Available online on 15.05.2019 at http://jddtonline.info



**Journal of Drug Delivery and Therapeutics** 

Open Access to Pharmaceutical and Medical Research

© 2011-18, publisher and licensee JDDT, This is an Open Access article which permits unrestricted non-commercial use, provided the original work is properly cited



# Open Access

**Research Article** 

# Phenotypic characterization and identification of bacterial isolates from smoked fish

Reyes Alvin T.\*, Reyes Alfred T., Bermudez Jay L., Atayde Jimbo D., Aguilar John Paul T., Estes Jojie G., Baltazar Luigi S., Calapardo Michael E. and Vallada Roval L.

College of Fisheries-Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

# ABSTRACT

This study aimed to phenotypically characterize and identify bacterial isolates from smoked fish. From the seven morphologically different bacterial colonies, four of them were identified using selective/differential media. The two isolates belong to genus *Pseudomonas* while the remaining two were confirmed under genus *Staphylococcus*. Results of gram-staining, motility and selected biochemical and physiological tests supported the identity of the four isolates. *Pseudomonas* isolates were found resistant to 30 µg tetracycline while *Staphylococcus* isolates were intermediate to susceptible on the said antibiotics. Unhygienic preparation of the smoked fish could be the possible reason for the product contamination.

Keywords: Smoked fish, phenotypic characterization, biochemical test, physiological test, antibiotic susceptibility

Article Info: Received 30 March 2019; Review Completed 06 May 2019; Accepted 11 May 2019; Available online 15 May 2019

# Cite this article as:



Reyes AT, Reyes AT, Bermudez JL, Atayde JD, Aguilar John Paul T, Estes JG, Baltazar LS, Calapardo ME, Vallada RL, Phenotypic characterization and identification of bacterial isolates from smoked fish, Journal of Drug Delivery and Therapeutics. 2019; 9(3):356-359 http://dx.doi.org/10.22270/jddt.v9i3.2680

\*Address for Correspondence:

Reyes Alvin T., College of Fisheries-Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

# **INTRODUCTION**

The world population is growing drastically and thus, the demand for food has been increasing likewise. In the modern era, the awareness on the benefits of consuming nutritious food has made the demand for certain nutritious food stuffs, such as fish, to rank at the top of the highly demanded food stuffs. Fish and fisheries products are the most important nutritious food all over the world which represents about 15 to 20% of all animal protein on a global basis<sup>1</sup>. Fish is also a good source of omega-3 polyunsaturated fatty acids (PUFAs), micronutrients like vitamin D and different minerals<sup>2</sup>.

However, fish is also known for its high perishability nature. The rapid deterioration of quality of fish and other seafoods after their harvest is mainly due to the various mechanisms of spoilage that takes place. The spoilage mechanisms associated with such deteriorations are grouped into microbial metabolic activities, endogenous enzymatic activities and chemical oxidation of lipids, all of which shorten the shelf life of seafoods<sup>3</sup>. According to Gram and Huss (1996), the high composition of non-protein nitrogen compounds and low acidity (pH>6) of the flesh of seafoods are the major cause of their spoilage, as these condition favor the growth of spoilage microorganisms. These microbes in turn produce metabolites that affect the

organoleptic properties of the products and render them undesirable attributes<sup>4</sup>.

The purpose of fish preservation is to reach the fish or fisheries product to an ultimate consumer in good and usable condition. Different types of fish preservation methods such as chilling, icing, freezing, sun drying, smoking, salting, fermentation and canning have been followed mostly in all the regions of Philippines to reach the fish or fisheries product to an ultimate consumer in good and usable condition and prevent or reduce the post-harvest losses<sup>5</sup>. Smoked fish are well accepted food items in the country. Smoking is the method of fish preservation effected by a combination of drying and deposition of naturally produced chemicals resulting from the thermal breakdown of wood<sup>6</sup>. Smoking gives the product a desirable color, taste and odor, a longer shelf-life through its antibacterial and oxidative effect, lowering of pH and acts as antagonist to spoilage<sup>1,8,9,10</sup>.

The nutrients present in fish provide a good medium for microbial growth. Spoilage and other disease-causing organisms are introduced during handling, processing, packaging and storage<sup>11</sup>. Smoked fish and shellfish products can be a source of microbial hazards including *Listeria monocytogenes, Salmonella* spp., *Clostridium botulinum* and coliform bacteria<sup>12</sup>.

