BSA conjugates at the test lines, which was observed in the absence of TTX, with increasing fluorescence measured in the presence of TTX, achieving an LOD of 0.2 ng/mL. Although these tests exhibit improved sensitivity compared to the ones based on colorimetric detection, fluorescence systems are generally expensive as they require expensive light sources to illuminate the fluorescent reporters, as well as needing interference filters and detection systems to process and capture the emitted light, and finally the data processing required to produce the result (Lee et al., 2013). The sandwich-type colorimetric test developed



Day	LOD (ng/mL)	EC50 (ng/mL)	R^2
1	0.306	1.252	0.9865
2	0.220	1.488	0.9858
3	0.185	1.571	0.9968
Average (MV ± SD)	**0.237 ± 0.062**	**1.437 ± 0.165**	

Fig. 2. Sensitivity and reproducibility of the LFA tests. (a) Representative images of the tests where TTX standard solutions of 0.04–40 ng/mL were analyzed. (b) Calibration curves constructed after measuring the test line intensities with the LFA reader. Three independent experiments were performed on different days with duplicate measurements for each TTX concentration.

in this work employs lower-cost material (ssDNA aptamer, antibody and AuNPs), provides fast analysis (<20 min) and is more sensitive than all the other colorimetric tests reported previously (LOD of 0.31 ng/mL TTX).

3.5. Specificity of the LFA test

The cross-reactivity of the developed LFA with other marine toxins was evaluated using saxitoxin (STX), okadaic acid (OA), domoic acid (DA), brevetoxin-2 (PbTx-2) and Pacific ciguatoxin 1B (CTX1B). In this study STX is the most relevant as this toxin can be found with TTX in pufferfish as well as in shellfish, and as it is hydrophilic, is often co-extracted with TTX. STX is also a paralytic neurotoxin and intoxication following ingestion could produce similar effects to TTX. DA, another hydrophilic neurotoxin, is not found in pufferfish but it does accumulate in shellfish like TTX, STX and OA. OA is a diarrhetic toxin commonly found in shellfish. Since it is lipophilic, it is unlikely to be extracted using the method of extraction for TTX and is thus not expected to be as potentially problematic in terms of false positives, as STX. Brevetoxins and ciguatoxins are lipophilic marine neurotoxins produced by dinoflagellates. Brevetoxins contaminate shellfish whereas CTXs accumulate in herbivorous and subsequently carnivorous fish, and both cause neurological and gastrointestinal symptoms after ingestion. Due to the high specificity of the hybrid aptamer-antibody sandwich system, no cross-reactivity or non-specific binding with the other marine toxins studied was observed when they were analyzed individually or in mixtures with TTX, and the detection of TTX was not hampered by the presence of other toxins present in the sample (Fig. 3). These results demonstrate that the test is specific for TTX, it exhibits no cross-reactivity with the other marine toxins studied and their concurrence with TTX in the same samples is not expected to interfere with the accurate detection of TTX.

3.6. Stability of the LFA test

The LFA tests were stored at 4 °C and 37 °C for 6 weeks and their

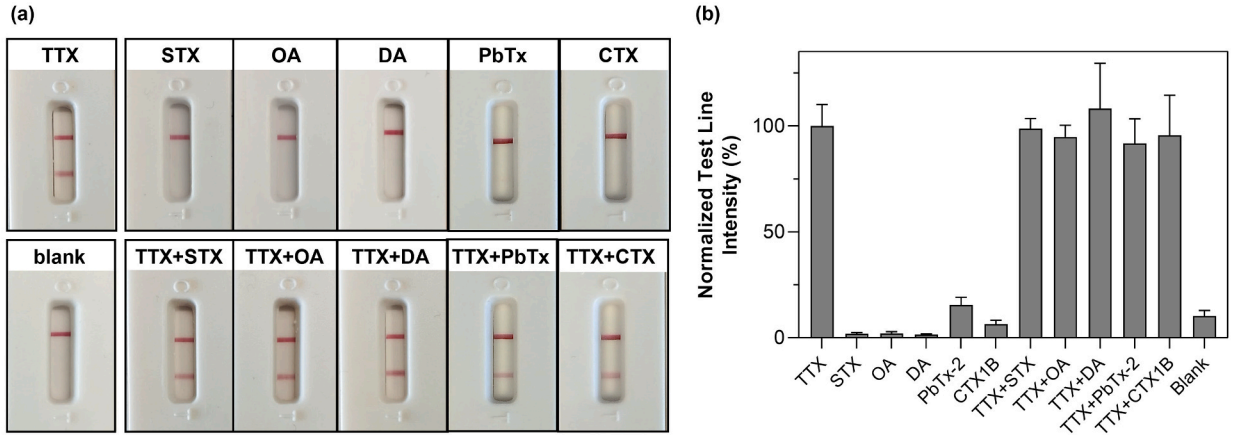


Fig. 3. Specificity of the LFA test. TTX or other marine toxins were analyzed with the tests, individually or in mixtures. (a) Representative images of the LFA tests. (b) Quantification of the tests with the Cube LFA reader. The test line intensities were normalized to the signal obtained for TTX alone. STX: saxitoxin; OA: okadaic acid; DA: domoic acid; PbTx-2: brevetoxin-2; CTX1B: Pacific ciguatoxin-1B. Duplicate measurements were performed and the error bars correspond to their standard deviation.

stability was evaluated by analyzing a TTX standard solution at different storage time intervals. No remarkable decrease in the intensity of the test lines was observed for the devices stored at 4 °C and 37 °C for 6 weeks and no false-positive signals were obtained after 6 weeks of storage at either temperature (Fig. 4). Based on the Arrhenius accelerated thermal stability study performed as described previously (Laboria et al., 2011; Bever et al., 2020), the tests can be stored for approximately 4.5 years at 4 °C. This long shelf life of the tests demonstrates their reliability and suitability for in field implementation.

