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Morphologic and Molecular Characterization of *Aspergillus flavus* Isolated from Smoked, Fermented and Dried Fishes Sold in Main Markets of Cotonou (Benin)

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Abstract

Fungal infection and aflatoxin contamination were evaluated on smoked or dried fishes sold in surrounding markets of Cotonou. These fishes are traditionally processed to avoid losses and rapid perishability and preserve them from a longer period. Fungal contamination was evaluated after plating on selective media with a total of 175 fungal isolated from 56 samples. Fungi identification shown high prevalence of *Aspergillus* genus that represent 44, 56% followed by *Mucors* (30%), *Rhizopus* and *Rhizomucor* (9.14%; 5.14%, respectively), *Penicillum sp.* (6.85%) and another minority. In *Aspergillus* genus, *A. flavus* represented 29.48% after *A. niger* that was 37.17%. *A. parasiticus, A. fumigatus, A. wentii, A.clavatus* and *A. ochraceus* had 10.25%, 7.69%, 5.12%, 3.84% and 2.56% respectively followed by *A. tamarii, A. candidus* and *A. versicolor* that had 1.28% each. In aflatoxin pathway gene cluster, we investigated three specific genes *AflD, AflO* and *AflP* to 18 stumps of *A. flavus* isolated. This shows that there are potential aflatoxins producers which were suspected by their fluorescence on CCA medium at visible UV and confirmed by determination in chloroformic extract by HPLC. TLC results of chloroformic extracts of samples confirmed the presence of AFB1 in some samples. This is the first study that shows mycotoxigenic fungi and mycotoxins in traditionally processed fishes sampled from Cotonou markets in Benin.

Keywords: *Aspergillus flavus*; Aflatoxins; Smoked fish; Dried fish; Fermented fish; Molecular characterization; Benin

Introduction

Fish is one of the very important foods in developing countries because of its nutritional value [1]. It is a real source of animal protein, vitamin A and D, minerals salt, phosphorus, magnesium, selenium and iodine [1]. The food and Alimentation Organization (FAO), in 2011 showed that the contribution of fish in food value is almost 17 kg per inhabitant and brings at least 15% of the average animal protein required for more than three billion people [2]. According to World Fish Center, fish can ameliorate food safety in Africa and the nutritional status of the people. It's the least costly source of animal protein and the most accessible for rural people [1]. Fish is a rapidly perishable commodity, especially in the tropical areas and hot climate where refrigeration technics is deficient. In Benin, like other countries in West Africa, post-harvest losses of fresh fish are estimated to be about 20% in spite of the great effort expended by more than 400 women to limit the losses through different kinds of traditional conservations process that are smoking, drying and traditional fermented fish called «*lanhouin*» [3]. Unfortunately, non-mastery of these process or bad conservation of transformed fish result in fish contamination by pathogenic micro-organisms among toxigenic molds [4,5] with the presence of aflatoxin B1 and G1 [6,7].

Natural occurrence of mycotoxins and fungal contamination on dried or smoked fish was investigated in multiple countries [4-7]. But none of these studies had been carried out in Benin. The objectives of this study are (1) to determine mycoflora and mycotoxins contamination specially aflatoxins of dried or smoked fish sold in surrounding markets of Cotonou (2) To identify and characterize *Aspergillus flavus*.

Materials and Methods

Survey

Dried, smoked or fermented fish were collected in main markets of Cotonou (Benin): Zogbo, Dantokpa, Degakon, Gbegamey, Cococodji and S[t] Michel. Samples were sampled and were conserved in sterile polyethylene bags in laboratory. Investigations were made by processing, storage and the eventual origin of product.

The origin of product, its storage and processing were investigated by a questionnaire.

In laboratory all the samples were stocked in a temperature of -20°C until fungal and mycotoxins analysis.

Chemical and reagents

All the media used were from Oxoid Ltd. and Hampshire, UK. All the reagents and solvents used were from Merck (Darmstadt, Germany).

