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Original Research

Isolation & Identification of Common Harmful Bacteria from Several Types of Dry Fishes from vicinity of Gono Bishwabidyalay Campus

Md. Rezaul Alam[1*], Ripa Khan[1], Md. Mokhlesur Rahman[2], Shyam Sundar Shaha[3] and Rokeya Parvin[1]

[1]Department of Microbiology, Gono Bishwabidyalay, Savar, Dhaka, Bangladesh.
[2]Department of Medical Physics and Biomedical Engineering, Gono Bishwabidyalay, Savar, Dhaka, Bangladesh.
[3]Department of Biochemistry and Molecular Biology, Gono Bishwabidyalay, Savar, Dhaka, Bangladesh.
* Corresponding Author: Md. Rezaul Alam; Email: rezaalam@gmail.com; Mobile: +8801712115200.
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Abstract

In a riverine country like Bangladesh fish consumption is widely popular. Various types of fishes are easily available and are relatively inexpensive. In a less developed country like Bangladesh access to this protein rich source is really a blessing. We have access to both freshwater and sea fish. Preservation of these protein source is easy. In our country dry fish market has huge customer base. Since dry fish consumption is really high in our country a detailed micro- biological analysis was performed. Presence of pathogenic bacteria was evaluated from fifteen (15) samples, which were collected from vicinity. Three dry fish tested were Mola, Loita and Chepa. Experiments were segmented into three parts, namely biochemical tests, gram staining test and antibiogram test. Tests were specifically performed for detecting pathogenic bacterial types. Confirmatory tests were observed for Bacillus cereus, Staphylococcus aureus, Klebsiella spp. Out of fifteen dry fish samples, eight (53%) were confirmed to contain Klebsiella spp, three (20%) were confirmed to contain Bacillus spp, four (27%) was confirmed to contain Staphylococcus spp. Chloramphenicol was sensitive to S. aureus, Bacillus spp and Klebsiella spp. Tetracycline was sensitive to all three organisms tested. Vancomycin was sensitive to all three organisms tested. Penicillin was only sensitive to Klebsiella a spp. CLSI guideline was followed for antibiotic and organism correlative inhibitory pattern. From this experiment it is evident that we should be careful for consuming local dry fish product.

Keywords: *Loita fish, Harpadon Nehereus, Chepa fish, Gudusia chapra, Mola fish, Amblypharyngodon Mola, Pathogenic bacteria, Biochemical tests*

Introduction

For maintaining good health human need proper balanced diet that comprised of protein, carbohydrate, lipid, minerals, etc. In Bangladesh, acquiring protein from meat source is expensive. An alternative of expensive meat source are fishes which are readily available [1]. Since Bangladesh has lots of rivers, ponds, lakes and there is no scarcity of fish source. But there is a problem of food preservation. Since electricity is scarce in rural areas and most of people don't have access to refrigerator. From this viewpoint fish preservation seems to be a vital problem but there is unique cheap alternative solution for preservation. This alternative solution is using available sunlight. In Bangladesh, we are extremely lucky that we live in a temperate zone where yearlong sunlight is available in contrast with other regions like Scandinavian countries (Norway, Sweden, and Finland) where yearlong sunlight is not available. Dry fishes are highly rich in protein and therefore have been in the menu for human from antiquity. From ancient times people from seaside and riverside has embraced the technique for dry fish preservation. Natural drying is a very cheap process in a country like Bangladesh where sunlight is abundant. In Bangladesh there is a huge market for dried fish. Dry food consumption requires lower energy which suits rural areas where electricity is scarce. In rural areas also there are acute shortages of refrigerators for long time fish preservation. Fish is extremely perishable food and require quick preservation. Without maintaining proper temperature fish is easily vulnerable to spoilage. Ancient people intelligently devised an easy process for fish preservation which is drying under sun [1]. Also adding salting process which is combined with sun drying as a curing process. Water activity (aw) is expressed as the ratio of the vapour pressure in a food (P) to the vapour pressure of pure water (P0). It predicts whether water is likely to move from the food product into the cells of micro-organisms that may be

present. aw= P/P0. When water activity is lowered in dried fish it is available for longer period of preservation (Ali et al, 2022). Since dried fish has high nutrient content it is highly susceptible for pathogenic bacterial attack. In tropical coastal regions where there is lack of electricity fish processing by sundried and salting is getting popular day by day [2] Women are worldwide employed in the processing of dry fishing [3] which is a positive sign for rural woman who has limited source of earning. So, women can take care of their families alongside earning since they don't have to move to other places for living.

