RESEARCH ARTICLE

Biomedicus: J. Biol. Biomed. Front., 1: 31-38, 2026

BIOMEDICUS
J. Biological &
Biomedical Frontiers

TSSBR

Prevalence of MDR and XDR in Clinically Relevant Enterobacteriaceae from Local Food Samples

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ABSTRACT

In this study, Enterobacteriaceae were isolated from food samples, either in raw or cooked form, including some bakery items, meat, fruits, vegetables, and sweets. This study aimed to obtain multidrug (MDR) and extremely drug-resistant (XDR) bacteria that have a high potential for biofilm formation and confer appreciable resistance to heavy metals, as foodborne microbes are becoming a menace even in the modern medicine era. In the current study, different raw and cooked food samples were evaluated for the prevalence of Enterobacteriaceae. A total of 40 food samples were used, and 33 bacterial isolates were assessed for their antimicrobial resistance potential, from which 7 isolates were later selected to determine their metal resistance and biofilm production profile. Out of 7 selected bacteria, 4 were XDR and 3 were categorized as MDR. All bacterial isolates were resistant to colistin, amikacin, gentamicin, cefexime, tetracycline, and sulphonamides. Among carbapenems, 50% of the isolates were resistant to meropenem, and 16% of the isolates were resistant to imipenem. The potential XDR bacteria were isolated from meat and milk, and fish and capsicum were also found as the main reservoirs of MDR bacteria. Ceftazidime-avibactam was found as a remarkable antibiotic, as all of the bacteria were sensitive to it. Nearly 80% of the isolates were sensitive to imipenem and levofloxacin. It was seen that those isolates that had high resistance against antibiotics had high minimum inhibitory concentration (MIC). Similarly, biofilm formation capacity also contributes greatly to developing mechanisms to resist antimicrobial drug penetration through the cell membrane. So, the majority of bacteria were biofilm producers and XDRs. Combined with it, the heavy metal resistance and biofilm-producing potential make the situation more challenging. There is a dire need to combat MDR and XDR pathogens to make the treatment of infectious diseases a complete reality and to call modern medicine an unchallengeable weapon.

KEY WORDS: Foodborne enterobacteriaceae, Multi-drug resistance, Extreme-drug resistance, Antimicrobial drugs, Metal sensitivity, Biofilm

INTRODUCTION

Foods originating from animals, such as milk, beef, and chicken flesh, are rich in proteins, which are vital for the growth and development of the body. Animal-based foods, however, have the potential to act as a carrier and medium for a variety of germs that can lead to illness, sickness, and even death. Foodborne infections are starting to pose a threat to public health worldwide[1]. Drug resistance in bacteria has escalated to risk-taking levels and poses a major problem in therapies for different diseases. The repercussive impacts of this obstacle are broadened life losses as well as putting a price on health maintenance[2]. Drug resistance becomes a menace when it comes to the treatment of chronic diseases. If any drug shows inefficacy, a constraint is put on treatment, as every patient may show different pharmacokinetics. Costly drugs and higher doses don't necessarily lead to a better response. Higher doses given to have early recovery pave the road to side effects as well as drug resistance[3].

Multidrug-resistant (MDR) bacteria are those that have insusceptibility to more than one drug, or if bacteria are not susceptible to at least one sub-category of two or

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Received 29 May 2025; Revised 05 August 2025; Accepted 17 August 2025; Published 30 April 2026

three classes of antibiotics. Extremely drug-resistant (XDR) bacteria are defined as those bacteria that show resistance to one sub-category in all antibiotic classes, or bacteria that are only sensitive to one or two classes. Pandrug-resistant bacteria are those that show no sensitivity to any sub-category in all classes of antibiotics[4]. Enterobacteriaceae are Gram-negative pathogens residing in the gut flora and intestines of humans and other animals. They can also reside in the soil or water. Common examples include *Escherichia coli, Salmonella, Shigella, Yersinia, Proteus, Klebsiella, Citrobacter, Providencia*, etc.[5]. Dairy products and meat have always been a center of concern for people around the globe. These sources are thought to be contaminated with Enterobacteriaceae. Indeed, they are used as a marker for evaluating the food quality. Therefore, the assessment of food quality is fundamental to prevent risks associated with food consumption[6].