3.7. Pufferfish sample analysis

The practical applicability of the LFA test for monitoring the TTX content in fish was demonstrated by analyzing pufferfish samples with known TTX content. Extracts from different tissues (muscle, skin, liver, intestinal tract, and gonads) of three *L. sceleratus* pufferfish specimens caught on the Crete coasts between March and May 2019 were prepared and analyzed by LC-MC/MS and CBA in a previous report (Alkassar et al., 2023). Based on the known TTX content in each extract, different dilutions were performed for analysis with the LFA. The test successfully detected TTX in all the tissue extracts from all three contaminated pufferfish, with test lines of different intensities depending on the dilution performed and the TTX content of the sample (Fig. 5). These results demonstrate the suitability of the tests to detect TTX in real samples with no interference from other fish matrix compounds since no signals were obtained when analyzing the tissue samples from the TTX-free pufferfish.

To quantify the TTX content in each extract, TTX standard solutions were analyzed in parallel and the test lines of all the tests were quantified with the Cube LFA reader. Based on the calibration curves constructed with the TTX standards, the TTX content in each extract were determined. The extracts were also analyzed with the microplate ELAA, LC-MS/MS and CBA analysis (Table 1). When comparing the different techniques used in this work, a good correlation of the results obtained between the LFA and ELAA was observed, which can be expected as they both use the same biorecognition elements, only changing the format and detection technique. This demonstrates the analytical benefits of the LFA assay which can be performed with a single step as opposed to the multi-step ELAA requiring consecutive binding/washing steps and a spectrophotometer for quantification. According to the results obtained with our LFA, all extracts contained TTX at levels higher than that considered safe for human consumption according to Japanese legislation (2 mg TTX equiv./kg), sometimes even reaching >60-fold higher contents. These values have also been found in other works (Christidis et al., 2021; Alkassar et al., 2023; Anastasiou et al., 2023), and highlights the probability of tetrodotoxication in the case of consumption of these fish specimens.

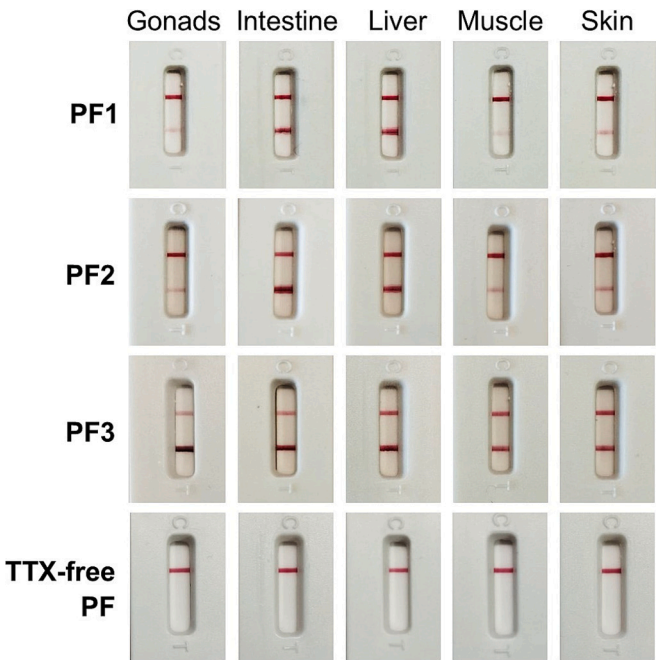


Fig. 5. Representative photos from the analysis of contaminated *L. sceleratus* pufferfish tissue extracts and of the TTX-free *L. lagocephalus* pufferfish with the LFA tests. Samples were diluted 1/500 (TTX-free PF), 1/1000 (all extracts from PF1 and liver, muscle, skin from PF3), 1/3000 (PF2) and 1/5000 (gonads and intestine from PF3). Duplicate measurements were performed.

There are >30 known analogues of TTX (Biessy et al., 2019; Reverté et al., 2023) and in the samples analyzed in this work, 13 different analogues were identified including TTX according to previous LC-MS/MS characterization (Alkassar et al., 2023). Table 1 shows the LC-MS/MS results for only TTX contents (which also includes 4-*epi*TTX, which is in chemical equilibrium with TTX) as well as for all TTXs after the application of the toxicity equivalency factors (TEFs) obtained by CBA to the TTX analogues, considering TEF = 0 for TTX analogues with unknown TEF. The actual TTX content varies significantly in the samples, from as low as 12 % *w/w* (gonads from PF3) to as high as 71 % *w/w* (gonads from PF1) within the total TTXs content. The differences observed in the quantification of TTX in the samples with the LFA as compared to LC-MS/MS are certainly due to the different detection principles. The gonads of PF3 contained unusually high levels of 5,6,11-trideoxyTTX and 4-*epi*-5,6,11-trideoxyTTX concentrations and therefore, this datapoint was considered as an outlier and it was excluded from all statistical analyses. The correlation between LFA and only TTX