This topic is selected for several reasons. The reasons are listed below.

- ❖ Consumption of dried fish is very high in our country.
- ❖ Health hazards are associated with consumption of dried fishes.
- ❖ Currently there is no HAACP (Hazard Analysis and Critical Control Point) procedure for maintaining safety standard for dried fish market in our country.
- ❖ There is huge potential for exporting of dried fish in foreign countries
- ❖ Many women are employed in the dried fish market.

From the listed points above it is very clear that dried fish market has huge potential in both domestic and international markets. To maintain safety, reliability and quality of hygienic dried fish, proper handling and marketing guidelines should be followed.

Since we have a trusted customer base market for dried fish in country and abroad microbiological testing of dried fish are essential. Dried fish product is perishable and need proper handling and storage. Between the two fish drying methods of sun drying and dehydration the latter is more scientific and should be followed in our country. Proper temperature, air velocity, relative humidity, proper shading, hygiene, timing can be maintained for dehydration process. Dehydrated fish has longer shelf life than sun dried fish. [2].

Literature Review

Worked on many aspects of fish handling, fish safety. They performed heavy metal identification test namely arsenic, cadmium and chromium, total viable bacterial count test, aerobic plate count test. Presence of heavy metals were within satisfactory range; bacterial count was within range. This experiment clearly indicated that if fish handling, drying, transport and storage done properly there were no problem in consuming dried fish [4]. Worked on women empowerment through dried fish preservation in global south. In long human history where use of refrigeration technique is merely a new event dried fish consumption is as old as human civilization. Dried fish consumption today is very popular in the world and especially in the global south. One very important aspect is that women are specially employed in this field [5]. Worked on microbiological quality of dried balm fish in sylhet region of Bangladesh. They collected 45 samples randomly. They found the following fungus species in samples, Aspergillus fumigatus, Fuserium proliferatum and Rhizopus stolonifer. They argued that their collected samples were better than commercially produced samples. Total fungal count and total plate count were performed [6]. Worked on microbiological quality of dried fish sold in local market in Harare, Zimbabwe. Their work clearly indicated that both streets sold dried fish and supermarket sold dried fish pose significant threat to consumer health [7].

Objectives of the study

- ❖ To find out morphological properties of harmful bacteria by gram staining and microbiological observation.
- ❖ To Identify and isolate harmful bacteria from dry fish by using different biochemical tests.
- ❖ To find out antibiotic sensitivity pattern of harmful bacteria by Kirby-Bauer method.

Materials and Methods

Location of study and Samples collection area.

The study was carried out in the Department of Microbiology Gono Bishwabidyalay, Savar, Dhaka. Sample was collected from vicinity. Total fifteen dry fish sample were collected from different region of greater Ashulia, Nayarhat, Pollybiddut, Nabinagar region, Savar, Dhaka. Only samples with good organolepic (appearance, aroma, texture and tests) character were selected. Samples were collected with utmost care. Sterilized glass containers, gloves were used to collect samples. Samples were brought to laboratory within half an hour from collection area.

After bringing the samples to lab immediately they were cut, crushed, mixed with peptone water incubated and was ready for use in culture media. (Mansur et al., 2013). The midsection of each sample was used for lab test.