To diagnose infections associated with *Enterobacter,* the involvement of the cultures is mandatory. To evaluate blood samples, two sets are used: One for the aerobic bottle and another for the anaerobic bottle. Urinalysis can also be performed. MacConkey agar is used for the indication of fermentation, and the indole test is crucial to differentiate between different species[7]. However, *Enterobacter* can be recovered from sputum samples for respiratory diseases, surgical wounds, and blood[8]. One of the weapons used by Enterobacteriaceae for its pathogenicity is its drug resistance. Beta-lactamase is a major agent for evaluating it. It can hydrolyze the beta-lactam ring in different drugs-approaching drug resistance. Talking about Enterobacteriaceae, *E. coli* is a key member. Usually, it is present as a commensal in the digestive tract, but due to certain virulence genes and antibiotic resistance genes, it can cause many infections, including digestive tract diseases, urinary system dysregulation, as well as inflammatory reactions in the brain, etc[9].

Among the Enterobacteriaceae family, the genus *Enterobacter* is highly associated with infections. Not all *Enterobacter* species cause infections; some of them lead to infections in the hospital environment; some are associated with community-borne infections, including airway infections, urinary and cystitis, cellulitis, heart and long bones problems, catheter-associated septicity, nosocomial sinusitis, etc. One of the weapons used by Enterobacteriaceae for its pathogenicity is its drug resistance. β-lactamase is a major agent for evaluating it. It can hydrolyze the beta-lactam ring in different drugs-approaching 'drug resistance[10].

Dairy products and meat have always been a center of concern for people around the globe. These sources are thought to be contaminated with Enterobacteriaceae. Indeed, they are used as a marker for evaluating the food quality. Therefore, the assessment of food quality is fundamental to prevent risks associated with food consumption[6]. The reason behind their efficiency to assess food quality with greater scope as compared to coliforms is because of one of the remarkable features i.e., resistance to the environment. So, Enterobacteriaceae have been used as a crucial element to gauge the sanitation of food and dairy products.

Biofilm aids bacteria in shielding bacteria from chemicals, protects bacteria from stressful conditions during food processing, and protects them from disinfectants[11]. It was seen that patients having bacteremia, septicemia, ulcers, and diabetes are more prone to MDR and XDRs, and these strains are also exhibiting biofilms. Also, members of Enterobactericaea family have membrane vesicles (MVs)[12]. These vesicles are coated with lipids, and they help the members of the biofilm to interact with each other for biofilm production. These vesicles have proved to be the agents for virulence and toxicity and perform their job in causing host infections[13]. To combat biofilm-producing capability, it is very crucial to check for its potential to show antibiotic resistance. Those bacteria that have the attribute of biofilm production are lost prone to the stresses imposed by the environment or even by the drugs. Biofilm producers are 10^3 times less prone to drug therapy as compared to non-producers[14]. Biofilms assist in drug resistance by acting as a permeability barrier to drugs, degrading enzymatic proteins, and drug-excluding capabilities[15].

Novel antibiotic discovery efforts are in progress. For some bacteria, there were very less treatment options i.e., *Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. Antimicrobial resistance poses a considerable challenge to even modern medicine. Pressurized by the regulatory bodies, scientists are having more work to get some new antibiotics to treat havoc-creating pathogens, including MDR and XDR bacteria[16].

Keeping in view the clinical implications of difficult-to-treat pathogens, it is foremost to develop antibiotics for such bacteria. Carbapenem resistance may occur due to membrane porins and efflux pumps. Aminoglycoside resistance may occur due to transposon elements harbored by bacteria. Fosfomycin exhibits the potential to inhibit cell wall synthesis, but it is not used for MDR infections. Ceftazidime-avibactam is used to treat abdominal infections, UTIs, and hospital-acquired infections that were very difficult to treat previously[17]. Avibactam prevents ceftazidime from degradation and ensures its longevity. Meropenem alone can confer no antibacterial activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* but when combined with a non-β-lactam-β-lactamase inhibitor can assure a good activity. Although vaborbactum is not antibacterial but it can restore the role of meropenem by protecting it from degradation[18].

MATERIALS AND METHODS

Sampling and isolation of bacteria

For the isolation of bacteria, different food samples were used, either from local vendors, private label brands, or confectioneries (see Table I).

