



Pooling of monoclonal antibodies for the rapid detection of commercially important finfish

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ABSTRACT

Fish are a valuable source of high-quality proteins, beneficial polyunsaturated fatty acids, and lipid-soluble vitamins in the human diet, but they also make up one of the “big eight” allergenic foods that are responsible for >90% of food allergy cases. A reliable and cost-effective analytical method is thus needed to detect fish protein in foods to protect fish-allergic individuals. Although immunoassays have been developed to detect many food allergens, there is no effective immunoassay capable of recognizing all common food fish species on the market. The selectivities of three monoclonal antibodies (mAbs), 8F5, 2G9 and 2F3, known to exhibit anti-fish activity against cooked crude fish protein extract from red snapper, yellow fin tuna and swordfish, respectively, were each tested for 63 commercially important fish species using indirect enzyme-linked immunosorbent assays (iELISA). Based on the results, none of the mAbs showed cross-reactivity with all fish species tested; the cross reactivity patterns of these 3 mAbs complemented each other and none cross reacted with any non-fish samples. Pooling these mAbs (1:1:1) in an iELISA successfully detected a wide spectrum of commercially important fish species, including those subjected to various processing conditions. This pooled mAbs based iELISA exhibited high sensitivity, specificity and reproducibility, suggesting its potential utility as a qualitative assay for the regulatory enforcement of the food laws that protect fish sensitive consumers.

1. Introduction

Finfish, commonly referred to simply as “fish”, play an important role in the human diet as a valuable source of high quality proteins, beneficial polyunsaturated fatty acids, and lipid-soluble vitamins. Due to the increased awareness of these nutritional benefits, the contribution of fish to global diets has now reached a record amount of approximately 20.3 kg per person per year on average, supplying over three billion people with at least 15 percent of their average animal protein intake (FAO, 2018).

Not everyone benefits from eating fish, however; approximately 10.8% of adults in the United States (Gupta et al., 2019) suffer from some type of food allergic disorder and fish are not always ‘innocuous’ or ‘harmless’, whether consumed or inhaled. Fish allergy is one of the eight major allergenic foods or food types that are responsible for more than 90% of food allergies in the United States (FAO, 2000). Fish allergens are usually proteins or glycoproteins that are resistant to heat and protease with a molecular mass ranging from 10 to 70 kDa (Griesmeier et al., 2010). Although only a small portion of the proteins in fish

are allergenic, the allergens that cause IgE-mediated responses belong to a number of different protein families (Besler, 2001). As parvalbumin triggers more than 50% of the cases of fish allergies, previous fish detection tools have generally focused on anti-parvalbumin probes (Gajewski et al., 2009; Sharp & Lopata, 2014; Wang et al., 2020). However, there are also numerous cases where patients are allergic to non-parvalbumin proteins (Kuehn et al., 2014), hence the most practical way of protecting consumers is to provide them with information on all fish ingredients that could potentially cause an allergic reaction. Since 2004, the Food Allergen Labeling and Consumer Protection Act (FALCPA) (U.S. FDA, 2004) and Food Safety Modernization Act (FSMA) (U.S. FDA, 2017) in the United States have required manufacturers to declare on the label any allergenic foods or ingredients derived from allergenic foods that may be present. Lacking other options, food manufacturers must depend on good manufacturing practices and hazards analysis and critical control point (HACCP) plan to avoid the presence of contaminating allergens in their products. This is clearly not a satisfactory situation and there is, therefore, an urgent need for a rapid test capable of detecting all common fish species that are potential sources of

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allergens in food products, which is the only way to protect fish allergic consumers (Vierk et al., 2002).

Immunoassays are widely accepted by regulatory agencies as a rapid and sensitive method for screening and monitoring substances in food and agricultural products (Masiri et al., 2016; O'Kennedy & Murphy, 2017). The usefulness of enzyme-linked immunosorbent assays (ELISA) for the quick detection and semi-quantification of allergens or target proteins of allergenic food has been clearly established (Hsieh & Rao, 2017; Su et al., 2015; Masiri et al., 2016; Wang et al., 2014). This study therefore sought to develop an effective and rapid immunoassay for the detection of commercially important fish.

Monoclonal antibodies (mAb) are favored as the standard reagents for immunodetection due to their uniformity and unlimited supply at low cost once successfully developed. The main obstacle to their widespread adoption is their cross reactivity, which has long been the reason for the failure to develop effective mono-species specific assays (Fæste & Plassen, 2008). Although this cross reactivity could be utilized to probe a group of fish species (Hsieh et al., 2009), the "desired cross reactivity" is not yet sufficient to cover all fish species within a group. Since no single mAb has yet been found that is capable of recognizing all the commercially important fish, we instead considered whether pooling a group of mAbs with complementary species-selectivity would provide a more comprehensive coverage of common fish species. Such pooled mAbs could then serve as detection antibodies for a future immunoassay that would be suitable for rapid, in-field screening. We, therefore, selected three previously developed mAbs known to exhibit complementary activities against cooked crude fish protein extracts from three common finfish, red snapper (8F5), yellow fin tuna (2G9) and swordfish (2F3), for this study. The specific objectives of this research were to: 1) verify the cross reactivity and identify the molecular weights of the target antigenic proteins of the three mAbs, 8F5, 2G9 and 2F3; and 2) demonstrate the usefulness of these pooled mAbs for the detection of common food fish using an indirect non-competitive ELISA (iELISA).