Table 1: Sampling number and types

Sample No.	Sampling Name	Sample collection No.	Total number of samples
01	Loita	5	15
02	Mola	5	

Procedure for lab Experiment

Extreme cautions were taken for collecting dry fish samples from vicinity of Gono Bishwabidyalay campus namely Polly biddut, Ashulia and Savar regions. Safety procedures were performed for bringing samples into the microbiology lab. Samples were collected into alkaline peptone water. Then the samples were incubated at 370 c for 24 hours. After overnight incubation samples were streaked onto the bacteriological media. Various media were used like Nutrient agar, MacConkey agar (MAC), Mannitol salt agar (MSA), Eosine methylene blue agar (EMB). Then further incubation for single colony pure culture at 370 c for 24h (Mansur et al., 2013). Morphological characterization by gram staining on pure culture were performed. Four reagents for gram staining were primary stain (crystal violet staining reagent), mordant (grams iodine), decolorizing agent (ethanol) and counterstain (safranin). Protocol for gram staining [8] Flood air-dried, heat-fixed smear of cells for 1 minute with crystal violet staining reagent. Wash slide in a gentle and indirect stream of tap water for 2 seconds. Flood slide with the mordant: Gram's iodine. Wait 1 minute. Wash slide in a gentle and indirect stream of tap water for 2 seconds. Flood slide with decolorizing agent. Wait 15 seconds or add drop by drop to slide unti decolorizing agent running from the slide runs clear. Flood slide with counterstain, safranin. Wait 30 seconds to 1 minute. Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper. Observe the results of the staining procedure under oil immersion using a Bright field microscope. At the completion of the Gram Stain, gram-negative bacteria will stain pink/red and gram-positive bacteria will stain blue/purple. Antibiogram test by disc diffusion method was done. Here total susceptibility pattern of pathogens was tested against four antibiotics out of which two were broad spectrum and two were narrow spectrum. Zone of inhibition measurement and analysis of antibiotic sensitivity was performed according to CLSI guidelines.

Culture Media

Following media were used. Mueller Hinton agar, Eosin Methylene Blue agar, Nutrient agar, MacConkey agar, Mannitol Salt Agar, TCBS (Thiosulfate–citrate–bile salts–sucrose agar), Cetrimide agar. Samples were diluted up to 10-5 and speeded on nutrient agar at 370C for 24 h, Then sub-cultured onto the MacConkey, TCBS, Cetrimide, agar by streak plate method [9]. The experiments were repeatedly sub cultured until obtaining homogenous colonies and pure culture morphology (shape, size, structure).

Nutrient agar medium

Most common microbiological growth medium used for growth of different kinds of bacteria. For total bacterial count and for growth of microorganism's different dilution factor were used.

MacConkey agar medium

It is a differential media specifically designed for growth of Gram-negative bacteria. It can also distinguish between lactose fermenting from nonfermenting bacteria. It contains bile salts (inhibit the growth of most Gram-positive bacteria, crystal violate dye (inhibits certain Gram-positive bacteria), neutral red dye (stains lactose fermenting microbes). Lactose fermenter turns pink to red whereas negative organisms does not change colour.

Eosin-Methylene Blue (EMB)

This media is specifically used for E. coli isolation after incubation period of 24-48h at 37oC and positive result clearly demonstrate green metallic sheen which is clearly visible.

Mannitol salt agar medium
It is a differential and selective media for Staphylococcus aureus. The organism fermenting lactose produce yellow colonies in medium after incubation at 37oC for 48h.

Media for biochemical test

MR-VP (Methyl Red- Voges Proskauer) broth.
Methyl red test in this test bacteria cleaves glucose lowering pH. The test was performed by inoculating a colony of the test organism in sterile glucose phosphate broth. After overnight incubation at 370C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red color, indicating of acidity. A yellow or orange color was negative.

VP broth
Acetone was produced pink color was formed. Methyl Red and Voges-Proskauer test are among the two various tests used in the biochemical identification of bacterial species. These tests were originally studied by Voges, Proskauer and subsequently by Clark and Lubs to differentiate between members of the coli- aerogens group. Both the tests were based on the detection of specific breakdown products of carbohydrate metabolism.