Isolation of Enterobacteriaceae

To specifically isolate Gram-negative bacteria, MacConkey agar (Peptone 17g, proteose peptone 3g, lactose monohydrate 10g, bile salts 1.5g, sodium chloride 5g, neutral red 0.03g, crystal violet 0.001g, agar 13.5g, 1000 mL distilled water) was used[19]. Food samples were first diluted in normal saline and then spread on poured MacConkey agar plates. MacConkey agar was autoclaved, and agar plates were prepared. Then, 50 µL of the prepared food sample was dispensed in the center of prepared plates with the help of a micropipette, and the food preparations were spread with the help of a sterile glass spread sunken in ethanol. Even distribution of the suspension was ensured throughout the surface of the poured agar plates. The plates were placed on the bench top for 10 min to ensure dryness and proper absorbance[20].

After the absorption of the suspension into the agar plates, the plates were inverted and placed in an incubator at 37°C for 24 h. After 24 h of incubation, plates were analyzed for growth. Colonies were counted semi-quantitatively, and from each plate, two well-isolated and different colonies were streaked on MacConkey agar plates and incubated for 18-24 h at 37°C.

Preparation of minimal salt medium

Minimal salt medium (MSM) (Glucose 0.25g, $MgSO_4$ 0.01g, $CaCl_2$ 0.013g, KH_2PO_4 1.25g, Na_2HPO_4 0.175g, $(NH_4)_2SO_4$ 0.25g, $FeSO_4$ 0.335g, and agar 3.75g were added up to 500 mL distilled water) was used for the growth of microorganisms in the absence of carbon source to evaluate growth upon stress conditions in the presence of heavy metals[21]. However, glucose was added to check if the organism relies upon salts after the complete utilization of glucose.

Kirby-Bauer method to evaluate antimicrobial potential of bacteria

Susceptibility tests were used to evaluate the antimicrobial potential of bacteria in the laboratory environment. Disc-diffusion (Kirby-Bauer technique) was used to determine the sensitivity or resistance of bacteria to antibiotic drugs[22].

Mueller Hinton (MH) agar (Beef infusion solids 2g, starch 1.5g, casein hydrolysate 17g, agar 17.5g, 1000 mL of distilled water, Final pH 7.3 +/- 0.2 at 25°C) was prepared and autoclaved under standardized conditions and poured into plates. The plates were allowed to solidify. Bacterial cultures grown in L-broth and swabbed on MH-agar plates after adjustment with a 0.5 McFarland standard[23]. Then, the following antibiotics were applied. Ciprofloxacin, gentamicin, cefepime, meropenem, tazobactam, tetracycline, sulphonamide, colistin, amikacin, levofloxacin, imipenem, aztreonam, ceftazidime-avibactum. Plates were incubated at 37°C for 24 h. After which zones of inhibition were measured with the ruler to measure the diameter around the disc. The size of zones of inhibition was measured in millimeters and compared with the Clinical and Laboratory Standards Institute (CLSI). Bacteria were categorized as resistant, intermediate, or sensitive with CLSI guidelines. MDR and XDR bacteria were identified.

Biochemical characterization

For biochemical characterization, catalase, oxidase, triple sugar iron, urease, indole, methyl red/Voges-Proskauer, and citrate tests were performed[24]. Gram staining was done to isolate Gram-negative rods.

Molecular identification

The phenol-chloroform-alcohol method was used for DNA isolation[25], which was then used for amplification of 16S rRNA using the following primers

27F= 5'AGAGTTTGATCCTGGCTCAG3' and

1492R= 5'GGTTACCTTGTTACGACTT3'.

The PCR reaction mixture (50 µL) comprised 2.5 mM $MgCl^2$, 1 µL of 10X Taq buffer with KCl (100 mM Tris-HCl pH 8.8, and 500 mM KCl), 200 mM deoxyribonucleotide triphosphate (dNTPs), 50 pmoles of each forward and reverse primers, 1 unit of Taq DNA polymerase and 15 µL of DNA (20 ng/µL DNA conc) while the thermal cycle consisted of initial denaturation at 95°C for 2 min, followed by 40 cycles each of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72 °C for 1 min. The final extension was done at 72°C for 10 min. The PCR product was visualized on 1% agarose gel and then purified using the Mo-Bio Laboratories purification kit 16S rRNA PCR product. The purified PCR product was sent for sequencing to Macrogen, Korea, and the sequence obtained was then aligned by utilizing BLAST analysis. Moreover, a dendrogram was created by using the MEGA7 program on the basis of homology.