### **Objectives of the Study**

This study aimed to phenotypically characterize and identify bacterial isolates from smoked fish. The characterization mainly focused on selected morpho-biochemical and physiological traits, and tetracycline susceptibility of the isolated bacteria. Selective media were used in the process of identification.

# **MATERIALS AND METHODS**

#### **Isolation of Bacterial Colonies**

Smoked fish sold in the public market of Muñoz, Nueva Ecija, Philippines was macerated using disinfected mortar and pestle. Two series of 10-fold dilutions (10<sup>-1</sup> and 10<sup>-2</sup>) of the macerated smoked fish was made in Trypticase Soy Broth (TSB). One hundred microliters (100  $\mu$ l) of the diluted sample was spread into Trypticase Soy Agar (TSA) plates. The plates were incubated at 37 °C for 18 to 24 hours.

## **Cultural Characterization of Bacterial Colonies**

Bacterial colonies grown in MHA plates were meticulously sorted based on their appearances on the medium. Colony characters such as size, color, optical property, shape, margin, elevation and texture were considered. Colonies that exhibit different cultural characters were grown in selective media, and were subjected to gram-staining, selected biochemical and physiological tests, and tetracycline susceptibility test.

# Presumptive Identification of the Isolated Colonies Using Selective/Differential Media

Three selective/differential media were used namely LS (Lactobacillus Streptococcus) Differential Agar for detection of *Lactobacillus* spp. and *Streptococcus* spp., Staphylococcus Selective Agar for detection of *Staphylococcus* spp. and GSP (Glutamate Starch Phenol) Agar for detection of *Pseudomonas* spp. and *Aeromonas* spp. The media were prepared following the manufacturer's instruction. The culturally characterized colonies were streaked in the prepared selective/differential media. The plates were incubated at room temperature for 24 to 48 hours. Bacterial growth was observed the following day.

# **Gram Staining**

The isolate was streaked on TSA plate and incubated at 37 °C for 18 to 24 hours. A smear was prepared by mixing a small amount of growth with a drop of distilled water. The smear was air-dried and fixed by heat. The glass slide was labeled properly. The dried smear was stained with crystal violet for 1 minute and was rinsed thoroughly with tap water. Afterwards, the smear was covered with Gram's iodine for 1 to 2 minutes and was washed with tap water. The smear was decolorized by dripping 95% ethanol and was washed immediately. Then, the smear was counterstained with safranin for 45 seconds and was washed by tap water. The slide was examined under microscope. Gram-positive bacterium should be colored blue while Gram-negative bacterium should be colored red. Cell size, shape and arrangement were also noted.

#### **Catalase Test**

The isolate was streaked on TSA plate and was incubated at 30  $^{\circ}$ C for 18 to 24 hours. A loopful of the bacterium was transferred to a clean slide. One to two drops of freshly prepared 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were dropped into the slide. Bubble formation indicates presence of catalase enzyme.

#### **Citric Acid Utilization Test**

The isolate was inoculated to Simmon Citrate Agar (SCA) slant using a wire loop by stabbing the butt and streaking on the surface. The slant was incubated at 33  $^{\circ}$ C for 48 hours. Growth and shift of the green color to Prussian blue color means positive utilization of citrate.

# **Urea Hydrolysis**

The isolate was inoculated heavily to Christensen's Medium (CM) Urea broth. The tube was incubated at 35  $^{\circ}$ C for 4 to 6 hours. A red to violet color means positive test for urea hydrolysis.

## **Phenylalanine Deamination Test**

The isolate was inoculated to Phenylalanine Agar (PA) slant. The slant was incubated at 33 °C for 18 to 24 hours. Four to five drops of 10% ferric chloride solution was be added. The immediate appearance of an intense green color indicates positive phenylalanine deamination.

# Hydrogen Sulfide (H<sub>2</sub>S) Production and Motility

The isolate was inoculated to SIM medium slant by stabbing the butt and streaking the surface. The slant was incubated at 30 °C for 48 hours. Browning on the surface and along the line of puncture means formation of lead sulfide which is an indication of  $H_2S$  production from amino acids. Diffused growth from the line of inoculation indicates that the bacterium is motile.

## Growth in 6.5% Sodium Chloride (NaCl)

The isolate was streaked in TSA plate supplemented with 6.5% NaCl. The plate was incubated at 30 °C for 24 hours. The plate was observed for the presence of bacterial growth.