Simmons' Citrate Agar
After inoculation and incubation at 24h only citrate utilizing had grown. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases the alkalinity. It turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

Triple Sugar Iron Agar
Utilization of three sugars such as sucrose, dextrose and lactose and hydrogen sulfide production and gas production was observed in this media. Blackening of media was observed due to presence of thiosulfate and ferrous sulfate in the media.

Phenol red dextrose, lactose and sucrose broth
Utilization of these sugars with pH change in media will cause color change of the broth. Gas formation observed through Durham tube. Color change to yellow yield positive result.

Antibiotic sensitivity test: protocol was followed [10].

Sample preparation
Middle portion of samples was taken, grinded, measured and diluted for fifteen fish samples.

Maintenance of Stock culture: Stock cultures were prepared by adding 1 ml 80% sterilized glycerol in 1 ml of pure culture in nutrient broth and it was stored at -200C for further use.

Serial dilution Procedure: Dilution factor was based on 10. Dilution was done up to 10-5.

Procedure of plate inoculation: Inoculation was done with 100 µl of sample with spreading. After incubation at 37ºC for 24 h colony enumeration and isolation were done.

Results and Discussion

Bacterial counts
Total viable counts of the isolates were performed. The mean colony count on the nutrient agar plates of each given dilution was used to estimate the total viable count for the samples in colony forming units per gram CFU g-1. Colony forming unit.

Table 2: TVC of dry fish sample collected from different area.

Sample No.	Dilution No	Numbers of colonies per plate	Result cfu\ml
1. Chepa	10-1	More than 360	TNTC
	10-2	270	2.7×103
	10-3	220	2.2×104
	10-4	120	1.2×105
	10-5	80	8×107
2. Loita	10-1	More than 320	TNTC
	10-2	More than 320	TNTC
	10-3	70	7×104
	10-4	50	5×105
	10-5	10	1×106
3. Mola	10-1	More than 360	TNTC
	10-2	More than 370	TNTC
	10-3	130	1.3×104
	10-4	50	5×105
	10-5	20	2×106

TNTC Too numerous to count

In the present study, 15 dry fish were collected from different region for isolation and identification of harmful bacteria.

Nutrient agar: Nutrient agar showed regular identifiable colonies.

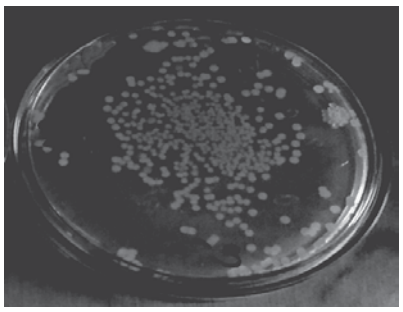


Figure-1: Culture of organism on Nutrient agar plate.

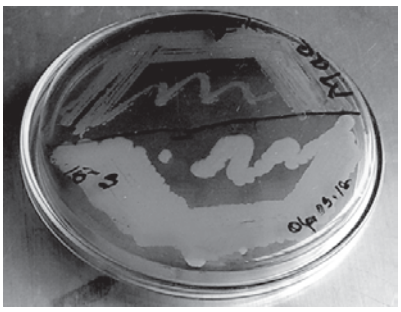


Figure-2: MacConkey Agar plate

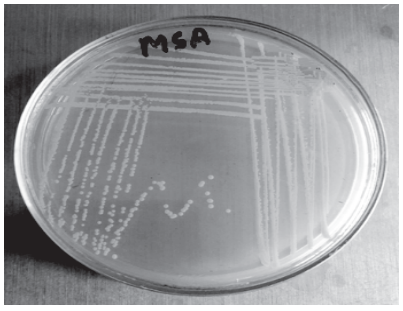


Figure-3: MSA Agar plate

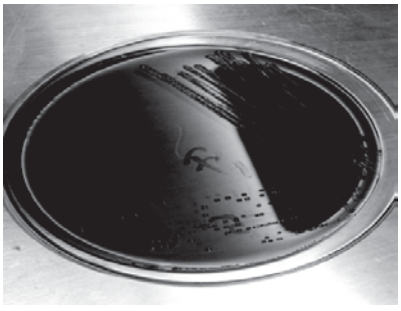


Figure-4: EMB Agar plate

MacConkey agar: After prolonged incubation pink colour colonies were observed.
MSA Agar: Mannitol fermentation showed yellow halo.
EMB Agar: Both normal colonies and sheen colonies

Table 3: Culture characteristic on different agar.