2. Materials and methods

2.1. Materials

Alkaline phosphatase (AP) conjugate substrate kit, dye reagent concentrate, porcine gelatin, goat anti-mouse IgG (H + L) AP (anti-IgG-AP) conjugate, Mini-Protein III electrophoresis cell, Mini Trans-Blot Electrophoretic Transfer Cell, nitrocellulose membrane, protein assay kit II, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl buffer (pH 8.8), 30% acrylamide/bis solution, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), 20 mM Tris-buffered saline (TBS, pH 7.5), Tris-glycine buffer, Tris-glycine-SDS buffer and Precision Plus Protein Kaleidoscope standards for Western blotting were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). 2,2'-azobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ammonium persulfate, EZBlue Gel Staining Reagent, hydrogen peroxide (H₂O₂) solution (30%, g/g), goat anti-mouse IgG (Fc-region specific) horseradish peroxidase (anti-IgG-HRP) conjugate and Ponceau S were purchased from Sigma-Aldrich Co. (St. Louis, MO). Other common laboratory chemicals, reagents, and 96-well microplates were purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals and reagents were of analytical grade. All solutions were prepared using distilled, deionized, and sterilized pure water from the NANOpure Diamond ultrapure water system (Barnstead International, Inc., Dubuque, IA). Soy protein concentrate was purchased from Central Soya Co. (Fort Wayne, Inc.). Fish (63 species), shellfish (4 species), beef, pork, chicken breast, chicken thigh, lamb, rabbit, turkey breast, turkey thigh, duck, porcine gelatin, and nonfat dry milk (NFDM) were purchased from local supermarkets. Deer, elk, and horse meat were obtained from a private source.

The three mAbs, 8F5 (IgG 2a), 2F3 (IgG1), and 2G9 (IgG1) used in this study were previously developed against crude thermostable protein extract from cooked (100 °C, 15 min) red snapper, yellow fin tuna,

and swordfish, respectively, according to published methods (Gajewski et al., 2009). They were selected for use in this study because of their complementary selectivity of fish species from our preliminary screening data during the original mAb development. Properly diluted cultural supernatants of these mAbs were used in the current study.

2.2. Preparation of protein extracts

Seafood (fish and shellfish) samples were stored at -20 °C prior to sample preparation. All the seafood products were cut into small pieces (2.5 cm × 2.5 cm) immediately before use. For the raw samples, about 8 g of each sample were mashed and weighed into a beaker and 9 fold saline (0.15 M sodium chloride) added to the mashed sample to extract the soluble proteins. For the cooked samples, about 8 g of each sample were weighed into a beaker covered with aluminum foil which was then sealed with autoclave tape and heated in a boiling water bath for 8 min at 100 °C. After cooling to room temperature, each cooked sample was mashed into fine particles together with its own liquid. A 2-fold (w/v) of saline solution was then added to the mashed cooked samples to extract the soluble thermostable proteins and the mixtures were centrifuged at 3220×g for 30 min at 4 °C, using an Eppendorf 5810R centrifuge (Brinkmann Instruments, Inc., Westbury, NY). The supernatants were filtered through Whatman no. 4 filter paper into 1.5 mL microcentrifuge tubes (Fisher) and stored at -20 °C.

Fat and connective tissues were trimmed off meat samples (pork, beef, chicken, turkey, elk, deer, rabbit, horse, duck and lamb) and the lean meat cut into small pieces and ground twice using a clean meat grinder (Waring Consumer Products, East Windsor, NJ) to ensure homogeneity. The raw and cooked meat samples were then treated following the same procedure as that used for the raw and cooked fish samples, described above.

The nonmeat proteins used in this study included bovine serum albumin (BSA), egg albumin, gelatin, NFDM and soy protein concentrate. About 2 g of each sample were weighed into a beaker and mixed with a 2-fold (w/v) of 0.15 M sodium chloride solution. The cooked samples were heated in a boiling water bath for 8 min at 100 °C, and then both the cooked and raw samples were each mashed with a 3-fold (w/v) of 0.15 M sodium chloride solution. The sample extracts were then centrifuged and the supernatants filtered and stored in the same manner as the cooked seafood samples, described above.

The protein concentration of each extract (seafood, meat and nonmeat) was determined using the Protein Assay Kit II (Bio-Rad). BSA was used as the protein standard.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot

To study the protein profile of the seafood samples and examine the molecular weight of the target antigenic protein of mAbs 8F5, 2G9 and 2F3, selected cooked fish protein extracts were separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking gel and 14% separating gel) under non-reducing conditions in a Mini-Protein III electrophoresis cell (Bio-Rad). The running buffer was 25 mM Tris buffer (pH 8.3) containing 192 mM glycine and 0.1% SDS (g/mL). Samples of extracted soluble protein were loaded 3 µg per lane. After running at 200 V for about 45 min at room temperature, the gels were stained with the EZBlue Gel Staining Reagent.

To further examine the antigenic protein in the cooked form of the extracts, the separated protein bands in the unstained gels were transferred to supporting nitrocellulose membranes using the Mini Trans-Blot electrophoretic transfer cell according to published methods (Gajewski et al., 2009). Briefly, the transferring running condition was 100 V for 1 h. The membranes were incubated in the blocking solution for 2 h at room temperature, and then incubated overnight at 4 °C with mAbs (8F5, 2G9 and 2F3) properly diluted in antibody buffer (1% BSA in TBST). The membranes were then incubated for 2 h with goat

anti-mouse IgG (H + L)-AP conjugate diluted 1: 3000 in 10 mL antibody buffer. Between each step, the membranes were washed several times in the washing buffer. Precision Plus Protein Kaleidoscope standards (Bio-Rad) were used to estimate the molecular weights on the gels and blots.

2.4. Indirect non-competitive enzyme-linked immunosorbent assay (iELISA)

iELISA was performed to: 1) test the fish species selectivity and cross-reactivity of the individual mAbs 8F5, 2G9, and 2F3 on samples of raw and cooked extracts from fish, nonfish (shellfish, meats, and common protein additives); and 2) demonstrate the utility of pooled mAb 8F5, 2G9 and 2F3 (1:1:1) for fish detection. Sample extracts were properly diluted in 50 mM carbonate-bicarbonate buffer (35 mM NaHCO₃ and 15 mM Na₂CO₃, pH 9.6) and coated onto a 96-well flat bottom polystyrene high bind microplate (Costar 9018) at 2 µg/100 µL per well, then incubated at 37 °C for 2 h. After three washing steps with a washing buffer (PBS containing 0.05% Tween 20 [PBST, mL/mL], pH 7.2), the remaining binding sites were blocked with a blocking buffer (PBS containing 1% BSA [g/mL], pH 7.2) for 2 h at 37 °C. After two washes, 100 µL/well of the mAbs diluted in antibody buffer (PBST containing 1% BSA [g/mL], pH 7.2) were added to the plate and incubated at 37 °C for 2 h. After another three washes, 100 µL/well of anti-IgG-HRP conjugate diluted 1:3000 in the antibody buffer were added to the plate and incubated at 37 °C for 2 h. Following five washing steps, the color was developed by adding 100 µL/well of 0.4 mM ABTS substrate solution (0.22 mg/mL of ABTS in 50 mM phosphate-citrate buffer [50 mM Na₂HPO₄ and 25 mM citric acid, pH 5.0] containing 0.15 µL/mL of H₂O₂) followed by incubation at room temperature for 30 min. The reaction was stopped by adding 100 µL/well of 0.2 M citric acid. The absorbance was read using a PowerWave XS microplate reader (Bio-Tek Instruments, Inc. Winooski, VT) at 415 nm.

