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# Detection of Potential Bacterial Pathogens and Aflatoxigenic Fungi from Grain Samples

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ABSTRACT

Current research work was carried out for the detection of potential bacterial pathogen and aflatoxigenic fungi *Aspergillus* spp. from grain comprising [Rice (5), Maize (5), Wheat (5), Khessari dal (5) and Anchora dal (5)] were collected from 3 different local markets of Dinajpur District, Bangladesh. 15 bacterial isolates comprising 4 genera of bacteria were found from a total of 25 samples. The isolated bacteria were *Staphylococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp. with 16%, 28%, 16% and 16% prevalence respectively. Antibiogram studies revealed that overall effective drugs against isolated bacteria were Ciprofloxacin followed by Gentamycin. But resistant drugs were Penicillin, Vancomycin, Erythromycin, Kanamycin, and Amoxicillin. The variation in the sensitivity of common antibiotic could be the result of extensive and indiscriminate use of these antibiotics. *Aspergillus* spp. was isolated from 4-grain samples with 16% prevalence. But aflatoxigenic *Aspergillus* spp. was isolated from 3 samples with 12% prevalence. From the wheat samples and maize, the aflatoxigenic fungus was isolated and their prevalence in maize, wheat was 40% and 20% respectively. Their early detection can help to take preventive measures to combat economic and health losses. The study showed that earlier detections can be made by simple traditional identifications using macro and micromorphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

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## Introduction

Cereals like corn (maize), wheat, barley, rice, oats, rye, millet & sorghum, and cereal products are significant and important human food resources and livestock feeds worldwide. In addition, cereal products are used as ingredients in numerous products, such as batters and coatings, thickeners and sweeteners, processed meats, infant foods, confectionary products, and beverages such as beer. Cereal crops can potentially become contaminated with pathogenic micro-organisms during growth and at the time of harvesting. A major cause of spoilage in stored food grain is fungus (Oliveira et al., 2013). There are two important groups of fungi: field fungi and storage fungi. First one is those that invade the seeds while the second is still in the field and require high moisture conditions (20-21%) (CAST, 2003). Fungi produce mycotoxins under stressful conditions such as temperature, moisture or aeration. There are some conditions which influence the fungal growth such as species, a high temperature, and moisture. Fungi grow at temperatures between 20–30°C. Normally, fungi grow in storage conditions at 13–18% moisture (Zviceviėius et al., 2005). Amongst the fungal genera, Fusarium and Alternaria are considered most important because of their toxigenic ability to produce mycotoxins and they are classified by some authors as field fungi, while, Aspergillus and Penicillium species are often considered storage fungi (Roige et al., 2009). Fumonisin, aflatoxin, ochratoxin, zearalenone, and trichothecenes such as deoxynivalenol, T-2 toxin, and nivalenol are appreciated as the most important mycotoxins (Shepard et al., 2008). A variety species of microorganisms often contaminates the wheat grains before harvesting or after harvesting and other species may develop when grains are in silo conditions. In all cases, they decrease the quality of wheat (Magan et al., 2003). There are a lot of factors that affect wheat grains health, but from all of them, the most important are fungi. Some of their effects on wheat grains consist in the fact that they reduce seed germination and vigor and cause decrease in quality during storage (Brennan et al., 2003).

Maize (*Zea mays* L.) is one main cereal crop that has been broadly cultivated worldwide for human consumption and animal feed. However, maize is susceptible to be infected with toxigenic fungi, such as *Fusarium* spp. (*F. verticillioides, Gibberella zeae, F. moniliformis, F. graminearum*) (Doohan et al., 2003) and Aspergillus spp. (*A. Niger, A. flavus, A. parasiticus*), in the field and/or post-harvest conditions. Different toxigenic fungi can produce relevant toxic metabolites, for example, aflatoxins by *A. flavus and A. parasiticus, ochratoxins by A. niger, fumonisins by Fusarium* spp. (Wilson, 2017). Among the various toxins, aflatoxin is highly toxic, carcinogenic and immunosuppressive to animals and humans (Oliveira et al., 2013; Edite et al., 2014). They also responsible for feed refusal and emesis, discoloration, off odors, fungal spoilage of foods and beverages also causes respiratory disease in man (Naresh et al., 2007).