Sample No.	Colour of colony		
	MacConkey	EMB	MSA
1.	Pink colour	No growth	Yellow
2.	Same	No growth	Yellow
3.	Same	No growth	Red
4.	Same	Sheen	Red
5.	Same	Sheen	Yellow
6.	Same	Sheen	Yellow
7.	Same	Sheen	Yellow
8.	Same	Sheen	Yellow
9.	Same	Sheen	Yellow
10.	Same	No growth	Red
11.	Same	No growth	Red
12.	Same	No growth	Red
13.	Same	No growth	Red
14.	Same	No growth	Red
15.	Same	No growth	Red

Microscopic Examination
All samplers were gram positive cocci. All were rod shaped.

Table: 4 Results of various Biochemical Tests.

SL. No.	GRAM'S REACTION	OXI	CT	IND	UR	SC	MR	VP	TSI	MIO	NR	Result
1. Chepa	+, rod	_	+	_	_	_	+	_	Acid butt,Alk slant	_	+	Bacillus spp
2. Chepa	_, rod	_	+	_	+	+	+	+	Alk butt,Alk slant, gas	+	+	Klebsiella spp
3. Chepa	+, cocci	_	+	_	_	_	+	+	Acid butt,Alk slant, gas	+	+	Staphylococcus spp
4. Chepa	_ , rod	_	+	_	+	+	+	_	Alk butt,Alk slant, gas	_	+	Klebsiella spp
5. Chepa	+ , rod	_	+	_	_	_	_	_	Acid butt,Alk slant	+	+	Bacillus spp
6. Mola	+ , cocci	_	+	_	_	_	+	+	Acid butt,Alk slant	_	+	Staphylococcus spp
7. Mola	_ , rod	_	+	_	+	+	_	_	Acid butt,Alk slant	+	+	Klebsiella spp
8. Mola	+ , cocci	_	+	_	_	_	+	_	Acid butt,Alk slant	+	+	Bacillus spp
9. Mola	+ , rod	_	+	_	_	_	_	+	Acid butt,Alk slant	_	+	Staphylococcus spp
10. Loita	+, cocci	_	+	_	_	_	+	+	Acid butt,Alk slant	+	+	Staphylococcus spp
11. Loita	_ , rod	_	+	_	+	+	+	+	Acid butt,Alk slant	+	+	Klebsiella spp
12. Loita	_ , rod	_	+	_	+	+	_	_	Acid butt,Alk slant	+	+	Klebsiella spp
13. Loita	_ , rod	_	+	_	+	+	+	+	Acid butt	+	+	Klebsiella spp
14. Loita	_ , rod	_	+	_	+	+	+	+	Acid butt	+	+	Klebsiella spp
15. Loita	_ , rod	_	+	_	+	+	+	+	Acid butt	+	+	Klebsiella spp

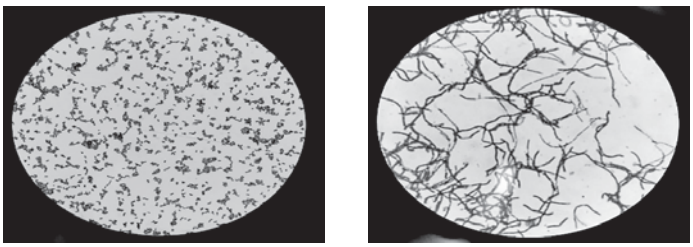


Figure-5: Gram positive cocci and Gram-positive rod shape

Biochemical Results

Results are summarized in the following table. Organisms were identified according to biochemical tests.