2.5. Assay validation

The specificity and sensitivity of the iELISA using the pooled mAbs was determined according to Dixon (1998):

$$\text{sensitivity} = A/B \times 100\% \quad (1)$$

where A is the number of positive samples with a positive result and B is the number of positive control samples with a positive result; and

$$\text{specificity} = C/D \times 100\% \quad (2)$$

where C is the number of negative samples with negative result and D is the number of negative control samples with a negative result. The assay precision was determined according to the FDA guidance for industry bio-analytical method validation (U.S. FDA, 2018). The intra-assay and inter-assay reproducibility of the iELISA results were examined to provide a statistical measure of the variation between replicate determinations in the same assay (intra-assay variability) and in the same assays performed on different days (inter-assay variability), represented by the coefficient of variation (%CV) (Crowther, 2001). Overall accuracy consisted of the combined ability to detect positive and negative samples (Dixon, 1998):

$$\text{overall accuracy} = \text{specificity} + \text{sensitivity} \quad (3)$$

computed as $E/F \times 100\%$, where F represents the total number of positive and negative samples tested and E the number of positive and negative samples correctly detected by the assay.

2.6. Statistical analysis

Each sample was tested in duplicate, and each experiment repeated three times. Results were analyzed using Microsoft Office 365 Excel, and

SAS University edition software. One-way analysis of variance (ANOVA) followed by a Duncan's multiple range test was performed; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Species selectivity of the three individual mAbs

3.1.1. mAb 8F5 recognizes 57 of 63 commercially important fish samples

The species selectivity of these three mAbs was tested against extracts of raw and cooked samples of 63 commercially important fin fish species. The iELISA results showed that mAb 8F5 reacted with 57 of the 63 species (the 6 exceptions being yellow fin tuna, blue fin tuna, albacore, escolar, wahoo and swordfish) when fish were cooked (Table 1), indicating a wide cross reactivity among the fish species tested. mAb 8F5 was produced using heated crude red snapper extract as the immunogen. Antibodies recognize relatively small regions on antigens (epitopes) and can thus on occasion find homologues epitopes on related molecules, which is the molecular basis of cross-reaction (Sarantopoulos, 2014). This is why an antibody produced using a single species of fish as the immunogen can react with other species.

Among these positively-reacted fish species, mAb 8F5 showed strong immunoreactivity to cooked samples but was negative to raw samples except three species (fresh water bream, Gag grouper and Speckled trout) (Table 1). Because the immunogen of the antibody was a cooked fish extract, it is reasonable to expect strong immunoreactivity with cooked fish. The ability of the antibody to react with the cooked samples indicates that the target epitope on the antigen molecule is thermostable, probably due to the exposure of these epitopes after heat-induced protein unfolding. Furthermore, the antigen was concentrated after the heat-labile proteins had been aggregated and precipitated. When loading the same amount of protein on the ELISA plate, the amount of target antigen in cooked sample extracts is much higher than that in raw samples, resulting in a stronger signal for the cooked fish samples. Although the raw fish cannot be directly detected by this assay, it can be detected after heating for several minutes.

Although it failed to recognize six of the species, mAb 8F5 did recognize most of the commercially important finfish, as shown in Table 1. Interestingly, the 6 species of fish that were not recognized by mAb 8F5 shared several common characteristics: these species have much lower soluble protein concentrations after cooking (see 3.1.2) and they are all dark muscle fish, which seem to have different allergenicity characteristics from white muscle fish (Kobayashi et al., 2016). The other two mAbs (2G9 and 2F3), which were raised against extracts of cooked dark muscle fish, were thus selected to fill this gap.

3.1.2. mAb 2G9 and mAb 2F3 recognize the remaining 6 fish species

The two mAbs chosen to supplement mAb 8F5, 2G9 and 2F3, were previously developed against cooked yellow fin tuna extract and swordfish extract, respectively. As the iELISA results in Table 1 show, mAb 2G9 reacted strongly with tuna species (yellow fin tuna, blue fin tuna, and albacore), escolar, and wahoo, but not with most of the fish species that were positive to mAb 8F5. Mahi-mahi, which was positive to mAb 8F5, only weakly reacted with mAb 2G9. mAb 2F3 reacted strongly with swordfish and escolar in iELISA. These two mAbs (2G9 and 2F3) also showed weak reactivity with raw fish samples compared to cooked samples, following the same pattern as mAb 8F5.

Species that positively reacted to either mAbs 2G9 or mAb 2F3 included all 6 of the fish species that mAb 8F5 failed to detect, exhibiting similar rapid decreases in their soluble protein concentration (<0.45 mg/mL, a 96% decrease from the uncooked raw samples) after cooking and hence similar precipitation characteristics for their myofibril proteins. Other fish species that did not react with mAbs 2G9 and 2F3 had much higher soluble protein concentrations after cooking (>1.8 mg/mL after cooking, a 80% decrease from the uncooked raw samples) comparing with these 6 fish species (data not shown).

Table 1

Immunoreactivity of individual mAbs 8F5, 2G9, 2F3 against 63 species of cooked and raw fish using iELISA.