Aflatoxins standards (AFB1, AFG1, AFB2, AFG2) were purchased from Sigma Aldrich Chemicals (St. Louis, MO). Taq DNA polymerase used for molecular characterization was purchased from Invitrogen (Carlsbad, CA, USA). Agarose gel and primers used for genes determination were purchased from Eurobio (Courtaboeuf, France).

Moisture determination

Moisture content was determined as described in AOAC 925.04

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method [8]. Ten grams (10 g) of ground sample in crucible porcelain were dried in vacuum oven at 105°C for 24 hours to constant weight and reweighing. Moisture (Te) in percentage was determinate by the formula below:

$$Te=(Ph - Ps) \times 100/Ph$$

Where: Ph=moist mass; Ps=drying mass.

Isolation of molds

The direct plating method described [9] was used with a slight modification. Briefly, twenty-five bits of each sample are surface sterilized by 10% bleach (potassium hypochlorite solution) for 1 min and rinsed twice with sterile distilled water. Five bits were placed in cluster on Dichloran Chloramphenicol 18% Agar (DG 18) (Oxoid Ltd, Hampshire, UK) in Petri dishes and incubated at 25°C for 7 days.

Identification of isolates

The total number of fungal isolated from each product was recoded. Identification of fungi was based on morphology. Macroscopic and microscopic features of each colony were used. A loop full of pure isolate cultured on PDA were sub-cultured on malt extract agar (MEA), *Aspergillus* Flavus Parasiticus Agar (AFPA) (Oxoid Ltd., Hampshire, UK) for identification. The MEA plates were incubated at 25°C for 7 days. Identification of fungi was based on gross morphology and microscopic features such as spores and fruiting structures using [10] descriptions.

Evaluation of *Aspergillus* section *Flavi* aflatoxin potential

Aflatoxins production: Aflatoxins production by the isolated fungi was estimated by culture of pure colony of each sample on coconut media. After 5 days the fluorescence on UV 365 nm is observed for the aflatoxigenic. The aflatoxins production is confirmed by HPLC-PDA after extraction of 7days culture by chloroform as described [11].

Gene characterization: *A. flavus* isolated were sub-cultured on (DG18) for molecular analysis. *Aspergillus* section *Flavi* isolated genes were characterized by extracting the DNA and performing a polymerase chain reaction for genes identification using the method described [12]. Genes amplification was done by using the method described [13]. Gene markers aflD, aflO, aflP, aflR, aflQ (Sigma Aldrich, UK) were used and their specifications are shown on Table 1 according to a study [14].

Aflatoxin detection

Aflatoxins were extracted from samples according to the method [15] with some modifications. Briefly, a ground of each sample (10 g) was mixed with 50 ml of chloroform and macerated for 30 min. The chloroform extracts (10 µl) were analyzed by using thin layer chromatography (TLC) on silica gel plate type (1.05553. DC-Alufolien Kieselgel 60 MERCK) to show the presence of different aflatoxins (B1, B2, G1, G2). The plates were developed first with diethyl ether and then with chloroform: acetone (9:1 v/v). Aflatoxin was identified based on co-migration with aflatoxins standards and by their fluorescent color characteristic under UV 360 nm illumination.

Data analysis

The data were analyzed with SPSS 16.0 (SPSS, Chicago, IL). Univariate analysis of variance (Tukey test) was used to compare the average of moisture content and fungal incidence.

Results

Survey data

Processed fish is highly consumed in Benin. Fifty-six samples of transformed fish have been sampled from forty-five sellers in six markets of Cotonou, Benin. Every seller can sell the three varieties of transformed fish that we studied. The investigation allowed to seller summarizes the processes of production of smoked, fermented and dried fish and the storage conditions. The sampling sites were chosen because of the importance of the customers frequency (main markets which serve secondary markets) and the situation in the city.