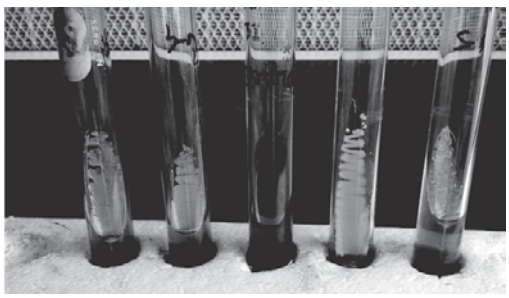


Figure-6: Triple sugar iron test.

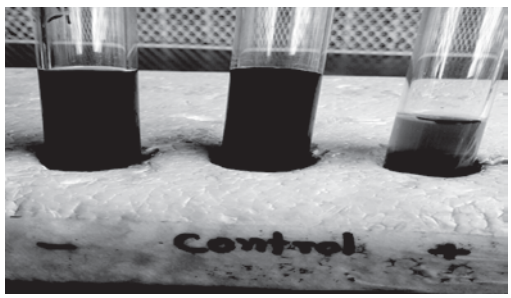
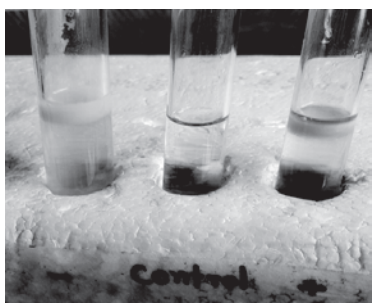


Figure-7: Motility Indole urease test.

Figure-8: Carbohydrate utilization test



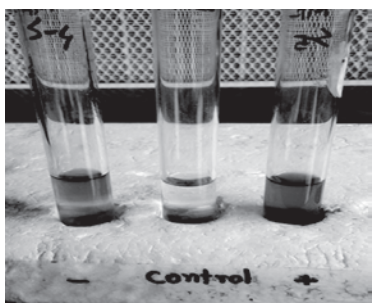


Figure-9: Methyl-red and Voges-Proskauer test.

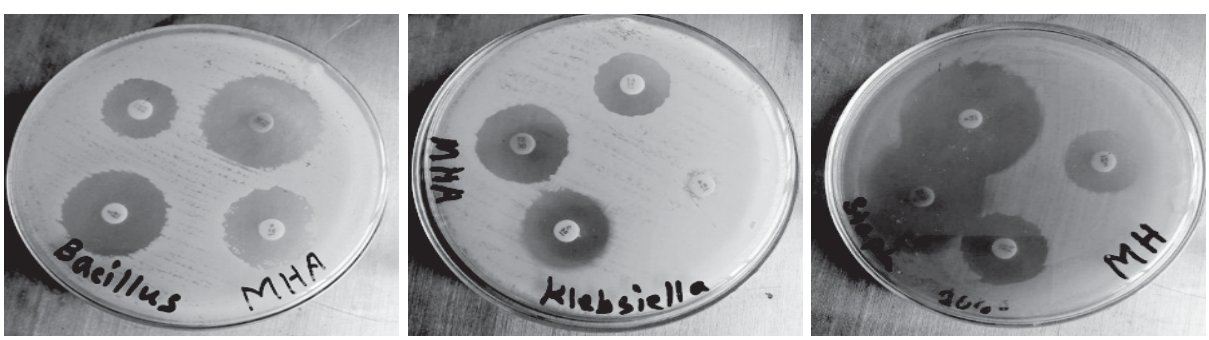


Figure-10: Antibiotic susceptibility test.

Antibiotic Sensitivity Test

Standard MacFarlane solution was used. Mueller Hinton agar (MHA) was used. After inoculation with different organisms' antibiotic discs were placed on agar [10]. After overnight incubation either clear zone or no clear zone observed. Standard antibiotic discs of Tetracycline (Tet) (5µg), Vancomycin (Van) (5µg), Chloramphenicol (Chl) (30µg) and Penicillin (Pen) (10µg) were used.