Therefore, there has been a mass of studies focusing on detection of aflatoxins and fungi on maize to prevent health threats to people resulting from consumption of infected kernels. So earlier detection of aflatoxigenic fungi can be made by simple traditional identifications using macro and micromorphological fungal features rather than adopting the time and cost consuming molecular identification techniques. Bearing in mind the above facts the present study was undertaken with the following specific objectives: a) Isolation and identification of potential bacteria of aflatoxigenic fungus from the grain sample. b) Isolation and identification of aflatoxigenic fungus from grain sample. c) Determination of antibiotic-resistant pattern of isolated bacteria.

## Materials and Methods

*Sample Collection and Processing*

A total of 25 samples approximately 300 g of each grain sample were collected from local market of Dinajpur district, Bangladesh for bacteriological and fungal examination. The samples kept in an ice-box during transportation to the laboratory and stored at 4°C until testing. They were analyzed within 24 hours of sampling. After that, samples were uniformly homogenized in mortar and pastel using a sterile diluent as per the recommendation of ISO (1995). A quantity of 10 gm homogenate samples transferred carefully into a sterile pastel containing 90 ml of PBS. Thus 1:10 dilution of the samples were obtained.

*Isolation and Identification of Bacteria*

After processing of samples, primary cultured of the sample in both nutrient agar and nutrient broth at 37°C for 24 hours. Then performed gram staining technique simultaneously bacteria inoculated on Mac-Conkey, Mannitol salt agar at 37°C for 24 hours. Observed by gram staining and sub-cultured onto Eosin Methyl Blue agar (EMB), Salmonella-Shigella agar (SS) and Mannitol Salt agar (MSA) at 37°C for 24 hours. Biochemical characterization of isolates using Indole, Citrate, TSI, MR-VP, and MIU. Incubate at 37°C for 24, 48 and 72 hours. After that, spreading of pure bacterial culture colony onto Mueller Hinton agar at 37°C for 24 hours and placed on it antibiotic disc to determine the sensitivity and susceptibility of isolates against the antibiotic disc by disc-diffusion method.

*Antibiotic Susceptibility Test*

To determine the drug sensitivity and resistance patterns of isolated organisms with different types of antimicrobial discs, commercially available antimicrobial discs (Oxoid Ltd., UK) were used. The method allowed for the rapid determination of the efficacy of the drugs by measuring the diameter to the zone of inhibition that resulted from different diffusion of the agent into the medium surrounding the disc. The list of commercially available antimicrobial disc used in this study with their concentration are Vancomycin (VA) 30 µg, Ciprofloxacin (CIP) 5 µg, Ciprofloxacin (CIP) 15, Kanamycin (K) 30 µg, Neomycin (N) 30 µg, Gentamicin (GEN) 10 µg, Erythromycin (E) 5 µg, Erythromycin (E) 15 µg, Amoxicillin (AMX) 30 µg, Penicillin(P) 10 µg and Chloramphenicol (C) 10 µg. The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting (Cappuccino et al., 2005).

*Isolation and Identification of Fungus*

With the help of a sterile inoculating loop, the processed samples were inoculated into Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 5-7 days. The incubated media were then examined for the growth of fungi. Colony, greenish colonies were found SDA. Colonies from SDA were a subculture in potato dextrose agar (PDA) and incubated at 37°C for 5-7 days. Observed specific colonies from SDA and PDA were subculture in *Aspergillus flavus parasiticus* agar (AFPA) medium. A bright orange color on the reverse side of the plates of AFPA medium will indicate a positive result. (Thilagam et al., 2016). Micromorphological characteristics of the pure culture colonies like conidia were observed at 40X objectives under the microscope as a wet mount in Lactophenol cotton blue stain for identification by the conidiophores appearance and arrangement (Thilagam et al., 2016).

*Rapid Aflatoxin Determination by Abraxis Aflatoxin Rapid Test Strip 20 ppb*

This Rapid Aflatoxin Test is designed solely for use in preliminary screening of grain samples. This test is a qualitative one-step competitive inhibition immunoassay for the detection of aflatoxin. It detects the presence of aflatoxin at 5 ppb or higher in grain samples by utilizing highly specific reactions between antibodies and aflatoxin in grain samples (Delmulle et al., 2005; Xiulan et al., 2005; Stubblefield et al., 1991).