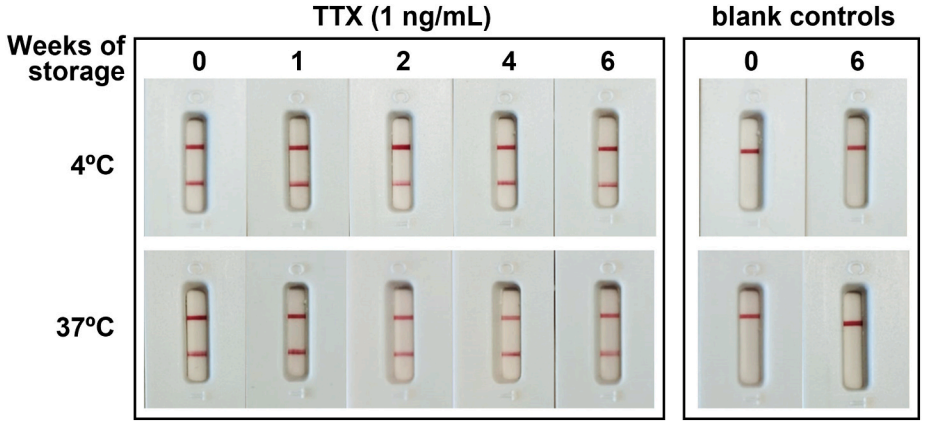


Fig. 4. Stability of the LFA tests. The tests were stored at 4 °C or 37 °C for up to 6 weeks and were tested at different storage time intervals with TTX (1 ng/mL) or assay buffer alone (blank controls). Duplicate measurements were performed.

Table 1
Detection of TTXs in pufferfish tissue extracts with the LFA test and comparison with other techniques. The ELAA on microtiter plates was performed using the same α-TTX IgG/D3 aptamer sandwich system also used in the LFA test. Quantification by LC-MS/MS and CBA were performed in a previous study (Alkassar et al., 2023). TTX equiv. values correspond to MV ± SD, $n = 3$.

Pufferfish	Tissue	LFA TTX equiv. (mg/kg)	ELAA TTX equiv. (mg/kg)	LC-MS/MS		CBA TTX equiv. (mg/kg)
				TTX (mg/kg)	TTX equiv. (mg/kg)	
1	Gonads	3.8 ± 0.3	2.8 ± 0.1	1.5	2.1	3.7
	Intestine	11.3 ± 0.4	6.7 ± 0.2	19.2	34.6	19.6
	Liver	12.5 ± 0.7	5.0 ± 0.2	20.6	38.9	21.5
	Muscle	2.5 ± 0.0	1.5 ± 0.1	4.6	7.6	5.6
	Skin	4.3 ± 0.1	1.8 ± 0.2	5.3	14.3	8.0
2	Gonads	9.8 ± 1.7	5.6 ± 0.5	5.4	10.5	6.4
	Intestine	109.6 ± 32.8	25.0 ± 4.3	69.9	205.8	92.4
	Liver	60.1 ± 0.4	19.6 ± 2.7	53.9	188.2	51.4
	Muscle	10.9 ± 0.1	7.0 ± 1.4	15.3	36.5	14.1
	Skin	12.9 ± 0.8	6.2 ± 0.8	13.5	63.2	16.1
3	Gonads	129.1 ± 40.1	91.3 ± 6.5	171.7	1324.4	228.9
	Intestine	95.1 ± 9.6	59.0 ± 2.9	80.4	210.9	113.1
	Liver	21.6 ± 0.6	6.4 ± 0.3	17.0	57.2	26.8
	Muscle	11.6 ± 1.0	3.5 ± 0.6	6.4	11.6	8.5
	Skin	10.4 ± 0.3	2.9 ± 0.2	9.2	34.7	14.7

by LC-MS/MS indicates that LFA would be overestimating the TTXs contents (Fig. S7a). Nevertheless, when all TTX analogues with their TEFs were included in the correlation, the agreement between LFA and LC-MS/MS was excellent (Fig. S7b). This result indicates that not only the LFA is able to properly detect TTX, but it also recognises TTX analogues, probably proportionally to their toxicity. In fact, the correlation between LFA and CBA, which measures toxicity on Neuro-2a cells, was also excellent (Fig. S7c). These results demonstrate that the LFA is a very appropriate tool to guarantee food safety from a toxicological point of view. Future research is focused on the establishment of cross-reactivity factors (CRFs) of TTX analogues with the LFA to understand the interactions with the two biorecognition molecules.

Finally, with regards to the sensitivity of the LFA test for detection of TTX in pufferfish, this can be estimated as follows: the gonads extract from PF1 contains 2.1 mg TTX equiv./kg according to LC-MS/MS, with >70 % being TTX. Considering the 1000-fold dilution performed prior to the analysis of this sample with the LFA, this sample contains 0.42 ng TTX equiv./mL and can be detected with the LFA since a discernible red band could be observed (Fig. 5). This is consistent with the visual LOD of the test in assay buffer (0.31 ng/mL TTX). Taking into account the LOD in buffer and a 500-fold dilution of samples, which has been observed not to cause any matrix effects, an LOD of 0.78 mg TTX/kg tissue for the test in sample extracts can be calculated, which is far below the Japanese regulatory limit of 2 mg TTX/kg. Moreover, currently there are only four available commercial tests for TTX and they all based on a competitive immunoassay (Table S1) and they all exhibit lower sensitivities than the LFA described here.