Fish species	Scientific name	mAb 8F5 1:20		mAb 2G9 1:3		mAb 2F3 1:3	
		Cooked	Raw	Cooked	Raw	Cooked	Raw
Amberjack	<i>Seriola dumerili</i>	+++	–	–	–	+/-	–
Atlantic croaker	<i>Micropogonias undulatus</i>	++	–	–	–	–	–
Basa	<i>Pangasius bocourti</i>	+++	–	–	–	–	–
Black grouper	<i>Mycteroperca bonaci</i>	+++	–	–	–	–	–
Black rock bass	<i>Mycteroperca phenax</i>	+++	–	–	–	–	–
Black sea bass	<i>Centropristis striata</i>	+++	–	–	–	–	–
Blue cat fish	<i>Ictalurus furcatus</i>	+++	–	–	–	–	–
California sea bass	<i>Stereolepis gigas</i>	+++	–	–	–	–	–
Caribbean red snapper	<i>Lutjanus purpureus</i>	+++	–	–	–	–	–
Cat fish	<i>Ameiurus catus</i>	+++	–	–	–	–	–
Chilean sea bass	<i>Dissostichus eleginoides</i>	+++	–	–	–	–	–
Cod	<i>Gadus morhua</i>	+++	–	–	–	–	–
Cubera snapper	<i>Lutjanus cyanopterus</i>	++	–	–	–	–	–
Farm raised salmon	<i>Oncorhynchus gorbuscha</i>	+	–	–	–	–	–
Fresh water breams	<i>Abramis brama</i>	+++	+++	–	–	–	–
Gag grouper	<i>Mycteroperca microlepis</i>	++	+++	–	–	–	–
Grass porgy	<i>Calamus arctifrons</i>	+++	–	–	–	–	–
Gulf Flounder	<i>Paralichthys albigutta</i>	+++	–	–	–	–	–
Haddock	<i>Melanogrammus aeglefinus</i>	+++	–	–	–	–	–
Halibut	<i>Hippoglossus Stenolepis</i>	+++	–	–	–	–	–
Hog snapper	<i>Lachnolaimus maximus</i>	+++	–	–	–	–	–
Idaho Rain bow trout	<i>Oncorhynchus mykiss</i>	+	–	–	–	–	–
Key west grunt	<i>Labrus plumieri</i>	+++	–	–	–	–	–
King mackerel	<i>Scomberomorus cavalla</i>	+++	–	–	–	–	–
Lady fish	<i>Elops saurus</i>	++	–	–	–	–	–
Lane snapper	<i>Lutjanus synagris</i>	+++	–	–	–	–	–
Mahi-mahi	<i>Coryphaena hippurus</i>	+++	–	+	–	–	–
Mangrove snapper	<i>Lutjanus griseus</i>	+++	–	–	–	–	–
Mississippi redfish	<i>Sciaenops ocellatus</i>	++	–	–	–	–	–
Mullet	<i>Agonostomus telfairii</i>	++	–	–	–	–	–
Nile perch	<i>Lates niloticus</i>	++	–	–	–	–	–
Ocean perch	<i>Sebastes alutus</i>	+++	–	–	–	–	–
Orange roughy	<i>Hoplostethus atlanticus</i>	+++	–	–	–	–	–
Pig fish	<i>Orthopristis chrysoptera</i>	+++	–	–	–	–	–
Pink snapper	<i>Pagrus auratus</i>	++	–	–	–	–	–
Pollock	<i>Pollachius pollachius</i>	+++	–	–	–	–	–
Pompano	<i>Trachinotus carolinus</i>	++	–	–	–	–	–
Red snapper	<i>Lutjanus campechanus</i>	+++	–	–	–	–	–
Red grouper	<i>Epinephelus morio</i>	+++	–	–	–	–	–
Rock bass	<i>Ambloplites rupestris</i>	+++	–	–	–	–	–
Sand perch	<i>Parapercis millepunctata</i>	++	–	–	–	–	–
Scamp grouper	<i>Mycteroperca phenax</i>	++	–	–	–	–	–
Sheep head	<i>Archosargus probatocephalus</i>	+++	–	–	–	–	–
Sockeye salmon	<i>Oncorhynchus nerka</i>	+	–	–	–	–	–
Southern flounder	<i>Paralichthys lethostigma</i>	+++	–	–	–	–	–
Spanish mackerel	<i>Scomberomorus maculatus</i>	+++	–	–	–	–	–
Speckled trout	<i>Cynoscion nebulosus</i>	++	++	–	–	–	–
Striped bass	<i>Morone saxatilis</i>	++	–	–	–	–	–
Tilapia	<i>Oreochromis niloticus</i>	+++	–	–	–	–	–
Tra	<i>Pangasius hypothalamus</i>	+	–	–	–	–	–
Vermillion snapper	<i>Rhomboplites aurorubens</i>	+++	–	–	–	–	–
Whiting	<i>Merlangius merlangus</i>	+++	–	–	–	–	–
White grunt	<i>Haemulon plumieri</i>	++	–	–	–	–	–
Wild cobia	<i>Rachycentron canadum</i>	++	–	–	–	–	–
Wild salmon	<i>Oncorhynchus gorbuscha</i>	+++	–	–	–	–	–
Wolf fish	<i>Anarhichas lupus</i>	+++	–	–	–	–	–
Yellow edge grouper	<i>Hyporhodus flavolimbatus</i>	+++	–	–	–	–	–
Yellow tail snapper	<i>Ocyurus chrysurus</i>	+++	–	–	–	–	–
Yellow fin tuna	<i>Thunnus albacares</i>	–	–	+++	–	++	–
Blue fin tuna	<i>Thunnus orientalis</i>	–	–	+++	–	+	–
Albacore	<i>Thunnus alalunga</i>	–	–	+++	–	+	–
Escolar	<i>Lepidocybium flavobrunneum</i>	–	–	+++	–	++	–
Wahoo	<i>Acanthocybium solandri</i>	–	–	+++	–	+	–
Swordfish	<i>Xiphias gladius</i>	–	–	–	–	+++	–

+++ : Very strong positive (O.D. higher than 1); ++ : Strong positive (O.D. 0.5 to 0.999); + : Weak positive (O.D. nm 0.2 to 0.499)+/- : (O.D. 0.15 to 0.199); - : negative (O.D. lower than 0.15).