Processing methods used to decrease post-capture perishability, included smoking, drying and fermentation: smocked fish is obtained by direct smocking on wood fire, dried fish by drying on the beach after fishing or salting and fermented fish by dry salting, fermented and two or four days of sun drying (Figure 1). All these products are stored on basket or cardboard. The contamination could be facilitated by unhygienic conditions at different processing sites, storage and display system in the markets. According to the trades, dried and smoked fish can be stored at least 6 months and fermented fish between 3 and 6 months maximum.

Moisture content

Moisture level varied according to the processing method. Dried fish have the lowest average between 10 and 15.48% at the normal rate of 12.74% followed by smocked fish 8.36 to 69.24 (normal rate: 38.8%) and fermented fish 35.16 to 59.71 (normal rate: 47.43%).

Fungal isolation and identification

The fungal species recovered from the samples are listed in Table 2. A total of 175 strains of fungi isolated on all the samples, ranging from 100 in smoked fish, 40 in fermented fish to 35 in dried fish, were identified during the study. Nine (9) fungal species were isolated from

Gene		Primer pair	Primers sequences (5' → 3')	PCR product length (bp)
Structural	AflD (Nor1)	Nor1-R Nor1-F	ACGGATCACTTAGCCAGCAC CTACCAGGGGAGTTGAGATCC	990
Structural	AflO (OmtB)	OmtB(F)-F OmtB(F)-R	GCCTTGACATGGAAACCATC CCAAGATGGCCTGCTCTTTA	1333
Structural	AflP (OmtA)	Omt1-F Omt1-R	GCCTTGCAAACACACTTTCA AGTTGTTGAACGCCCCAGT	1490
Structural	AflQ (OrdA)	OrdA-R OrdA-F	TCGTCCTTCCATCCTCTTG TGTGAGTAGCATCGGCATTC	757
Regulator	AflR	AflR-F AflR-R	CGAGTTGTGCCAGTTCAAAA AATCCTCGCCCACCATACTA	999

Table 1: Primers sequences.

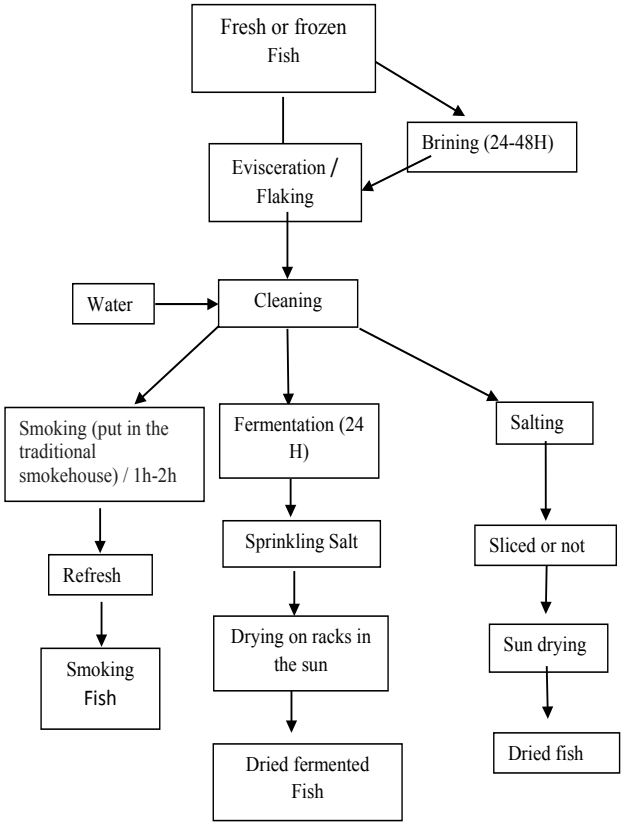


Figure 1: Diagrams of production of smoked fish, fermented fish and dried fish. Process described by the sellers on the various markets. The production of fermented fish varies a little and depending on the producers.