#### **Tetracycline Susceptibility Testing**

About 2 to 3 colonies of the presumptively identified bacterium were suspended in TSB. The bacterial suspension was incubated for 1 to 2 hours at 37 °C and then adjusted to 0.5 McFarland turbidity standards. The adjusted suspension was streaked in TSA plate using a sterilized cotton swab. The antibiotics discs, tetracycline (30  $\mu$ g), were placed on the surface of the inoculated plate using sterile forceps. The plates were incubated at 37 °C for 24 hrs. The zone of inhibition was measured using a ruler. The susceptible, intermediate and resistant categories of the isolates to tetracycline were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute (CLSI).

#### **Statistical Analysis**

Statistical difference in zone of inhibitions was compared using One Way Analysis of Variance. Comparison of means was done using Tukey's Test.

# **RESULTS AND DISCUSSION**

#### **Cultural Characterization of Bacterial Colonies**

Seven bacterial colonies were isolated from the  $10^{-1}$  and  $10^{-2}$  dilutions of macerated smoked fish sample bought in the market. The bacterial colonies were chosen based upon its colonial characteristics in TSA as shown in Table 1. Colony size ranged from pinpoint (D) to small (F and G) to medium (A, B, C and E). All colonies were white in color. Based upon optical property, majority of the isolates were shiny (A, B, C, E, F and G). The colony shape varied from round (B, C, D, F and G) to complex (A and E). Three of the isolated colonies had entire margin (D, F and G), some were wavy (B and C), curled (A) and lobate (E). Colony elevation ranged from

convex (A, D, F and G) to umbonate (B and C) to flat (E). Based on texture, isolates B, C and D were mucoid, isolates A

and E were rough, and the remaining isolates were smooth (F and G).

Table 1. Colonial characteristics of the seven bacterial isolates from smoked fish.

Isolates	Size	Color	Optical Property	Shape	Margin	Elevation	Texture
Α	Medium	White	Shiny	Complex	Curled	Convex	Rough
В	Medium	White	Shiny	Round	Wavy	Umbonate	Mucoid
С	Medium	White	Shiny	Round	Wavy	Umbonate	Mucoid
D	Pinpoint	White	Opaque	Round	Entire	Convex	Mucoid
Е	Medium	White	Shiny	Complex	Lobate	Flat	Rough
F	Small	White	Shiny	Round	Entire	Convex	Smooth
G	Small	White	Shiny	Round	Entire	Convex	Smooth

# Presumptive Identification of the Isolated Colonies Using Selective/Differential Media

All of the isolated colonies failed to grow in LS Differential Agar. Isolates F and G were confirmed as *Staphylococcus* spp.

because of its luxurious and cream growth in Staphylococcus Selective Agar<sup>13</sup>. Meanwhile, isolates B and C were confirmed as *Pseudomonas* spp. because of its luxurious and red-violet growth in GSP agar as opposed to the yellow growth of *Aeromonas* spp. in the same agar (Table 2)<sup>14</sup>.

Table 2. Growth confirmation of the isolates in three selective/differential media.

Isolates	LS Differential Agar	Staphylococcus Selective Agar	GSP
А	-	-	-
В	-	Dell'Vere de la	+
С	- DU/E		+
D	- 10 - L	- '''(').	-
Е	· · ·		15 -
F	- I	+	1207 - E
G	-	+	97. <del>;</del>

# Gram Staining, Motility, and Selected Biochemical and Physiological Tests

To further elucidate the phenotypic characteristics of the presumptively identified isolates, Gram-staining, catalase test, and selected biochemical and physiological tests were performed (Table 3). *Pseudomonas* spp. isolates had rod cells, Gram-negative, motile and positive to catalase test and citric acid utilization, negative to urea hydrolysis, phenylalanine deamination and hydrogen sulfide production. One of the *Pseudomonas* spp. isolate could live in a medium supplemented with 6.5% NaCl. Meanwhile, *Staphylococcus* spp. had spherical clustered cells, Grampositive, non-motile, positive to catalase test and citric acid utilization and negative to urea hydrolysis, phenylalanine

deamination and hydrogen sulfide production. Both of the *Staphylococcus* spp. isolates could withstand 6.5% NaCl.