## Results

*Prevalence of Bacterial Pathogens in Grain Samples*

The total prevalence of *E. coli*, *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus* spp.in the grain samples was 16%, 28%, 16%, and 16% respectively. From the wheat samples E. coli, *Salmonella* spp. was isolated from one sample and their prevalence was 20% separately. But no *Klebsiella* spp. and *Staphylococcus* spp. was found. From khessari dal *E. coli* and *Staphylococcus* spp. were isolated and their prevalence were 40% and 20% respectively. In maize the prevalence of *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus* spp. were 40%, 20%,

and 20% respectively. No *Klebsiella* spp. was found from maize. From rice no *E. coli* and *Staphylococcus* spp. was isolated but *Salmonella* spp. .and *Klebsiella* spp. was found and their prevalence was 20% and 40% respectively. From anchor dal *E. coli*, *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus* spp. was isolated and their prevalence was 20%, 60%, 20%and 40% respectively (Table 1).

*Prevalence of Aflatoxigenic Aspergillus spp. in Grain Samples*

Among the 25 grain samples, *Aspergillus* spp. was isolated from 4 grain samples with 16% prevalence. But aflatoxigenic *Aspergillus* spp. was isolated from 3 samples with 12% prevalence. From the wheat samples and maize, the aflatoxigenic fungus was isolated and their prevalence in maize and wheat was 20% and 40% respectively (Table 1).

*Bacterial Species*

Four bacterial species like *E. coli*, *Salmonella* spp., *Staphylococcus* spp., *Klebsiella* spp. are isolated based on cultural, staining and biochemical technique. For *E. coli*, smooth metallic sheen color colonies were produced on EMB and bright pink color smooth colonies were produced on Mac-Conkey agar. Gram-negative, pink colored, small rod-shaped organisms arranged in single, pairs or short chain observed by gram staining technique. For *Salmonella* spp. samples inoculated onto Mac-Conkey agar plates produced colorless, smooth, transparent and raised colonies, on SS agar suspected isolated produced translucent, smooth, small round black centered colonies. The thin smears prepared with the colony from SS agar and MC agar for grams staining revealed gram-negative, pink colored, very small plump rod-shaped appearance, arranged in single, paired under the microscope examination. Isolated was found to be motile when examined using hanging drop slide under a microscope. For *Staphylococcus* spp., on MSA plates streaked separately with the organism from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and were indicated by the growth of circular, small, yellowish colonies (Plate 6). Blood agar plates streaked separately with the organism and incubated at 37ºC aerobically for 24 hours and β-type of hemolysis were produced. Gram-positive Cocci arranged in grape-like cluster observed under a microscope by gram staining technique. Lastly for *Klebsiella* spp. Mac-Conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and were indicated by the growth of bright-pink colored smooth mucous colonies. Then EMB agar plates streaked separately with the organisms from Mac-Conkey (MC) agar revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically and were indicated by the growth of smooth mucoid pink color colonies. The microscopic examination of grams stained smears from MC and EMB agar revealed gram-negative, pink colored, small rod-shaped organisms arranged in single, pairs or short chain. Biochemical properties of isolated species observed and result shown in Table 2.

*Result of Antibiotic Sensitivity Pattern of Isolated Bacteria*

The isolated bacterial pathogens were tested for the antibiotic sensitivity and resistance pattern against commonly used antibiotic. The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistance, intermediate, sensitive. The results of antibiotic sensitivity are given in percentage shown in Table 3.

*Identification of Fungus*

After inoculation on SDA according to methodology, greenish colonies were found on SDA (Figure 1). On PDA the colonies were yellow-green with white to cream mycelia and yellow-green edges and also in some plates greenish colony were found (Figure 2). Characteristics conidia were found under a microscope by lactophenol cotton blue stain (LPCB) method (Figure 3). In the present study, AFPA was used to detect the aflatoxin-producing ability of the fungal isolates. A bright orange color on the reverse side of the plates of AFPA indicated a positive result (Figure 4).

*Screening of Aflatoxin by Abraxis Aflatoxin Rapid Test Strip 20ppb*

Aflatoxigenic *Aspergillus* spp. was isolated from 3 samples with 20 ppb aflatoxin with no test line. (Figure 5).