The rapid test developed in this work is based on an aptamer-antibody sandwich and to the best of our knowledge, is the first signal-on colorimetric test for TTX, which can be assessed by simple visual inspection. The test can be performed in a single step in <20 min and the presence of TTX in the sample is easily confirmed by the appearance of a red line. The test is extremely sensitive and specific, reproducible and with long storage stability. Its compatibility with pufferfish analysis for evaluation of potentially toxic samples was confirmed by the excellent correlation between the results from the LFA and ELAA, LC-MS/MS and CBA.

3.8. Ecotoxicological and human health implications

Invasive alien species such as toxic pufferfish are a global threat to biodiversity, ecosystem services (e.g., tourism, fisheries, fisherpersons' livelihoods) and human health. Invasive pufferfish are known to reduce native fish and some invertebrate stocks, depredate and destroy both catches and gear, and cause events impacting human health. In the Eastern Mediterranean, 28 records of physical attacks, at least 144 non-lethal poisoning episodes, and 27 human fatalities resulting from consumption have been reported between 2004 and 2023 (Ulman et al., 2024) (Fig. 6a). These ecological, economic, and public health consequences demonstrate the necessity for research not only in the Mediterranean hot spot, but also in other regions where these toxic species have always been present or are emerging. In Japan, intoxication cases are very common due to the traditional consumption of pufferfish. Although the Ministry of Health, Labour and Welfare of this country is continuously updating the list of cases (https://www.mhlw.go.jp/topics/syokuchu/poison/dl/animal_det_01-02.pdf; accessed on 11/11/2024), these are not always reported in the scientific literature in English and the incidence is probably higher. In a study from 2019, which reviewed global cases of TTX intoxication in humans but excluded Japan to avoid bias, 3042 cases were identified, 341 of which were fatalities (Guardone et al., 2019). The vast majority of the cases were described in Asia (even excluding Japan), followed by Africa, America, Oceania and Europe (Fig. 6b). Therefore, tetrodotoxication is a global threat that we will all face in the next years.

Several fields are still lacking investigation. For example, pufferfish consume fish as the main diet component, followed by crustaceans and mollusks (mostly gastropods and cephalopods), which are known to contain TTX or its precursors (Gomes et al., 2025). Nevertheless, although some works demonstrate that the diet of *L. sceleratus* may be responsible, at least in part, for the TTX contents, the symbiosis with TTX-producing bacteria belonging to the intestinal microflora cannot be ruled out (Christidis et al., 2021). Certain bacteria, such as those from the *Vibrio* and *Pseudomonas* genera, can produce TTX. The exact contribution of these bacteria and others to the TTX levels in pufferfish remains an area of active investigation. Analyzing the gastrointestinal microbiome of pufferfish will be crucial in understanding the relationship between bacterial communities and variations in toxicity levels among fish from different geographical regions. Additionally, environmental conditions such as salinity, temperature and water depth have been suggested as possible factors that affect pufferfish diet habits (Gomes et al., 2025) as well as bacterial production of TTX and presence of TTX in host organisms (Anastasiou et al., 2023). Specifically, changes in those parameters can affect bacterial cell growth and metabolism, enzyme activity and gene expression related to TTX biosynthesis. Additionally, fluctuations in environmental parameter affect toxin accumulation, metabolism, and detoxification kinetics in animals. In any case, prediction of TTX contents according to the morphological/biological characteristics of pufferfish or to environmental-related data is not straightforward (Anastasiou et al., 2023). Understanding the role of different environmental parameters in TTX production requires

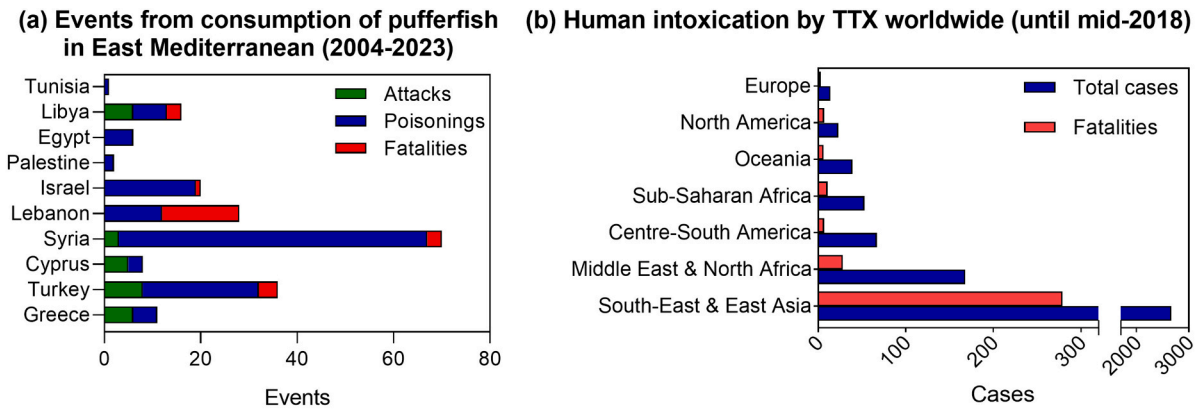


Fig. 6. Intoxications caused by consumption of TTX-contaminated (a) pufferfish in East Mediterranean and (b) seafood worldwide. Data was adapted from (a) Ulman et al., 2024 and (b) Guardone et al., 2019.

further investigation, particularly regarding how microbial communities contribute to toxin synthesis and transfer through the food web. Therefore, to better understand the trends and provide accurate predictions, it would be beneficial to have more data on TTX contents in food and environmental samples, and analytical tools such as the one proposed in this work are thus crucial.