Table 5: Measurement of Zone of inhibition against antibiotic discs with reference to CLSI guidelines.
CLSI (Clinical and. Laboratory Standards Institute)

Name of the Microorganisms	Broad Spectrum				Narrow Spectrum			
	Chl	Ref	Tet	Ref	Van	Ref	Pen	Ref
S.aureus	S 21mm	20 mm	S 31mm	19 mm	S 19mm	15mm	R 10 mm	12
Bacillus spp	S 27mm	20 mm	S 30mm	19 mm	S 18mm	15 mm	R 11 mm	12
Klebsiella	S 21mm	20 mm	S 23mm	18 mm	S 17mm	15 mm	S 15 mm	12

From the study of total volume count of dried fish was found. In our study total count was higher than that study. So relative contamination was higher [11]. So, we have to be careful in dry fish consumption unless good measures are taken by Fisheries dept. tin our country to alleviate fish pollution. In a poor country like Bangladesh where protein rich diet is expensive dried fish can be an alternative cheap source. Dried fish is a very good source of essential amino acids and protein. Bangladesh is a riverine country and fish source is abundant. Rural people can easily get their nutrition from fish source. Total 15 samples were collected from different local area. Gram staining, microbiological tests, biochemical tests and antibiotic sensitivity tests were performed. Some samples showed too much growth and TNTC (too numerous to count) were observed. Among the three fishes Chepa is better in all dilutions except the first one than Loita and Mola in bacterial count. Mola is better than Loita in bacterial count. So, to categorize from high value to low value the numbering is Chepa, Mola Loita. The reasons for presence of harmful bacteria may be due to handling procedures problems. Some samples showed good qualities and showed less count then recommended value. This experiment showed varied positive bacterial isolates. Bacillus spp, S aureus and Klebsiella spp were identified. Both S. aureus spp and Bacillus spp are resistant against Penicillin but showed zone of inhibition against Chloramphenicol, Tetracycline and Vancomycin. Klebsiella was sensitive to both broad spectrum and narrow spectrum antibiotics. Various biochemical tests were performed to identify harmful bacteria. Gram staining was performed to find out class of bacteria. S. aureus and Bacillus are gram positive and Klebsiella is gram negative spp. Since these three bacteria are harmful, we should be cautious to consume dried fish from unhygienic shop. Comparative data from Indian dry fish market gives similar result. From the work of [12]. aureus was found in dried sea fish in the Indian market. The strength of using dry fishes is several namely cost effectiveness, easy access, high yield and popularity. The limitations are lack of hygiene, presence of potentially pathogenic microorganisms. The advantages are high yield, less cost, women empowerment. From the work of presence of vibrio spp in eastern China seafood was observed [13]. In our study we did not detect any vibrio species so that was one satisfactory aspect. No Salmonella was detected either. From the study of, Zimbabwe fish market had less bacterial load compared to our study [7].

Conclusion and Future Directions

Present experiment was carried out to find contamination in dried fishes in greater Ashulia regions. Since dried fish is a protein rich source there are chances of bacterial contaminations. Total samples were fifteen. Confirmatory tests were observed for Bacillus cereus, Staphylococcus aureus, Klebsiella spp. Out of fifteen dry fish samples, eight (53%) were suspected to contain Klebsiella spp, three (20%) were suspected to contain Bacillus cereus, one (6%) was suspected to contain Staphylococcus aureus. Since there are presence of harmful bacteria people should be cautious of consuming dried fish. Dept. of fisheries, Bangladesh should take steps to safeguard health and hygiene of common people. Recommendation for better fish processing would be to encourage following scientific dehydration process. Coastal and riverside dry fish dehydration industries and facilities should be established.

Authors Contribution's

Md. Rezaul Alam Supervised and guided the research process, providing overall direction and expertise. Ripa Khan and Rokeya Parvin Collected data and performed the isolation and identification of bacteria from the dry fish samples used in the study. Shyam Sundar Shaha and Mokhlesur Rahman Evaluated, reviewed the study and contributed to paraphrasing and enhancing the research manuscript.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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