Table 1 Prevalence of bacteria, fungus and aflatoxigenic *Aspergillus* spp. in grain samples

| Samples | E | % | S | % | K | % | St | % | A | % | AA | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wheat (5) | 1 | 20 | 1 | 20 | | | | | 2 | 40 | 2 | 40 |
| Khesari dhal (5) | 2 | 40 | | | | | 1 | 20 | 1 | 20 | | |
| Maize (5) | | | 2 | 40 | 1 | 20 | 1 | 20 | 1 | 20 | 1 | 20 |
| Rice (5) | | | 1 | 20 | 2 | 40 | | | | | | |
| Anchor dhal (5) | 1 | 20 | 3 | 60 | 1 | 20 | 2 | 40 | | | | |
| Total (25) | 4 | 16 | 7 | 28 | 4 | 16 | 4 | 16 | 4 | 16 | 3 | 12 |

(Legends: S= No. of isolated Salmonella spp., E= No. of isolated E. coli., K= No. of isolated Klebsiella spp., St= No. of isolated Staphylococcus spp., A= No. of isolated Aspergillus spp. (fungus), AA= No. of isolated Aflatoxigenic Aspergilus spp.)

Table 2 Biochemical properties of isolated species

| Organisms | CUT | SC | Catalase | IT | TSI | MR | VP | MIU |
|---|---|---|---|---|---|---|---|---|
| *E. coli* | — | N/A | N/A | + | S-A, B-A, Gas +, $H_2S$ — | + | — | + |
| *Salmonella* spp. | — | N/A | N/A | — | S-Al, B-A, Gas +, $H_2S$ — | + | — | — |
| *Staphylococcus* spp. | N/A | + | + | + | S-Al, B-A, Gas —, $H_2S$ — | + | + | + |
| *Klebsiella* spp. | + | + | + | — | S-A, B-A, Gas +, $H_2S$ — | — | + | + |

(Legends: S=Slant, B=Butt,A= Acid, Al- Alkaline, CUT = Citrate utilization test, IT = Indole test, TSI = Triple sugar iron test, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility indole urease, SC= Simon citrate,+ = Positive reaction, — = Negative reaction, N/A= Not done).

Table 3 Antibiotic sensitivity pattern of isolated bacteria

| Isolates | Diameter of zone of inhibition(%) | Antibacterial agents and Disc concentration (µg/disk) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GEN (10) | AMX (30) | C (10) | CIP (5) | CIP (10) | E (5) | E (15) | P (10) | N (30) | VA (30) | K (30) |
| *E.coli* | Sensitive | 66.7 | | | | 100 | 0 | | 0 | | 0 | |
| | Resistant | 33.3 | | | | 0 | 100 | | 100 | | 100 | |
| *Salmonella* spp. | Sensitive | 40 | | | 100 | | | 0 | 0 | 40 | | |
| | Resistant | 60 | | | 0 | | | 100 | 100 | 60 | | |
| *Staphylococcus* spp. | Sensitive | 66.7 | 0 | | 100 | | | | 0 | | 0 | |
| | Resistant | 33.3 | 100 | | 0 | | | | 100 | | 100 | |
| *Klebsiella* spp. | Sensitive | 50 | | 50 | 100 | | | | | 50 | | 0 |
| | Resistant | 50 | | 50 | 0 | | | | | 50 | | 100 |

(Legends: GEN = Gentamicin, AMX = Amoxicillin, C = Chloramphenicol, CIP = Ciprofloxacin, E = Erythromycin, P = Penicillin G, N = Neomycin, VA = Vancomycin, K= Kanamycine,0 = No zone of inhibition, N/A= Not done)