Metal resistance activity

To check metal resistance or minimum inhibitory concentration (MIC), MSM was used. Minimal salt media were prepared and autoclaved as per standard conditions. Five metals i.e., cadmium ($CdCl_2$), arsenic ($NaAsO_2$), lead ($PbNO_3$), chromium ($K_2Cr_2O_7$), and zinc ($ZnSO_4.7H_2O$), were used. Firstly, a stock of 10 mM of each metal was made, and then working concentrations of 0.5 mM, 1 mM, 3 mM,5 mM, and 7 mM were prepared. For each strain (MT st1, MT st2, MK, SM st2, GP, CO st1, and SP; isolated from food samples), five plates using MSM were prepared. After pouring, the media was allowed to solidify. Then, quadrant streaking was done on MSM agar plates. Plates were incubated at 37°C for 24 h and growth was noted.

Biofilm production test

Congo-red media was prepared (Glucose 60g, Brain

Heart Infusion agar 11.1g, Congo red 0.24g, agar 6g, and added to 300 mL of distilled water)[26] and autoclaved, poured into autoclaved Petri plates, and inoculated with bacterial strains by the quadrant streaking method. The plates were incubated at 37°C for 24 h. Results were visualized as crystal-textured black colonies, which serve as a qualitative indicator of biofilm production[27].

RESULTS AND DISCUSSION

Table I gives an overview of bacterial prevalence in raw and cooked foods. It was found that raw chicken, raw milk, carrot, green chilli, spinach, mutton, fish, and grapes had enormous bacterial communities from the raw food group, whereas corn, sugarcane, brinjal, banana, and potato showed negligible, and ketchup, egg shell, black pepper, pomegranate, peas, and onion showed no bacterial growth. Among the cooked foods, tremendous bacteria were obtained from Kabab and chestnut, whereas other samples like boiled chicken, fried eggs, pizza, snacks, and sauces showed no growth.

From a total of 40 food samples, almost 69-70 strains were isolated. Based on the antimicrobial resistance (MDR and XDR profile), 11 strains were selected on which further antimicrobial resistance was gauged using five more antibiotics i.e., colistin, amikacin, levofloxacin, Imipenem, and aztreonam. Out of these 11 strains, 1 strain was selected for 16S rRNA sequencing, and it was designated as extremely drug-resistant bacteria (XDR), having accession number PP978759.

Bacterial identification

Various biochemical tests were performed to identify the bacteria. The partial 16S rRNA gene was amplified with general primers, and bands appeared in parallel with a 1500bp ladder sequence. The phylogenetic tree was also constructed based on 16S rRNA sequencing (Fig. 1). It was found that the query sequence was of *Escherichia coli*, having the accession number PP978759.

Antibiotic resistance profile

It was found that the subject strains were highly resistant to ciprofloxacin and less resistant to levofloxacin, which both belong to the fluoroquinolone. There was a high resistance shown against gentamicin and amikacin by all strains, which both belong to the aminoglycosides. Among carbapenems, 50% of strains were resistant to meropenem and 16% of strains were resistant to imipenem. Among cephalosporins, 100% of strains were resistant to cefexime. For tetracyclines, 100% of strains were resistant to tetracycline. 16% of strains were resistant to aztreonam, and 33% of strains were resistant to ceftazidime-avibactam. Similarly, almost 70% of strains were resistant to sulphonamides. For bacterial infections of the urinary system,

Table I. Semi-quantitative assessment of bacterial prevalence in raw and cooked foods.

Raw foods	Growth
Raw chicken	++
Raw milk	+++
Banana	+
Ketchup	No growth
Egg shell	No growth
Black pepper	No growth
Rice	No growth
Carrot	+++
Potato	+
Spinach	+++
Mutton	+++ (*E. coli* isolated)
Phallian	+++
Fish	+++
Grapes	++
Grapefruit	+
Pomegranate	No growth
Peas	No growth
Onion	No growth
Brinjal	+
Green chilli	+++
Beef	++
Sugarcane	+
Fenugreek	++
Corn	+
Apple	No growth
Capsicum	+
Coconut	+++
Paan	+++
Cooked Food	Growth
Fried egg	-
Pizza	-
Sweets	-
Salan	-
Boiled chicken	-
Samosa	-
Macaroni	-
Water chestnut	+++
Chapati	-
Naan	-
Kebab	++
Snacks	-
Fruit chat	-
Sauces	-
Biscuit	-