*Pseudomonas* spp. and *Staphylococcus* spp. possessed catalase enzyme that mediates the breakdown of hydrogen peroxide into oxygen and water. Therefore, the two bacteria had the ability to protect itself from the lethal effect of hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism. Both of them had the capability to consume citrate as carbon source. *Pseudomonas* spp. and *Staphylococcus* spp. lacked the enzymes amino acid oxidase, urease and cysteine desulfonase because of negative results on phenylalanine deamination test, urea hydrolysis and hydrogen sulfide production, respectively<sup>15</sup>.

Isolates	Gram Staining	Shape	Motility	Catalase	Citric Acid Utilization	Urea Hydrolysis	Phenylalanine Deamination	Hydrogen Sulfide Production	Growth in 6.5% NaCl
Pseudomonas 1	-	Rod	Motile	+	+	-	-	-	-
Pseudomonas 2	-	Rod	Motile	+	+	-	-	-	+
Staphylococcus 1	+	Spherical (cluster)	Non- motile	+	+	-	-	-	+
Staphylococcus 2	+	Spherical (cluster)	Non- motile	+	+	-	-	-	+

# **Tetracycline Susceptibility Testing**

The two *Pseudomonas* spp. isolates were resistant to 30 µg tetracycline while *Staphylococcus* 1 and *Staphylococcus* 2 were categorized as susceptible and intermediate, respectively based upon the critical points recommended by the Clinical and Laboratory Standards Institute (CLSI). Highest and significant zone of inhibition was recorded in

Staphylococcus 1 (Table 4). It is well known that most species of *Pseudomonas* showed significant degrees of intrinsic resistance to a wide variety of antimicrobial agents such as  $\beta$ -lactams, tetracyclines, chloramphenicol and fluoroquinolones. The main cause of the resistance of *Pseudomonas* spp. was due to the low non-specific permeability of its outer membrane to small, hydrophilic molecules<sup>16,17</sup>. Kelman et al. (2011) found out that

*Staphylococcus* isolated from ground meats showed 69% resistance to tetracycline<sup>18</sup>. In a separate study, *S. aureus* isolates revealed 56.7% resistance to tetracycline<sup>19</sup>.

The result of this study was contradicting to the published reports of Kelman et al. (2011) and Akanbi et al.  $(2017)^{18,19}$ .

Table 4. Zone of inhibition and CLSI category of the presumptively identified isolates.

Isolates	Zone of Inhibition (mm) to 30 µg Tetracycline	CLSI Category
Pseudomonas 1	8.0±0.0¢	Resistant
Pseudomonas 2	8.0±0.0¢	Resistant
Staphylococcus 1	21.0±1.4ª	Susceptible
Staphylococcus 2	15.8±0.5b	Intermediate

Different superscript was significant at p<0.05 Resistant = < 14 mm; Intermediate = 15 to 19 mm; Susceptible = > 20 mm

# Occurrence of *Pseudomonas* spp. and *Staphylococcus* spp. in Smoked Fish

In the past, smoking was a form of food preservation because large amounts of salt and long smoking times were practiced. Today fish is smoked more for flavor and appearance, and the amounts of salt and smoke used are not sufficient to prevent bacterial spoilage. Unclean, insufficiently or inadequately cleaned processing equipment have been identified as a source of bacterial contamination<sup>20</sup>. During handling and preparation, bacteria are transferred from contaminated hands of food workers to food and subsequently to other surfaces<sup>21</sup>. Poor hygiene, particularly deficient or absence of hand washing has been identified as the causative mode of transmission<sup>20</sup>. Insects, birds and rodents have been recognized as important carries of pathogens and other microorganisms<sup>22,23</sup>.

# **CONCLUSION**

From the seven morphologically different bacterial colonies isolated from smoked fish, two of them belong to genus *Pseudomonas* and the other two to genus *Staphylococcus*. The bacteria were identified using selective/differential media. Results of Gram-staining, motility and selected biochemical and physiological tests supported the identity of the four isolates. *Pseudomonas* isolates were found resistant to 30  $\mu$ g tetracycline while *Staphylococcus* isolates were susceptible and intermediate to the said antibiotics. Unhygienic preparation of the smoked fish could be the possible reason for the bacterial contamination.

# ACKNOWLEDGEMENT

The authors expressed their profound gratitude to the College of Fisheries-Freshwater Aquaculture Center in Central Luzon State University for providing the reagents and media used in this study.