3.1.3. All three mAbs showed complementary species selectivity without any cross reactivity with non-fish proteins

iELISA was also used to determine whether any of these three mAbs have cross reactivity with any of the non-fish proteins likely to be present in food products. The non-fish proteins tested were categorized into

two groups, namely protein food additives (non-fat dry milk, gelatin, soy protein and egg albumin) and non-fish meat proteins (raw and cooked meat from non-fin fish, shell fish, poultry, and mammalian animals). Because BSA was used in both the blocking and antibody buffers in the iELISA, this part of the study also sought to confirm whether any of these

mAbs cross-reacted with BSA. The results clearly showed that none of these mAbs (8F5, 2G9 and 2F3) cross-reacted with any of the food additive proteins tested, nor with any of the heat-treated meat samples tested (Fig. 1). We therefore continued to utilize BSA in both the blocking buffer and antibody buffer for all the subsequent iELISA procedures.

In summary, mAb 8F5 reacted strongly with 57 of the 63 commercially important fin fish, with the exceptions being yellow fin tuna, blue fin tuna, albacore, escolar, wahoo and swordfish. mAb 2G9 reacted strongly with tuna (yellow fin, blue fin and albacore), escolar, and wahoo. mAb 2F3 reacted strongly with swordfish and escolar, and weakly with yellow fin tuna, blue fin tuna, albacore, and wahoo. Therefore, a combination of these three mAbs could reasonably be expected to recognize all the 63 commonly used fish species with a strong ELISA signal and no cross reactivity with any of the non-fin fish proteins tested. Pooling of these three mAbs would thus provide effective detection of all commercially important fin fish. The characterization studies described below yielded further information to help understand the unique binding characteristics of these mAbs.

3.2. Characterization of the antigenic proteins of mAbs 8F5, 2G9 and 2F3

Cooked protein extracts of representative species were resolved by 14% SDS-PAGE (Fig. 2). These selected fish samples consisted of the five species that were positive to mAb 2G9 (yellow fin tuna, blue fin tuna, albacore, escolar, and wahoo), one species that was only positive to mAb 2F3 (swordfish) and two representative mAb 8F5 positive fish species (mahi-mahi and amberjack). Since mAb 8F5 is cross reactive across a wide range of 57 fish species, the protein profile of its positive fish species was used as a reference for comparison with the six mAb 8F5 negative fish species that reacted with either mAb 2G9 or mAb 2F3. Protein banding patterns (Fig. 2) were established from substantial amounts of thermostable proteins extracted from these cooked fish species. As expected, the gel patterns showed similarities and differences among the eight fish species because patterns of muscle proteins in the

different species of fish are affected by physiological and environmental factors such as seasons, stress, starvation, breeding season and migration (Gomez-Guillen et al., 2000; Ladrat et al., 2000; Stoliar & Lushchak, 2012).

Blotting these protein bands with different mAbs revealed the unique antigenic components of each of the mAbs (Fig. 3). The Western blot results show that mAb 8F5 recognized a 36-kDa antigenic protein specific in both mahi-mahi and amberjack but did not react with any other species (Fig. 3a), which is in accord with the iELISA results. This 36 kDa muscle protein has been reported to be thermally stable, abundantly distributed in most common fish species, and highly antigenic (Chen & Hsieh, 2014).

mAb 2G9 recognized a major protein band around 23 kDa (Fig. 3b) and several minor bands among yellow fin tuna, blue fin tuna, escolar and wahoo, along with a faint band in mahi-mahi. These results also agree with those obtained from iELISA, where mAb 2G9 strongly reacted with yellow fin tuna, blue fin tuna, albacore, escolar, and wahoo. This may be because the antigenic protein for mAb 2G9 is universally present in fish muscle, although its folding characteristics may differ slightly among species. Based on its molecular weight, troponin I might be a possible candidate for the antigenic protein; several studies have found anti-troponin antibodies are produced by injections of crude protein extracts and troponin I is reported to be a thermostable species marker (Chen & Hsieh, 2001). Further research is needed to fully characterize the antigenic proteins of these mAbs, however.

mAb 2F3 strongly recognizes multiple bands in the range of 30–150 kDa (Fig. 3c) in swordfish and escolar, and weakly binds to wahoo, yellow fin tuna, blue fin tuna and albacore. This finding implies that the antigenic peptides involved might be a group of degraded muscle proteins in these fish species that is encoded slightly differently as a result of the evolution process. The myosin light chain, a subunit of actin, or the myosin or troponin isoforms are possible antigen candidates due to the close proximity to this molecular weight range (Xiong, 2017), although there is as yet no solid evidence to support this assumption. These recognized polypeptides could also be a result of heat-induced large