+++, Enormous growth; ++, Good growth; +, Less growth.

sulphonamides and fluoroquinolones can be used, but in this research, 100% strains were resistant to ciprofloxacin. The antimicrobial resistance profile of *E. coli,* a potential isolate from the mutton sample, elucidated as being resistant to ciprofloxacin, meropenem, tazobactam, sulphonamide, tetracycline, colistin, levofloxacin, imipenem, and aztreonam, and was sensitive to gentamicin, cefexime, and amikacin. It was categorized as XDR as it was resistant to one sub-category in all antimicrobial classes.

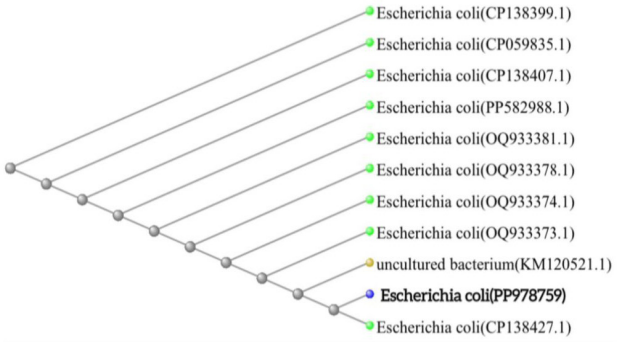

Fig. 1. Sequencing-based phylogeny obtained through 16S rRNA sequencing.

It was found that the subject strains were highly resistant to ciprofloxacin and least resistant to levofloxacin, which both belong to the fluoroquinolone. A high resistance pattern was shown by all strains against gentamicin and amikacin, which belong to the aminoglycosides. Among carbapenems, 50% of strains were resistant to meropenem and 16% of strains were resistant to imipenem. Among cephalosporins, 100% of strains were resistant to cefexime. For tetracyclines, 100% of strains were resistant to tetracycline. The aztreonam was resistant to 16% of the strains, and 33% of the strains were resistant to ceftazidime-avibactum. Similarly, almost 70% of strains were resistant to sulphonamides (Fig. 2).

Among the different food samples used, pathogens from mutton were thought to be highly resistant to almost all antibiotics, hence being XDR. Similarly, the pathogen extracted from milk was also found to be heavily resistant to ciprofloxacin, gentamicin, cefexime, meropenem, tazobactum, sulphonamides, amikacin, and aztreonam and sensitive to levofloxacin, imipenem, and ceftazidime-avibactum. The pathogen isolated from grapes was found to be resistant to all antibiotics mentioned but sensitive to meropenem, levofloxacin, imipenem, and ceftazidime-avibactam. Similarly, the pathogen isolated from fish was resistant to aminoglycosides, cephalosporins but sensitive to carbapenem and aztreonam. The pathogen isolated from capsicum was only sensitive to imipenem, hence termed as XDR.

Among the studied strains, 66% strains were XDR and 33% of the strains were MDR. Among the studied strains, 66% strains were XDR and 33% of the strains were MDR. The figure describes well the healthcare burden imposed by the heavy isolation of Enterobacteriaceae from different departments of the hospital, from the study done in 2016. 79% of *E. coli* strains were MDR, and 22% of them were XDR. Similarly, out of a total of 212 strains, 66% of *Pseudomonas aeruginosa* were MDR and 29% of them were XDR[4]. In the current study, XDRs were only found in mutton, milk, and capsicum samples, while bacteria in fish and grape samples were MDR (Fig. 3).

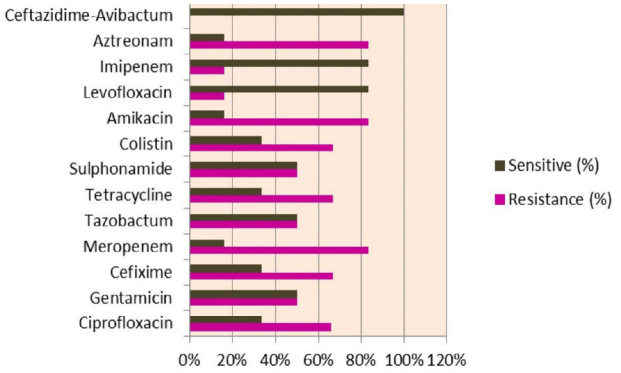

Fig. 2. Bacterial sensitivity and resistance (%) against the antibiotics used.