The emerging presence of TTX in shellfish worldwide is also of concern. The operational performance of the LFA developed and validated for pufferfish in this work demonstrates the great value of this tool for the rapid screening of these types of samples, and work is ongoing to implement signal amplification strategies to attain even lower limits of detection, whilst also eliminating matrix effects, thus allowing the test to be used for the analysis of shellfish. The use of this tool for the analysis of seafood samples, together with environmental data, would contribute not only to guaranteeing food safety, but also to unlocking the unknowns regarding the origin, presence and distribution of TTX in marine organisms.

Finally, effective management strategies with the objective of reducing human health threats as well as the negative environmental impact on coastal areas are necessary and urgent. In this regard, data collection on the occurrence and exposure is needed to better understand the geographical distribution of toxic organisms, conduct a proper risk assessment and management, as well as predict potential areas of risk. Collaborative research across ecological, toxicological, analytical and socioeconomic sectors is imperative to protect food safety, human health and well-being.

4. Conclusions

The expanse of toxic fish species in temperate waters around the world resulting in intoxication after contaminated fish consumption highlights the urgent need for rapid, sensitive and accurate tests to monitor toxin content and prevent intoxications. This work describes the development of the first LFA device for TTX detection in pufferfish based on a sandwich antibody-aptamer assay. The format of this test enables facile result interpretation since the presence of TTX in the sample is indicated by the generation of a coloured line signal, as opposed to the signal-off detection provided by competitive assays typically developed for small molecule target analyte. Most relevant parameters affecting the performance of the test were optimized and the final device allowed for the fast detection of TTX within 20 min with a visual limit of detection of 0.3 ng/mL TTX in buffer, way below legal safety limits, and 0.2 ng/mL when combined with a portable LFA reader. The sensitivity of the test is higher compared to commercial kits and similar works described in literature, avoiding the use of complex and costly methodologies. No cross-reactivity or interference from other marine toxins was observed, whereas an Arrhenius accelerated thermal stability study suggested a long storage stability of the test devices of more than four years under refrigeration. Naturally contaminated pufferfish samples were finally analyzed with the LFA tests and excellent agreement with other analytical techniques was demonstrated. The tool still suffers from some limitations depending on the applicability purpose. For instance, if shellfish analysis is conducted, it will be essential to achieve lower detection limits in order to meet the EFSA's recommendation of 44 μg TTX equiv./kg of shellfish meat. This will involve enhancing the inherent performance of the assay through proper optimization of the experimental parameters and/or adapting the system to function under high shellfish matrix loadings. In the latter case, clean-up and/or preconcentration strategies will certainly be required, and work is in progress to achieve this. Another potential limitation is the cross-reactivity of the aptamer and the antibody with the TTX analogues, which is currently being evaluated.

Abbreviations

TTX	Tetrodotoxin
AuNPs	gold nanoparticles
α-TTX IgG@AuNPs	mouse anti-tetrodotoxin IgG antibody conjugated with gold nanoparticles
NeuA	neutravidin
LFA	lateral flow assay
ELISA	enzyme linked immunosorbent assay
ELAA	enzyme linked aptamer assay
STX	saxitoxin
OA	okadaic acid
DA	domoic acid
TEM	transmission electron microscopy
DLS	dynamic light scattering
LC-MS/MS	liquid chromatography with tandem mass spectrometry
CBA	cell-based assay
TEF	toxicity equivalency factor

CRediT authorship contribution statement

Ulises G. Díaz-Avello: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Vasso Skouridou:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Xhensila Shkembi:** Investigation. **Jaume Reverté:** Investigation. **Manolis Mandalakis:** Writing – review & editing, Resources. **Panagiota Peristeraki:** Writing – review & editing, Resources. **Mònica Campàs:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis. **Ciara K. O'Sullivan:** Writing – review & editing, Writing – original draft, Supervision, Funding

acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Design and optimization of the LFA tests; performance of the LFA tests and comparison of with other methods. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179419.

Data availability

Data will be made available on request.

References

Ali, M., Sajid, M., Khalid, M.A.U., Kim, S.W., Lim, J.H., Huh, D., Choi, K.H., 2020. A fluorescent lateral flow biosensor for the quantitative detection of vaspin using upconverting nanoparticles. Spectrochim. Acta A Mol. Biomol. Spectrosc. 226, 117610. https://doi.org/10.1016/j.saa.2019.117610.

Alkassar, M., Sanchez-Henao, A., Reverté, J., Barreiro, L., Rambla-Alegre, M., Leonardo, S., Mandalakis, M., Peristeraki, P., Diogène, J., Campàs, M., 2023. Evaluation of toxicity equivalency factors of tetrodotoxin analogues with a neuro-2a cell-based assay and application to puffer fish from Greece. Mar. Drugs 21, 432. https://doi.org/10.3390/md21080432.