# REFERENCES

- 1. Abolagba, OJ, Melle OO, Chemical composition and keeping qualities of a scaly fish tilapia (*Oreochromis niloticus*) smoked with two energy sources, Afr. J. Gen. Agric., 2008; 4:113-117.
- 2. Mohanty, B, Mahanty A, Ganguly S, Nutritional composition of food fishes and their importance in providing food and nutritional security, J Food Chemistry, 2017.
- Mahmud, A, Abraha B, Samuel M, Mohammedidris H, Abraham W, Mahmud E, Fish preservation: A multi-dimensional approach, MOJ Food Process Technol., 2018; 6(3):303–310. DOI: 10.15406/mojfpt.2018.06.00180
- 4. Gram, L, Huss HH, Microbiological spoilage of fish and fish products, Int. J. of Food Microbiol., 1996; 33(1):121–137.
- 5. Nowsad, AKMA, Participatory training of trainers: A new approach applied in the fish processing. Bangladesh Fisheries Research Forum, Bangladesh, 2007.
- 6. Rawson, GC, A short guide to fish preservation, U.S Food and Agriculture Organization, Rome, 1996.

- 7. Eyo, AA, Fish processing technology in the tropics, National Institute for Freshwater Fisheries Research (FIFR), New Bussa, Nigeria, 2001.
- Olokor, JO, Ihuahi JA, Omojowo FS, Falayi BA, Adelowo, EA, Handbook of practical fisheries technology, Fisheries Technology Division, National Institute for Freshwater Fisheries Research (NIFFR), PMB 6006, New Bussa, Niger State, Nigeria, 2007.
- Horner, WFA, Preservation of fish by curing, drying, salting and smoking, In: Fish Processing Technology, G.M. Hull (edn) Blackie Academic and Professional, New York, 1992.
- 10. Clucas, IJ, Ward AR, Post-harvest fisheries development: A guide to handling preservation processing and quality
- guide to handling, preservation, processing and quality, Natural Resource Institute Chatham Maritime, Kent, United Kingdom, 1996.
- 11. Codex Alimentarius, Recommended international code of practice for smoked fish CAC/RCP 25-1979, 1963.
- 12. Heintz, ML, Johnson JM, The incidence of *Listeria* spp., *Salmonella* spp., and *Clostridium botulinum* in smoked fish and shellfish, J. Food Prot., 1998; 61:318-323.
- 13. Chapman GH, J. Bact., 1952; 63:147-150.
- 14. Stainer, RY, Palleroni NJ, Doudoroff M, The aerobic *Pseudomonas*, a taxonomic study, J. Gen. Microbiol., 1996; 42:159.
- 15. Reyes, AT, Morpho-biochemical aided identification of bacterial isolates from Philippine native pig, Adv. Pharmacol. Clin. Trials, 2018; 3(5):000148.
- 16. Yoshimura, F, Nikaido H, Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes, J. Bacteriol., 1982; 152:636-642.
- Angus, BL, Carey AM, Caron DA, Kropinski AMB, Hancock REW, Outer membrane permeability in *Pseudomonas aeruginosa*: Comparison of a wild-type with an antibiotic supersusceptible mutant, Antimicrob. Agents Chemother., 1982; 21:299-309.
- Kelman, A, Soong YA, Depuy N, Shafer D, Richbourg W, Johnson K, Brown T, Kestler E, Li Y, Zheng J, McDermott p, Meng J, Antimicrobial susceptibility of *Staphylococcus aureus* from retail ground meats, Journal of Food Protection, 2011; 74:1625–1629, doi:10.4315/0362-028X.JFP-10-571
- Akanbi, OE, Niom HA, Fri J, Otigbu AC, Clarke AM, Antimicrobial susceptibility of *Staphylococcus aureus* isolated from recreational waters and beach sand in Eastern Cape Province of South Africa, Int. J. Environ Res. Public Health, 2017; 14(9): 1001, doi: 10.3390/ijerph14091001
- Reij, MW, Den Aantrekker ED, ILSI Europe Risk Analysis in Microbiology Task Force, Recontamination as a source of pathogens in processed foods, International Journal of Food Microbiology, 2003.
- Montville, R, Chen Y, Schaffner DW, Risk assessment of hand washing efficacy using literature and experimental data, International Journal of Microbiology, 2002; 73:305-313.
- 22. Olsen, AR, Hammack TS, Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the damp fly, *Hydrotea aenescens*, at caged layer houses, Journal of Food Protection, 2000; 63:958-960.
- 23. Urban, JE, Broce A, Killing of flies in electrocuting insects traps releases bacteria and viruses, Current Microbiology, 2000; 41:267-270.