	Incidence							
	Zogbo	**St Michel**	**Gbégamey**	**Dégakon**	**Cococodji**	**Tokpa**	**Total**	**%**
A. flavus	6/11	2/8	5/9	3/8	3/10	4/10	23	13.15
A. niger	6/11	5/8	5/9	4/8	4/10	5/10	29	16.57
A. parasiticus	1/11	3/8	2/9	1/8	-	1/10	8	4.57
A. fumigatus	-	-	-	2/8	4/10	-	6	3.43
A. wentii	-	2/8	2/9	-	-	-	4	2.28
A. clavatus	-	-	2/9	1/8	-	-	3	1.71
A. ochraceus	-	1/8	1/9	-	-	-	2	1.14
A. tamarii	-	-	-	1/8	-	-	1	0.57
A. candidus	-	-	1/9	-	-	-	1	0.57
A. versicolor	-	-	1/9	-	-	-	1	0.57
Mucor ssp	5/11	7/8	8/9	8/8	10/10	5/10	53	30.28
Penicillium decumbens	1/11	-	5/9	2/8	1/10	3/10	12	6.85
Penicillium restrictum	2/11	-	-	-	-	-	2	1.14
Emericella nidulans	-	-	-	-	1/10	-	1	0.57
Rhizopus sp	4/11	2/8	2/9	3/8	2/10	3/10	16	9.14
Rhizomucor sp	5/11	1/8	1/9	-	1/10	1/10	9	5.14
Scopulariopsis brevicaulis	-	-	-	-	-	1/10	1	0.57
Syncephalastrum racemosus	1/11	-	-	-	-	-	1	0.57
Penicillium janthinellum	-	-	-	-	1/10	-	1	0.57
Paecilomyces sp.	-	-	-	-	-	1/10	1	0.57
Isolat totale							175	100

Table 2: Total of molds isolated in processed fish and their occurrence in each market.

the different samples. The genus *Aspergillus* was most prevalent with ten (10) different species. Twenty-three (23) out of the samples were contaminated by both *A. flavus* and *A. niger*. These two species were present indifferently on 29 samples follow by *Mucor* genus which almost contaminates all the samples and *Penicillium* genus with 15 samples contaminated (Table 2). The ten (10) species of genus *Aspergillus* appear with following occurrence: *A. flavus* (29.48%), *A. niger* (37.17), *A. parasiticus* (10.25), *A. fumigatus* (7.69), *A. wentii* (5.12), *A. clavatus* (3.84), *A. ochraceus* (3.84), *A. tamarii* (1.28), *A. candidus* (1.28) and *A. versicolor* (1.28). Thirty-eight (38) strains of *Aspergillus* section *Flavi* have been isolated and identified. Figure 2 shows some of *Aspergillus flavus* strain morphologically different.

Aspergillus section *Flavi* aflatoxigenic potential

Aflatoxins genes research: The molecular characterization of the strains of *Aspergillus* section *Flavi* isolated from the products showed that all of them have genes tested. Figure 3 shows some genes amplification result on agarose gel.

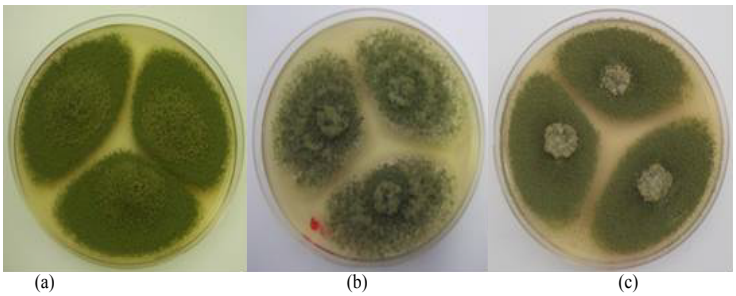
Secondary metabolites production: The production of aflatoxins determined by culture on coconut media has shown that thirty-height (38) strains of *Aspergillus* section *Flavi* are aflatoxins producers and confirmed by HPLC. Twenty (20) toxinogenic strains of *Aspergillus* section *Flavi* have been isolated from the thirty-four (34) samples of smoked fish, eleven (11) on the fourteen (14) samples of fermented fish and seven (7) on the height (8) dried fish, All the strains isolated have produced aspergillic acid.