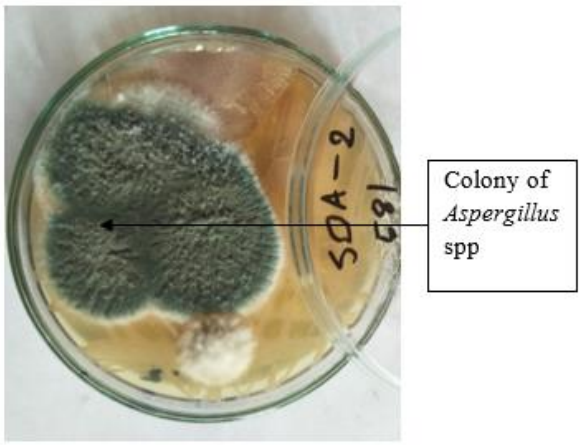


Figure 1 *Aspergillus* spp. on SDA agar

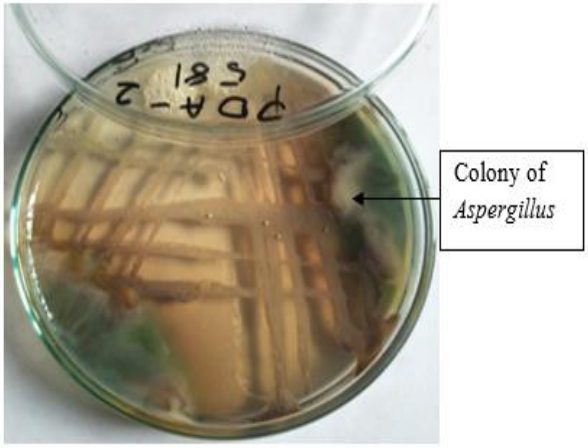


Figure 2 *Aspergillus* spp. on PDA agar

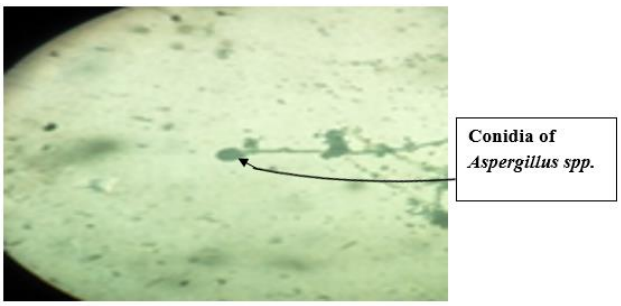


Figure 3 Microscopic view (10 X 40 object) of conidia of *Aspergillus* spp.by lactophenol cotton blue stain

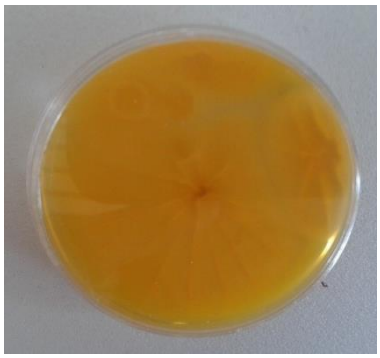

Figure 4 *Aspergillus* spp. on AFPA medium

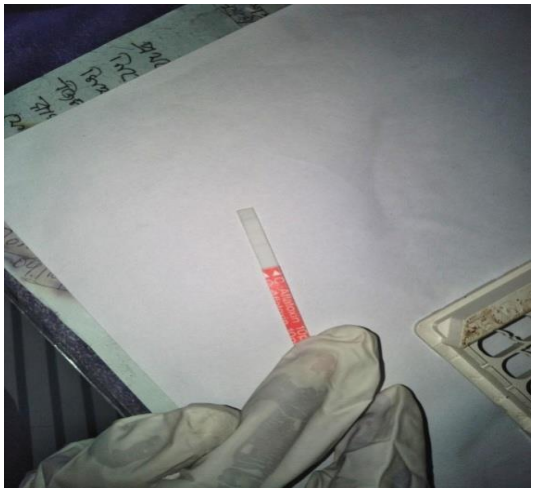

Figure 5 Aflatoxine test strip

**Discussion**

In this study, the 15 bacterial isolates comprising 4 genera of bacteria were found from a total of 25 samples. The Isolated bacteria were *Staphylococcus* spp., *Escherichia coli, Klebsiella* spp. *and Salmonella* spp. Among the 25 grain samples, 4 *Staphylococcus* spp., 7 *Salmonella* spp.,4 *Escherichia coli* and 4 *Klebsiella* spp. were isolated with 16%, 28%, 16%, and 16% prevalence respectively. The similar study was made by Haque et al., (2017) and Munaomar et al. (2018) in which they reported the *Escherichia coli* and *Staphylococcus* spp. from the positive grain samples with a prevalence of 100%, 50%, and 80% respectively. So above report was less similar to the findings of this study. Among the 25 grain samples, *Aspergillus* spp. was isolated from 4-grain samples with 16% prevalence. But aflatoxigenic *Aspergillus* spp. was

isolated from 3 samples with 12% prevalence. From the wheat samples and maize, the aflatoxigenic fungus was isolated and their prevalence in maize and wheat was 20% and 40% respectively which was more or less similar to the study of Bakr (1992).