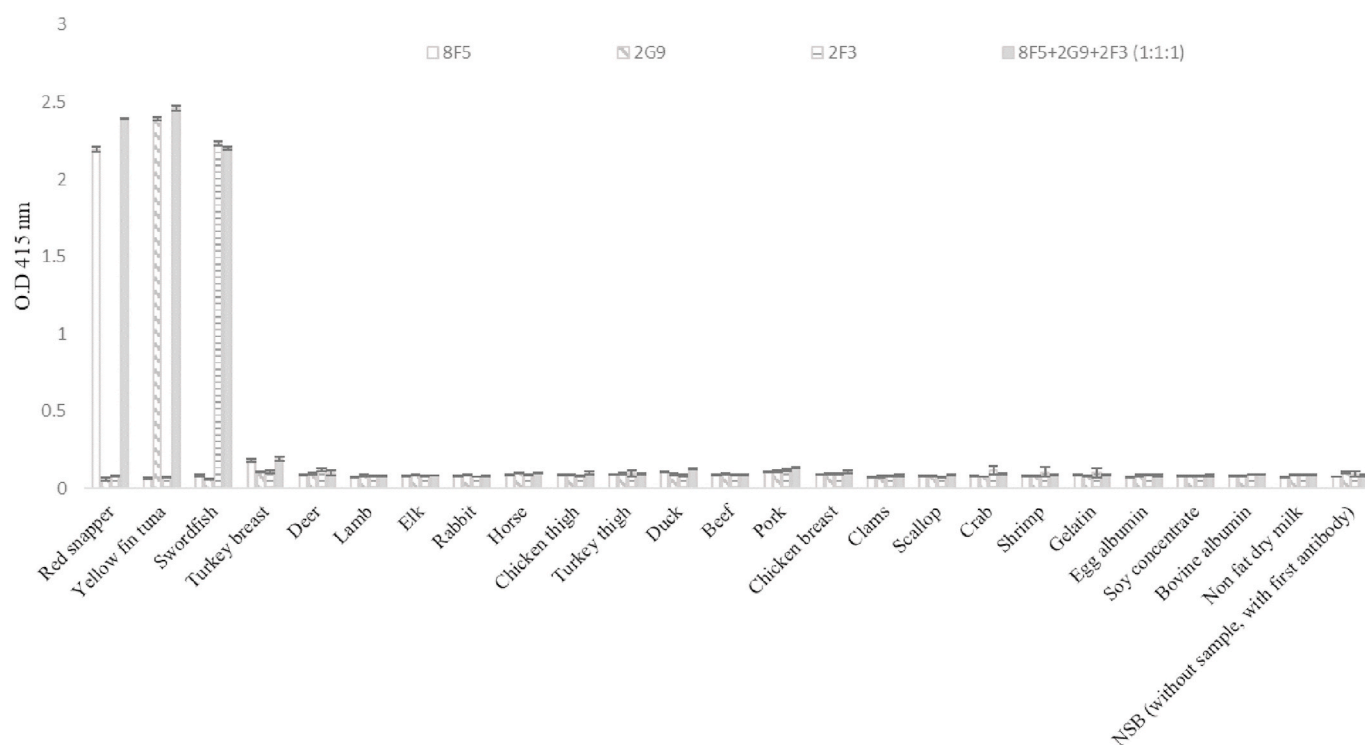


Fig. 1. Immunoreactivity of mAbs 8F5, 2G9 and 2F3 to non-fish cooked samples using iELISA. Results are expressed as the mean of O.D. 415 nm $\pm$ SD. Protein loaded on each well was 2 μ g/100 μ L.

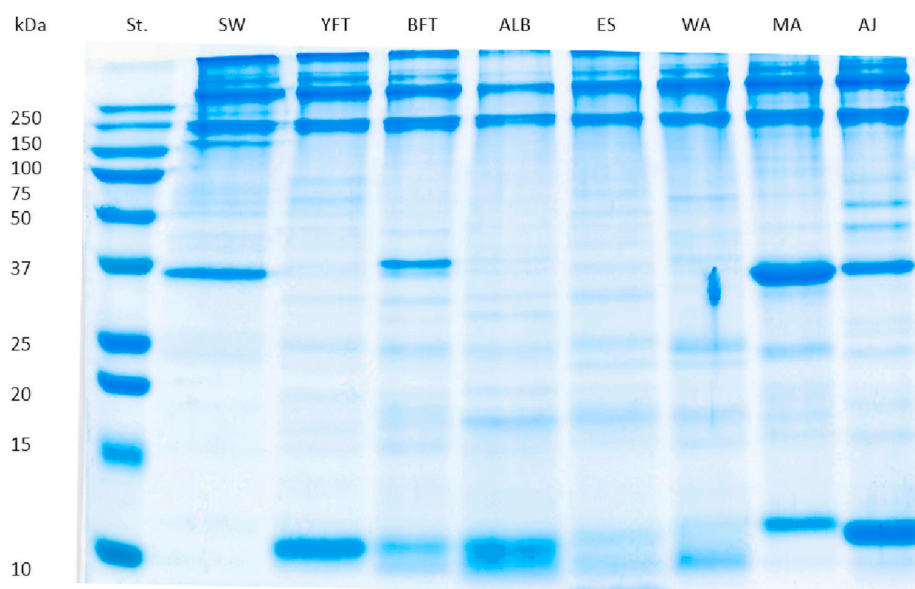


Fig. 2. SDS-PAGE protein banding profile of extracts from 9 species of cooked (100 °C, 8 min) fish under non-reducing condition. Samples of extracted soluble protein were loaded 3µg/lane onto 14% separating gel and 5% stacking gel. St.—Protein Standards; SW—Swordfish; YFT—Yellow fin tuna; BFT—Blue fin tuna; ALB—Albacore (White tuna); ES—Escolar; WA—Wahoo; MA—Mahi-mahi; AJ—Amberjack. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein degradation or small protein aggregation. The results also confirmed mAb 2F3's cross reactivity with some fish species that also positively reacted to mAb 2G9 in iELISA. The escolar showed a minor band around 30 kDa when blotted with mAb 2G9 (Fig. 3b), suggesting a possibly shared antigenic peptide between mAb 2G9 (30 kDa, minor band) and mAb 2F3 antigens (30kD). The Western blot results demonstrated that all three mAbs, 8F5, 2G9 and 2F3, have their own antigenic proteins with different target fish species, providing coverage for the complete range of species tested and showing great promise for the detection of fish species as a whole.

3.3. Detection of 28 representative food fish species using pooled mAbs

Twenty-eight representative fish species, selected from mAb 8F5 negative, weak positive and strong positive samples, were tested using both the pooled mAbs (8F5, 2G9, 2F3) reagent and the three individual mAbs in iELISA to provide a side-by-side comparison of their immunoreactivity. The results (Table 2) show that the pooled mAbs retained the same or higher immunoreactivity compared to their individual use in iELISA. This indicates little interference or inhibition among these three mAbs when pooled together in the ratio of 1:1:1. While mahi-mahi strongly reacted with mAb 8F5 and weakly with mAb 2G9, no species was found to react with both mAb 8F5 and mAb 2F3, indicating their non-shared and non-inhibitive epitopes. All of these results are in accordance with the Western blot patterns of the three mAbs.