Alkassar, M., Reverté, J., Fragoso, A., Torréns, M., Klijnstra, M., Gerssen, A., Diogène, J., Campàs, M., 2024. B-Cyclodextrin polymer as tetrodotoxins scavenger in oyster extracts. Microchem. J. 201, 110585. https://doi.org/10.1016/j.microc.2024.110585.

Anastasiou, T.I., Kagiampaki, E., Kondylatos, G., Tselepides, A., Peristeraki, P., Mandalakis, M., 2023. Assessing the toxicity of Lagocephalus sceleratus pufferfish from the southeastern Aegean Sea and the relationship of tetrodotoxin with gonadal hormones. Mar. Drugs 21, 520. https://doi.org/10.3390/md21100520.

Arshavsky-Graham, S., Heuer, C., Jiang, X., Segal, E., 2021. Aptasensors versus immunosensors – which will prevail? Eng. Life Sci. 22, 319–333. https://doi.org/10.1002/elsc.202100148.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., Furey, A., 2014. Tetrodotoxin: chemistry, toxicity, source, distribution and detection. Toxins 6, 693–755. https://doi.org/10.3390/toxins6020693.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to Lagocephalus sceleratus in the eastern Mediterranean. Toxicon 52, 964–968. https://doi.org/10.1016/j.toxicon.2008.10.001.

Bever, C.S., Adams, C.A., Hnasko, R.M., Cheng, L.W., Stanker, L.H., 2020. Lateral flow immunoassay (LFIA) for the detection of lethal amatoxins from mushrooms. PLoS ONE 15, e0231781. https://doi.org/10.1371/journal.pone.0231781.

Bhattacharjee, S., 2016. DLS and zeta potential - what they are and what they are not? J. Control. Release 235, 337–351. https://doi.org/10.1016/j.jconrel.2016.06.017.

Biessy, L., Boundy, M.J., Smith, K.F., Hardwood, D.T., Hawes, I., Wood, S.A., 2019. Tetrodotoxins in marine bivalves and edible gastropods: a mini-review. Chemosphere 236, 124404. https://doi.org/10.1016/j.chemosphere.2019.124404.

Bordbar, M.M., Sheini, A., Hashemi, P., Hajian, A., Bagheri, H., 2021. Disposable paper-based biosensors for the point-of-care detection of hazardous contaminations – a review. Biosensors 11, 316. https://doi.org/10.3390/bios11090316.

Campàs, M., Reverté, J., Rambla-Alegre, M., Campbell, K., Gerssen, A., Diogène, J., 2020. A fast magnetic bead-based colorimetric immunoassay for the detection of tetrodotoxins in shellfish. Food Chem. Toxicol. 140, 111315. https://doi.org/10.1016/j.fct.2020.111315.

Chen, L., Yang, G., Qu, F., 2024. Advances of aptamer-based small-molecules sensors in body fluids detection. Talanta 268, 125348. https://doi.org/10.1016/j.talanta.2023.125348.

Christidis, G., Mandalakis, M., Anastasiou, T.I., Tserpes, G., Peristeraki, P., Somarakis, S., 2021. Keeping Lagocephalus sceleratus off the table: sources of variation in the quantity of TTX, TTX analogues, and risk of tetrodotoxication. Toxins 13, 896. https://doi.org/10.3390/toxins13120896.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen, H.K., Alexander, J., Barregard, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., et al., 2017. Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA J. 15, 4752. https://doi.org/10.2903/j.efsa.2017.4752.

Gomes .R., V.C., Christidis, G., Peristeraki, P., Somarakis, S., Tserpes, G., 2025. The diet and feeding habits of Lagocephalus sceleratus in the eastern Mediterranean: a case study carried out off the island of Crete (southern Greece). Mediterr. Mar. Sci. 26, 97–114. https://doi.org/10.12681/mms.38444.

Guardone, L., Maneschi, A., Meucci, V., Gasperetti, L., Nucera, D., Armani, A., 2019. A global retrospective study on human cases of tetrodotoxin (TTX) poisoning after seafood consumption. Food Rev. Int. 36, 645–667. https://doi.org/10.1080/87559129.2019.1669162.

Huang, L., Tian, S., Zhao, W., Liu, K., Ma, X., Guo, J., 2021. Aptamer-based lateral flow assay on-site biosensors. Biosens. Bioelectron. 186, 113279. https://doi.org/10.1016/j.bios.2021.113279.

Huang, Y., Xu, A., Xu, Y., Wu, H., Sun, M., Madushika, L., Wang, R., Yuan, J., Wang, S., Ling, S., 2023. Sensitive and rapid detection of tetrodotoxin based on gold nanoflower- and latex microsphere-labeled monoclonal antibodies. Front. Bioeng. Biotechnol. 11, 1196043. https://doi.org/10.3389/fbioe.2023.1196043.

Jing, L., Xie, C.Y., Li, Q.Q., Yao, H.F., Yang, M.Q., Li, H., Xia, F., Li, S.G., 2022. A Sandwich-type lateral flow strip using a split, single aptamer for point-of-care detection of cocaine. J. Anal. Test. 6, 120–128. https://doi.org/10.1007/s41664-022-00228-w.

Kaiser, L., Weisser, L., Kohl, M., Deigner, H.P., 2018. Small molecule detection with aptamer based lateral flow assays: applying aptamer-C-reactive protein cross-recognition for ampicillin detection. Sci. Rep. 8, 5628. https://doi.org/10.1038/s41598-018-23963-6.