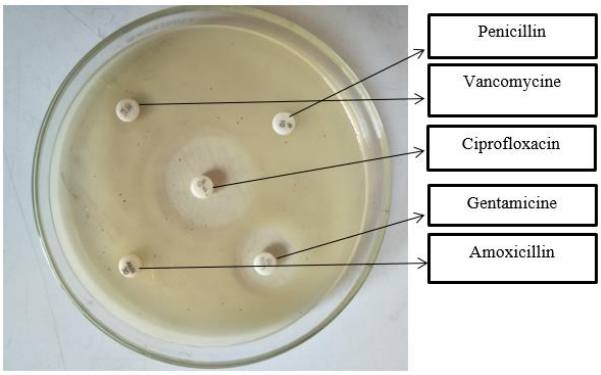


Figure 6 Antibiogram of *Staphylococcus* spp

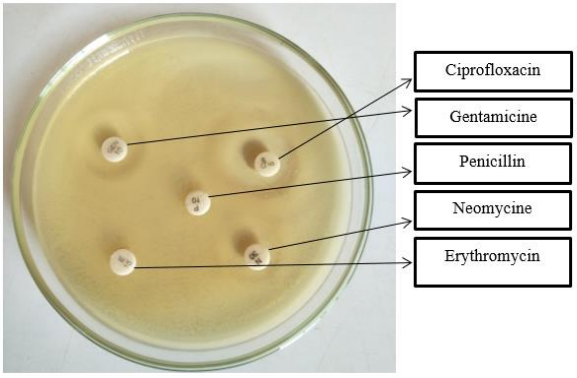


Figure 7 Antibiogram of *Salmonella* spp

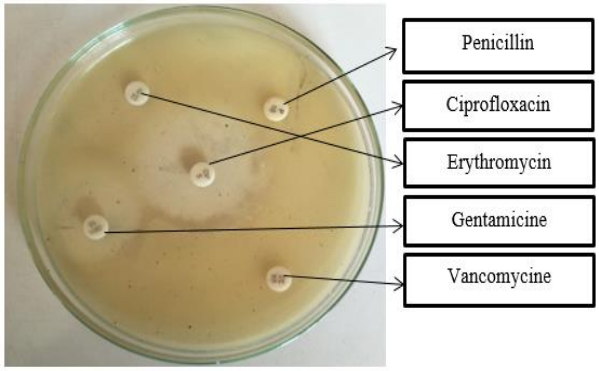


Figure 8 Antibiogram of *E. coli.*

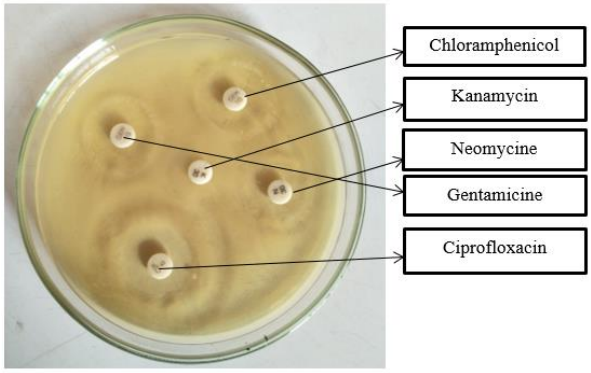


Figure 9 Antibiogram of *Klebsiella* spp.

Many infectious agents might be present in grains but *Staphylococcus* spp., *E. coli, Klebsiella* spp., *Salmonella* spp. were isolated from grain samples in this study. The frequency distribution of different bacterial isolates in different grain samples was found variable. The result of the present study indicates that all the four different genera of bacteria were not present in the same grain sample collected from the different market. From the wheat samples, *E. coli* and *Salmonella* spp. were isolated from one sample and their prevalence was 20% separately. But, no *Klebsiella* spp. and *Staphylococcus* spp. was found. From khessari dal, *E. coli* and *Staphylococcus* spp. were isolated and their prevalence were 40% and 20% respectively. In maize, the prevalence of *Salmonella* spp., *Klebsiella* spp. and *Staphylococcus* spp. was 40%, 20%, and 20% respectively. No *Klebsiella* spp. was found from maize. From rice no *E. coli* and *Staphylococcus* spp. was isolated but *Salmonella* spp. and *Klebsiella* spp. was found and their prevalence was 20% and 40% respectively. From anchor dal *E. coli, Salmonella* spp., *Klebsiella* spp. and *Staphylococcus* spp. was isolated and their prevalence was 20%, 60%, 20% and 40% respectively. The isolated *Staphylococcus* spp., *E. coli, Klebsiella* spp., *Salmonella* spp., showed an identical result in different biochemical tests of including catalase test, indole test, methyl-red, Voges-Proskauer test, motility indole urease test, triple sugar iron test, and citrate utilization test.