The uncooked raw sample extracts of these selected fish were also examined and all showed no signal stronger than the negative control except Speckled trout. However, as uncooked fish samples can always be detected using the same assay simply by applying heat to the raw samples for a few minutes this should not be a problem. The 21 non-fish samples, including land animal, shellfish and common food additive proteins, were also tested by the iELISA using the pooled mAbs (Fig. 1). As expected, no cross reactivity with any of these non-fish samples was found. Overall, these results clearly demonstrate the accurate fish-selectivity and effectiveness of employing the pooled mAbs as a standard ELISA reagent for the detection of raw and cooked fish.

3.4. Validation of the iELISA

The validation results for this newly developed iELISA using the 3 pooled mAbs are summarized in Table 3. The assay had a low intra assay variability of 0.15%–0.80% CV, and an inter assay variability of 0.44 %–

0.50% CV, indicating high reproducibility. These CV values are well below the level generally deemed acceptable, 15% and below (U.S. FDA, 2018). The sensitivity of the assays using individual and pooled mAbs were 99.2% and 100%, respectively. This new iELISA also achieved a 100% specificity in tested samples using individual mAbs and pooled mAbs, as all 732 of the negative samples tested were correctly identified as negative. The assay had an overall accuracy of 99.7%, computed as $E/F \times 100\%$ where F (1318) was the total number of positive and negative samples tested and E (1314) was the number of positive and negative samples correctly detected by the assay. Overall, this newly developed iELISA demonstrated high reproducibility, sensitivity, specificity and overall accuracy.

4. Conclusions

A new immunoassay, iELISA, utilizing pooled mAbs 8F5, 2G9 and 2F3 for the accurate detection of commercially important food fish proteins has been developed and its effectiveness for fish detection demonstrated in this study. Three mAbs, 8F5, 2G9 and 2F3, were individually used to study their species selectivity against 63 commercially important food fish species. mAb 8F5 reacted with 57 of the 63 species tested, while mAbs 2G9 and 2F3 each reacted with several of the six remaining negative species to fill the gaps in mAb 8F5's coverage. mAb 8F5, mAb 2G9 and mAb 2F3 recognize different thermostable antigenic proteins at approximately 36 kDa, 23 kDa, and 30–150 kDa, respectively, in their positively recognized species. Pooling of the three mAbs at a ratio of 1:1:1 made it possible to detect all the fish samples without incurring cross reactivity with any of the non-fish samples. The complementary species selectivity of the three mAbs, together with their unshared and noninhibitive epitope binding characteristics, contributed to the success of the assay. This newly developed iELISA using the pooled mAb reagent exhibited excellent reproducibility, sensitivity and specificity and appears to be highly suitable for the qualitative detection of fish proteins, whether raw or cooked.

Credit author statement

Yaozhou Zhu: performed the research including, Methodology, Data curation, Validation, Formal analysis, and wrote the draft of the manuscript. **Yun-Hwa Peggy Hsieh:** was responsible for the project, Conceptualization, and administration, Supervision, of the research activity, Funding acquisition, Resources, Writing - review & editing, of