Katikou, P., Vlamis, A., 2017. Tetrodotoxins: recent advances in analysis methods and prevalence in European waters. Cur Op Food Sci. 18, 1–6. https://doi.org/10.1016/j.cofs.2017.09.005.

Katikou, P., Gokbulut, C., Kosker, A.R., Campàs, M., Ozogul, F., 2022. An updated review of tetrodotoxin and its peculiarities. Mar. Drugs 20, 47. https://doi.org/10.3390/md20010047.

Laboria, N., Fragoso, A., O'Sullivan, C.K., 2011. Storage properties of peroxidase labeled antibodies for the development of multiplexed packaged immunosensors for cancer markers. Anal. Lett. 44, 2019–2030. https://doi.org/10.1080/00032719.2010.539732.

Lee, L., Nordman, E., Johnson, M., Oldham, M., 2013. A low-cost high-performance system for fluorescence lateral flow assays. Biosensors 3, 360–373. https://doi.org/10.3390/bios3040360.

Leonardo, S., Kiparissis, S., Rambla-Alegre, M., Almarza, S., Roque, A., Andree, K.B., Christidis, A., Flores, C., Caixach, J., Campbell, K., Elliott, C.T., Aligizaki, K., Diogène, J., Campàs, M., 2019. Detection of tetrodotoxins in juvenile pufferfish Lagocephalus sceleratus (Gmelin, 1789) from the North Aegean Sea (Greece) by an electrochemical magnetic bead-based immunosensing tool. Food Chem. 290, 255–262. https://doi.org/10.1016/j.foodchem.2019.03.148.

Li, Y., Xu, X., Liu, L., Kuang, H., Xu, L., Xu, C., 2020. A gold nanoparticle-based lateral flow immunosensor for ultrasensitive detection of tetrodotoxin. Analyst 145, 2143. https://doi.org/10.1039/d0an00170h.

Ling, S., Chen, Q.A., Zhang, Y., Wang, R., Jin, N., Pang, J., Wang, S., 2015. Development of ELISA and colloidal gold immunoassay for tetrodotoxin detection based on monoclonal antibody. Biosens. Bioelectron. 71, 256–260. https://doi.org/10.1016/j.bios.2015.04.049.

Ling, S., Li, X., Zhang, D., Wang, K., Zhao, W., Zhao, Q., Wang, R., Yuan, J., Xin, S., Wang, S., 2019. Detection of okadaic acid (OA) and tetrodotoxin (TTX) simultaneously in seafood samples using colloidal gold immunoassay. Toxicon 165, 103–109. https://doi.org/10.1016/j.toxicon.2019.04.011.

Liu, L.S., Wang, F., Ge, Y., Lo, P.K., 2021. Recent developments in aptasensors for diagnostic applications. ACS Appl. Mater. Interfaces 13, 9329–9358. https://doi.org/10.1021/acsami.0c14788.

Mills, C., Dillon, M.J., Kulabhusan, P.K., Senovilla-Herrero, D., Campbell, K., 2022. Multiplex lateral flow assay and the sample preparation method for the simultaneous detection of three marine toxins. Environ. Sci. Technol. 56, 12210–12217. https://doi.org/10.1021/acs.est.2c02339.

Parolo, C., Sena-Torralba, A., Bergua, J.F., Calucho, E., Fuentes-Chust, C., Hu, L., Rivas, L., Álvarez-Diduk, R., Nguyen, E.P., Cinti, S., Quesada-González, D., Merkoçi, A., 2020. Tutorial: design and fabrication of nanoparticle-based lateral-flow immunoassays. Nat. Protoc. 15, 3788–3816. https://doi.org/10.1038/s41596-020-0357-x.

Quesada-González, D., Merkoçi, A., 2015. Nanoparticle-based lateral flow biosensors. Biosens. Bioelectron. 73, 47–63. https://doi.org/10.1016/j.bios.2015.05.050.

Reverté, J., Alkassar, M., Diogène, J., Campàs, M., 2023. Detection of ciguatoxins and tetrodotoxins in seafood with biosensors and other smart bioanalytical systems. Foods 12, 2043. https://doi.org/10.3390/foods12102043.

Reverté, L., de la Iglesia, P., del Rio, V., Campbell, C., Elliott, C.T., Kawatsu, K., Katikou, P., Diogène, J., Campàs, M., 2015. Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based immunoassay and comparison with surface Plasmon resonance, LC-MS/MS, and mouse bioassay. Anal. Chem. 87, 10839–10847. https://doi.org/10.1021/acs.analchem.5b02158.

Reverté, L., Campbell, K., Rambla-Alegre, M., Elliott, C.T., Diogène, J., Campàs, M., 2017a. Immunosensor array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxins in puffer fish. Anal. Chim. Acta 989, 95–103. https://doi.org/10.1016/j.aca.2017.07.052.

Reverté, L., Campàs, M., Yakes, B.J., Deeds, J.R., Katikou, P., Kawatsu, K., Lochhead, M., Elliott, C.T., Campbell, K., 2017b. Tetrodotoxin detection in puffer fish by a sensitive

planar waveguide immunosensor. Sensors Actuators B Chem. 253, 967–976. https://doi.org/10.1016/j.snb.2017.06.181.