Aflatoxin contamination in fish

The Thin Layer Chromatography (TLC), showed the presence of high concentration of AFB1 in 2 samples.

Discussion

Agricultural products in tropical Africa, are very often contaminated by molds and mycotoxins because of climate and environmental conditions in this region. But the risks associated with storage conditions and commercialization of all foods make them potential targets to fungal and mycotoxins contamination. Fungal and mycotoxins contamination in African food products is rarely



(a) (b) (c)

Figure 2: Macroscopic aspect of some isolated strains on potatoes dextrose agar (5 days culture). (a) AFP 18; (b) AFP27; (c) AFP 35.

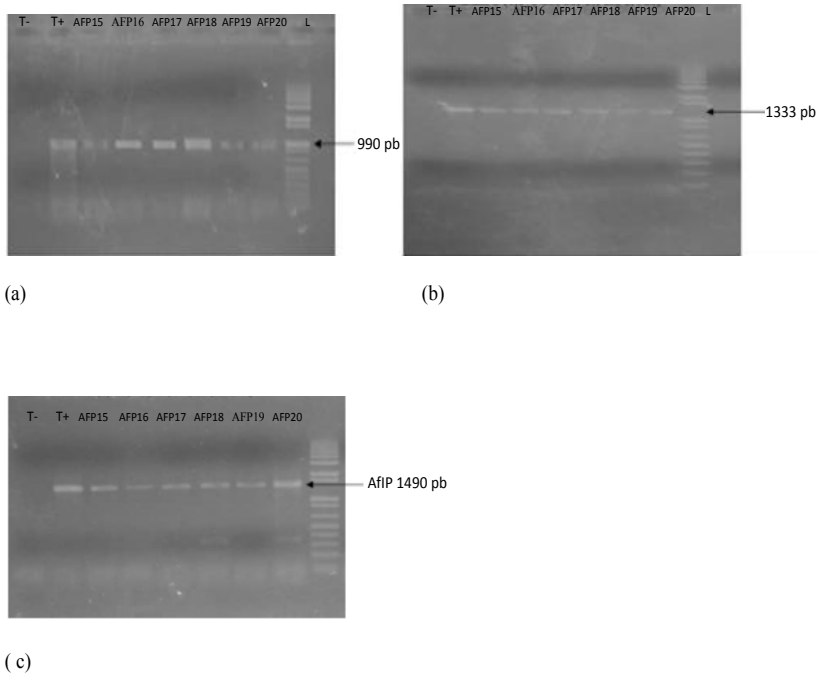


Figure 3: Some PCR results on agarose gel. (a) AFLD; (b) AFLO; (c) AFLP. Agarose gels of amplified sequences of strains AFP15, AFP16, AFP17, AFP18, AFP19, AFP20, negative and positive bystanders. AFLD amplified sequence have 990bp; AFLO have 1333 bp and AFLP 1490 bp.

assessed and almost never controlled. This study therefore focuses on one of the most consumed food products in Black Africa especially in Benin: transformed fish. Fish is a highly perishable product, to ensure its preservation in Benin it is transformed into semi-preserved, either smoked, dried or fermented.

During the exploration phase of this study, different methods were listed by sellers encountered. The conditions of storage and production sites of these products can promote cross contaminations between agricultural products, particularly the cereals.