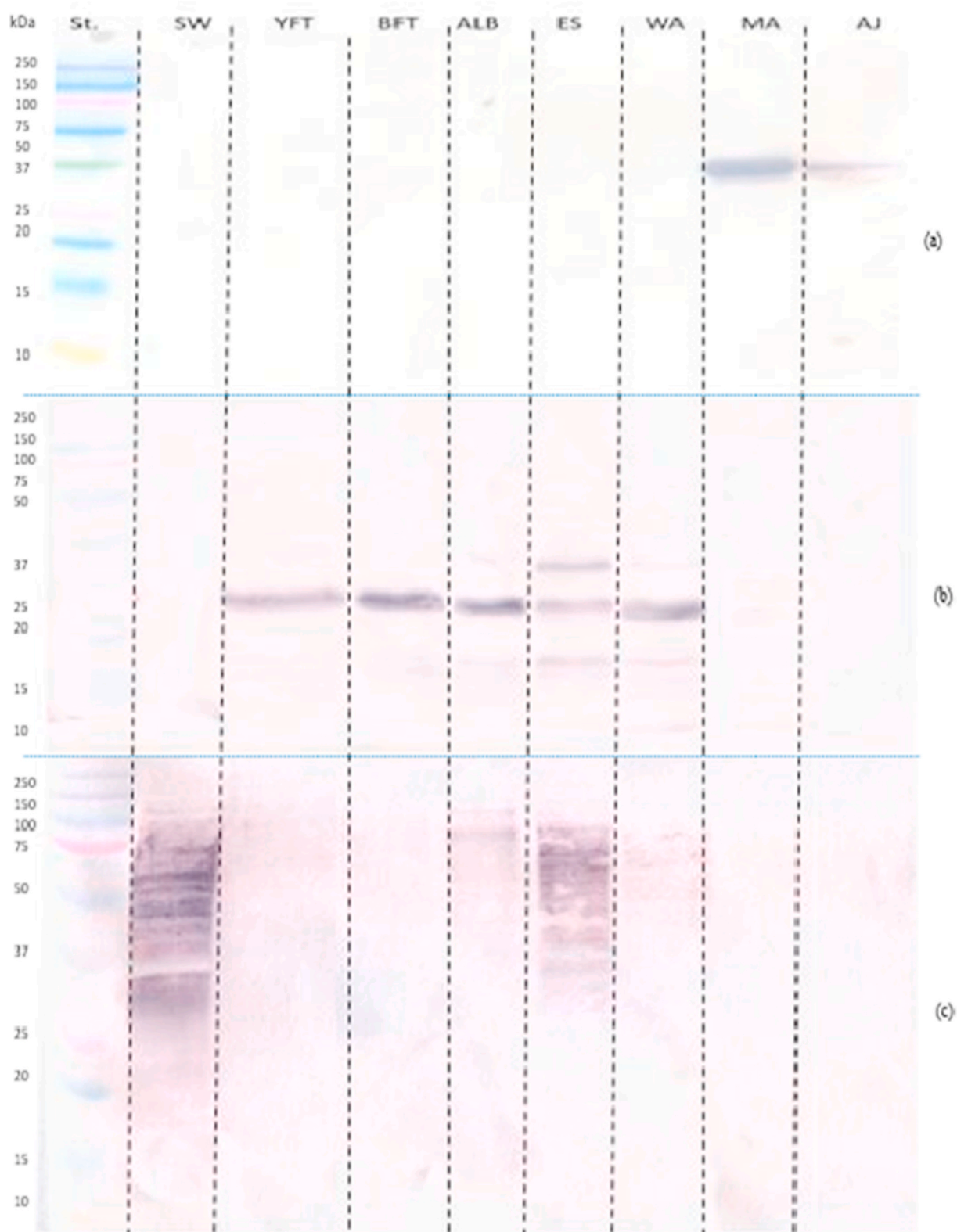


Fig. 3. Antigenic protein banding patterns of cooked fish extracts determined by Western blots using mAb 8F5 (a), 2G9 (b) and 2F3(c). mAbs were diluted as described ratio in 1% BSA-TBST. Samples of extracted soluble protein (3 μ g in 15 μ L per lane) were loaded alongside the Precision Plus protein kaleidoscope standard. Bands correspond to molecular weights of bio-engineered proteins. Goat Anti-Mouse Ig G (H + L)-AP conjugate, diluted 1: 3000 in 10 mL antibody buffer (1% w/v BSA in TBST) was used as the secondary antibody. St.—Protein Standards; SW— Swordfish; YFT—Yellow fin tuna; BFT—Blue fin tuna; ALB—Albacore (White tuna); ES— Escolar; WA—Wahoo; MA—Mahi-mahi; AJ— Amberjack. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Immunoreactivity of individual mAbs 8F5, 2G9, 2F3 and three pooled mAbs against 28 representative species of cooked and raw fish using iELISA.

Fish species	Scientific name	mAb 8F5 1:20		mAb 2G9 1:3		mAb 2F3 1:3		Pooled mAbs (8F5 +2G9 +2F3) 1:1:1	
		Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw
Amberjack	<i>Seriola dumerili</i>	+++	–	–	–	+/-	–	++	–
Basa	<i>Pangasius bocourti</i>	+++	–	–	–	–	–	+++	–
California basa	<i>Stereolepis gigas</i>	+++	–	–	–	–	–	+++	–
Catfish	<i>Ictalurus punctatus</i>	+++	–	–	–	–	–	+++	–
Cod	<i>Gadus macrocephalus</i>	+++	–	–	–	–	–	+++	–
Cubera snapper	<i>Lutjanus cyanopterus</i>	++	–	–	–	–	–	++	–
Lady fish	<i>Elops saurus</i>	++	–	–	–	–	–	++	–
Mahi-mahi	<i>Coryphaena hippurus</i>	+++	–	+	–	–	–	+++	–
Mullet	<i>Agonostomus telfairii</i>	++	–	–	–	–	–	++	–
Pink snapper	<i>Pagrus auratus</i>	++	–	–	–	–	–	++	–
Pollock	<i>Pollachius virens</i>	+++	–	–	–	–	–	+++	–
Red snapper	<i>Lutjanus campechanus</i>	+++	–	–	–	–	–	+++	–
Sand perch	<i>Parapercis millepunctata</i>	++	–	–	–	–	–	++	–
Seawolf	<i>Anarhichas lupus</i>	+++	–	–	–	–	–	+++	–
Sockeye salmon	<i>Oncorhynchus nerka</i>	+	–	–	–	–	–	+	–

Fish species	Scientific name	mAb 8F5 1:20		mAb 2G9 1:3		mAb 2F3 1:3		all 3 mAbs (8F5 +2G9 +2F3) 1:1:1	
		Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw
Spanish mackerel	<i>Scomberomorus maculatus</i>	+++	–	–	–	–	–	+++	–
Speckled trout	<i>Cynoscion nebulosus</i>	++	++	–	–	–	–	++	–
Tilapia	<i>Oreochromis mossambicus</i>	+++	–	–	–	–	–	+++	–
Tra	<i>Pangasius hypophthalmus</i>	+	–	–	–	–	–	+	–
White grunt	<i>Haemulon plumieri</i>	++	–	–	–	–	–	++	–
Whiting	<i>Merlangius merlangus</i>	+++	–	–	–	–	–	+++	–
Wild salmon	<i>Oncorhynchus tshawytscha</i>	+++	–	–	–	–	–	+++	–
Albacore	<i>Thunnus alalunga</i>	–	–	+++	–	+	–	+++	–
Blue fin tuna	<i>Thunnus orientalis</i>	–	–	+++	–	+	–	+++	–
Escolar	<i>Lepidocybium flavobrunneum</i>	–	–	+++	–	++	–	+++	–
Wahoo	<i>Acanthocybium solandri</i>	–	–	+++	–	+	–	+++	–
Yellow fin tuna	<i>Thunnus albacares</i>	–	–	+++	–	++	–	+++	–
Swordfish	<i>Xiphias gladius</i>	–	–	–	–	+++	–	+++	–

+++ : Very strong positive (O.D. higher than 1); ++ : Strong positive (O.D. 0.5 to 0.999); + : Weak positive (O.D.m 0.2–0.499)+/- : O.D. 0.15 to 0.199; - : negative O.D. nm lower than 0.15.

Table 3

Validation of the iELISA using pooled mAbs 8F5, 2G9 and 2F3.

Intra assay variability	0.15%–0.18%
Inter assay variability	0.44%–0.50%
Sensitivity (individual mAbs)	99.2%
Sensitivity (pooled mAbs)	100%
Specificity	100%
Number of negative samples tested	732
Number of negative samples tested correctly as negative	732
Number of positive samples tested	586
Number of positive samples tested correctly as positive	582
Total number of negative and positive samples tested (F)	1318
Number of positive and negative samples correctly identified (E)	1314
Overall accuracy = $E/F \times 100 = 1314/1318 \times 100 = 99.7\%$	

the manuscript.

Declaration of competing interest

The authors declare no conflict of interest in this research.

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