The in vitro antibiotic sensitivity test of isolated bacteria was done with 8 different antibiotics, such as Gentamycin, Ciprofloxacin, Kanamycin, Neomycin, Erythromycin, Vancomycin, and Amoxicillin, Chloramphenicol. The antibiotic study (Figure 6) revealed that isolated *Staphylococcus* spp. were sensitive to Ciprofloxacin (100%), Followed by Gentamycin (66.7%) and the isolates were found resistant to penicillin (100%), Amoxicillin (100%) and Vancomycin (100%) These findings were more or less similar to the findings of Haque et al. (2017). The antibiotic study (Figure 7) revealed that isolated *Salmonella* spp. were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to Penicillin (100%), Erythromycin (100%), followed by Gentamycin (60%), Neomycin (60%). These findings were more or less similar to the findings of Haque et al. (2017). The antibiotic study (Figure 8) revealed that isolated *E. coli* were sensitive of Ciprofloxacin (100%) followed by Gentamycin (66.7%), the isolates were found resistant to Penicillin (100%), Vancomycin (100%) and Erythromycin (100%). These findings were more or less similar to the findings of Haque et al. (2017). The antibiotic study (Figure 9) revealed isolated *Klebsiella* spp. were sensitive of Ciprofloxacin (100%) and the isolates were found resistant to Kanamycin (100%) followed by Neomycin (50%), Gentamycin (50%) and Chloramphenicol (50%). These findings were more or less similar to the findings of Haque et al. (2017) and Munaomar et al. (2018).

Overall effective drugs against isolated bacteria were Ciprofloxacin followed by Gentamicin. But resistant drugs were Penicillin, Vancomycin, Erythromycin, Kanamycin, and Amoxicillin. The variation in the sensitivity of common antibiotic could the result of extensive and indiscriminate use of these antibiotics.

So, for effective treatment of infection caused by consumption of such grain food, the medicinal formulation

should preferably contain antibiotics that have a good spectrum of inhibition against all species of these bacteria. In this context, it is interesting to note Ciprofloxacin and Gentamycin should be the antibiotic of choice and these antibiotics appear to be promising for the treatment of the infection caused by grain food in Bangladesh.

Grain samples were also found to be contaminated with *Aspergillus* spp. Not only *Aspergillus* spp. but also aflatoxigenic *Aspergillus* spp. For aflatoxin the action levels of 20 parts per billion (ppb) for grain and feed products, and 0.5 ppb for milk established by The Food and Drug Administration (FDA). Mixing aflatoxin contaminated grains with sound grains for sale is illegal. In the U.S. corn and other grain with less than 20 ppb aflatoxin can be sold as normal grain.

But according to this study in our maize samples and in two of our wheat samples, the aflatoxins are found to be as 20 ppb. So it was a great concern for human health because aflatoxin is a potent liver toxin known to cause cancer in animals like reproductive disturbances caused by the T-2 toxin in sows (Placinta et al., 1999) also in human consumer (Richard, 2000)**.** The study showed that earlier detections can be made by simple traditional identifications using macro and micromorphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

## Conclusion

The recent study was conducted for detection of potential bacterial pathogens and aflatoxigenic fungi from grain samples. Properties and antibiotic sensitivity pattern of the bacterial and fungal isolated from infected grain food. Their early detection can help to take preventive measures to combat economic and health losses. Antibiogram result indicated the Ciprofloxacin and Gentamycin in optimum doses would be the drug of choice to treat the most cases of infection caused by consumption of contaminated grain foods. Antibiotic sensitivity test revealed that Kanamycin, Vancomycin, Neomycin, and Erythromycin would not be recommended because isolated bacteria were resistant to these drugs.

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