According to FAO (1984), the current study showed that transformed fish can be contaminated by fungi. It reveals highest fungal contamination levels in smoked fish, dried fish and fermented fish. The most prevalent fungi genus was *Aspergillus* spp (Table 2). Previously isolated from similar conserved fish in other studies [4-7] and the species *A. niger* and *A. flavus* are predominant. Transformed fish are regularly contaminated by *A. flavus* in Vietnam [16]. But variations between the fungal contamination and those found in another existing literature are to be expected, because of sampling variability, differences in country of origin, related environmental factors, divergent processing and storage practices [17,18]. All in all, the climate conditions in West Africa are favorable for fungal development with high relative humidity [19], high temperature [20] and little aeration [21]. All conditions that accelerate fungal development and mycotoxins production during our prospection, we observed unhygienic condition on the traditional fish processing site, which could be a favorable factor for micro-organisms contamination of transformed products [3] showed that fermented fish from Benin called "*Lanhouin*" was produced by fermentation in a widely uncontrolled condition and generally in an unhygienic environment. According to our prospection results on the markets, inadequate conservations are used, and the products were exposed to air particles which potentially contain microbial spores because of the movement of people and vehicles, unloading and loading of trucks in confined spaces. Fungi can grow in a low water activity about 0.65, but in this study, processed fish have moisture between 12 to 47%. This parameter associated with the unhygienic and climatic conditions could justify the presence of mold and mold spores on the transformed fish. The presence of *A. flavus* on this processed fish, as described in 1971 by [22]. Can be explained by a cross contamination during storage and selling conditions. Indeed, various studies have shown the contamination of agricultural products and spices by molds. Considering that processed fish are stored and sold in Benin with fresh or dried spices, whether ground or not, the possibility of cross-contamination among these products is high [23] have shown the contamination of spices sold in Benin by *A. flavus* and aflatoxins.

Though this study, thirty-nine (39) strains of *Aspergillus* section *Flavi* have been isolated morphologically. Investigation of *AflD, AflO, AflP, AflQ* and *AflR* genes in aflatoxins partway genes cluster showed that *A. flavus* species isolated on survey samples are potential aflatoxins producers. Indeed, it has been demonstrated that 25 identified genes clustered within 70-kb DNA region in the chromosome are involved in aflatoxins biosynthesis [24,25] but the expression of the genes *AflD, AflO, AflP, AflQ* and *AflR* was consistently correlated with strain's ability to produce aflatoxins [11,26]. Strains aflatoxinogenicity is confirmed by the fluorescence of aflatoxins on coconut media and by HPLC. Thirty-eight (38) isolated strains are aflatoxin B1 producers. Thirty-eight (38) of the *A. flavus* strains isolated produce aspergillic acid. The last strain AFP 39 which did not have all the tested genes, and which did not produce AFs on coconut agar and aspergillic acid on AFPA, was identified after the sequencing of the internal transcribed spacers (ITS) rRNA and 5.8S rRNA (ITS 4 and ITS 5) as described [11] as *Aspergillus* Section *Tamarii* under species.

TLC analysis of chloroformic extract of samples showed the presence of aflatoxin B1 in some samples. This showed that fish are favorable substratum for *A. flavus* growth and production of aflatoxins. Similar results have been observed [6,7].

Several scientific studies carried out in Benin have shown that some basic foodstuff of Benin citizens such as maize [27] yam chips [28] hot chili [29] Spices [23] Bambara nut are contaminated by aflatoxins. Associating these results with ours, it is obvious that Benin people are strongly exposed to aflatoxins and therefore exposed to sanitary risk.

As the climate conditions of Benin are favorable for molds development on this foodstuff when preservation, stocking and unhygienic conditions are more like successful, contaminated mold can produce mycotoxins on its own. Therefore, it will be very important to inform the population about the simple and easy precautions resulting in an important reduction of aflatoxins level in this foodstuff.

Conclusion

The present study focuses on the characterization of *Aspergillus* section *Flavi* strains isolated from smoked, fermented and dried fish produced by various artisanal processing techniques and the search for aflatoxins. This study collected samples of dried fish, fermented fish and dried fish in the main markets of the city of Cotonou in Benin. The results of this study show that artisanally processed fish (smoked, fermented or dried) marketed in the main markets of Cotonou is contaminated by molds that can potentially produce mycotoxins. The storage conditions and the nature of the fish substrate favor the production of mycotoxins on these fish products examined. Thus, these studies showed the presence of toxigenic strains of *Aspergillus flavus* and aflatoxins in two of the samples. This study is the first in relation to the contamination of fish products marketed by aflatoxins and *Aspergillus flavus*.

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