Reverté, L., Rambla-Alegre, M., Leonardo, S., Bellés, C., Campbell, K., Elliott, C.T., Gerssen, A., Klijnstra, M.D., Diogène, J., Campàs, M., 2018. Development and validation of a maleimide-based enzyme-linked immunosorbent assay for the detection of tetrodotoxin in oysters and mussels. Talanta 176, 659–666. https://doi.org/10.1016/j.talanta.2017.08.058.

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell Charonia lampas lampas in Europe. Anal. Chem. 80, 5622–5629. https://doi.org/10.1021/ac800769e.

Shen, H., Zhang, S., Fu, Q., Xiao, W., Wang, S., Yu, S., Xiao, M., Bian, H., Tang, Y., 2017a. A membrane-based fluorescence-quenching immunochromatographic sensor for the rapid detection of tetrodotoxin. Food Control 81, 101–106. https://doi.org/10.1016/j.foodcont.2017.06.001.

Shen, H., Xu, F., Xiao, M., Fu, Q., Cheng, Z., Zhang, S., Huang, C., Tang, Y., 2017b. A new lateral-flow immunochromatographic strip combined with quantum dot nanobeads and gold nanoflowers for rapid detection of Tetrodotoxin. Analyst 142, 4393–4398. https://doi.org/10.1039/c7an01227f.

Shkembi, X., Skouridou, V., Svobodova, M., Leonardo, S., Bashammakh, A.S., Alyoubi, A. O., Campàs, M., O'Sullivan, C.K., 2021. Hybrid antibody-aptamer assay for detection of tetrodotoxin in pufferfish. Anal. Chem. 93, 14810–14819. https://doi.org/10.1021/acs.analchem.1c03671.

Sun, J., Shi, Z., Zhang, T., Wang, L., Dong, R., Zhang, Y., Sun, X., 2023. Highly sensitive and quantitative fluorescent strip immunosensor based on an independent control system for rapid detection of tetrodotoxin in shellfish. Food Control 145, 109403. https://doi.org/10.1016/j.foodcont.2022.109403.

Thattiyaphong, A., Unahalekhaka, J., Mekha, N., Nispa, W., Kluengklangdon, P., Rojanapantip, L., 2013. Efficiency of a rapid test for detection of tetrodotoxin in puffer fish. J. Immunoassay Immunochem. 35, 111–119. https://doi.org/10.1080/15321819.2013.802698.

Turner, A.D., Dean, K.J., Dhanji-Rapkova, M., Dall'Ara, S., Pino, F., McVey, C., Haughey, S., Logan, N., Elliot, C., et al., 2023. Interlaboratory evaluation of multiple LC-MS/MS methods and a commercial ELISA method for determination of tetrodotoxin in oysters and mussels. J. AOAC Int. 106, 356–369. https://doi.org/10.1093/jaoacint/qsad006.

Ulman, A., Abd Rabou, A.F.N., Al Mabruk, S., Bariche, M., Bilecenoğlu, M., Demirel, N., Galil, B.S., Hüseyinoğlu, M.F., Jimenez, C., Hadjioannou, L., Kosker, A.R., Peristeraki, P., Saad, A., Samaha, Z., Stoumboudi, M.T., Temraz, T.A., Karachle, P.K., 2024. Assessment of human health impacts from invasive pufferfish (attacks, poisonings and fatalities) across the eastern Mediterranean. Biology 13, 208. https://doi.org/10.3390/biology13040208.

Vijitvarasan, P., Cheunkar, S., Oaew, S., 2022. A point-of-use lateral flow aptasensor for naked-eye detection of aflatoxin B1. Food Control 134, 108767. https://doi.org/10.1016/j.foodcont.2021.108767.

Wang, X., Cohen, L., Wang, J., Walt, D.R., 2018. Competitive immunoassays for the detection of small molecules using single molecule arrays. J. Am. Chem. Soc. 140, 18132–18139. https://doi.org/10.1021/jacs.8b11185.

Wu, S., Liu, L., Duan, N., Li, Q., Zhou, Y., Wang, Z., 2018. Aptamer-based lateral flow test strip for rapid detection of zearalenone in corn samples. J. Agric. Food Chem. 66, 1949–1954. https://doi.org/10.1021/acs.jafc.7b05326.

Yu, H., Alkhamis, O., Canoura, J., Liu, Y., Xiao, Y., 2021. Advances and challenges in small-molecule DNA aptamer isolation, characterization, and sensor development. Angew. Chem. Int. Ed. 60, 16800–16823. https://doi.org/10.1002/anie.202008663.

Zhao, L., Huang, Y., Dong, Y., Han, X., Wang, S., Liang, X., 2018. Aptamers and aptasensors for highly specific recognition and sensitive detection of marine biotoxins: recent advances and perspectives. Toxins 10, 427. https://doi.org/10.3390/toxins10110427.

Zhao, L., Li, L., Zhao, Y., Zhu, C., Yang, R., Fang, M., 2023. Aptamer-based point-of-care-testing for small molecule targets: from aptamers to aptasensors, devices and applications. Trends Anal. Chem. 169, 117408. https://doi.org/10.1016/j.trac.2023.117408.

Zhou, Y., Li, Y., Lu, S., Ren, H., Li, Z., Zhang, Y., Pan, F., Liu, W., Zhang, J., Liu, Z., 2010. Gold nanoparticle probe-based immunoassay as a new tool for tetrodotoxin detection in puffer fish tissues. Sensors Actuators B Chem. 146, 368–372. https://doi.org/10.1016/j.snb.2010.02.049.