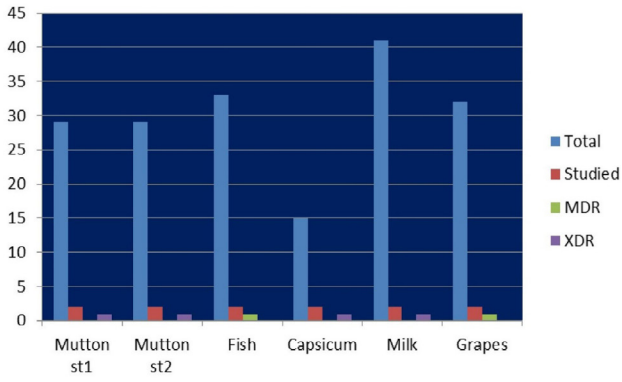

Fig. 3. Incidence of MDR and XDR in different food samples containing *E. coli*.

Widely distributed MDR bacteria threaten animal and human health. According to the study done[28] in China, 1,468 (97.54%) *Escherichia coli* strains were isolated from 1,505 pig (1,060) and chicken (445) anal swab samples. These isolates had a high resistance to tetracycline (92.92%), sulfisoxazole (93.05%), florfenicol (83.11%), and ampicillin

(78.27%). More than 88.68% of the strains were MDR bacteria. A low AMR ratio to the last-resort antimicrobials tigecycline (0.75%), colistin (1.36%), and meropenem (0.75%) was found. The AMR of *E. coli* from pigs was higher than that of chickens. For biofilm production, 83% of the isolates had the potential for biofilm formation. According to the study done in 2022 in Egypt of different food samples from different markets, it was found that out of a total of 30 strains, 25 strains of Enterobacteriaceae were having the ability to form the strongest biofilm, and 5 strains were late biofilm-formers[29]. However, in this current research, it was seen that *E. coli* was found in 90% of raw food samples, and the most frequent one was mutton.

Metal resistance profile

For metal resistance activity, out of 7 isolates, one strain was sensitive to all metals at all concentrations. The rest of the isolates were resistant to all metals, at specific MIC. Isolates MK, MT st1, GP, and SP resisted all concentrations of cadmium. MK, GP, SM, and SP resisted all concentrations of arsenic. MT st1, MK, and GP resisted all concentrations of Zinc. The SM and SP were moderately resistant to arsenic, lead, chromium, and zinc. MT st1 was also weakly resistant to arsenic and lead but strongly resistant to zinc and sensitive to chromium. *E. coli* was resistant to cadmium and zinc at all working concentrations. MIC for arsenic and lead was 3 mM, whereas it was entirely sensitive to chromium.

According to the study done in Brazil in 2022, MDR Enterobacteriaceae were highly resistant to zinc, copper, chromium, and mercury, with varying minimum inhibitory concentrations. It was found that for MDR, the MIC was higher for toxic metals as compared to those bacteria that didn't confer any resistance to antibiotics. For the latter group, MIC was lower[30]. According to the study done on chicken meat in Dhaka in Bangladesh in 2022, all strains (n=12) formed biofilms and were very highly resistant to ciprofloxacin, levofloxacin, ampicillin, meropenem, and colistin. These bacteria were highly resistant to zinc, copper, and nickel. It was found that the resistance to copper and zinc was interrelated with antibiotic resistance[31].

MT ST1 was later sequenced and identified as *E. coli*, one of the potent pathogens isolated from the mutton sample.

Biofilm formation

For biofilm production, 83% of the strains had the potential for biofilm formation. Samples isolated from mutton, milk, and grapes were biofilm producers, whereas the bacterial isolate from capsicum was not a biofilm producer. Interestingly, isolates from mutton and milk were XDR, emphasizing the combating potential to resist antibiotics, but isolates from grapes were MDR. Although an isolated bacterium from capsicum was not a biofilm producer, it was XDR, ruling out that there is not always a combined potential (Fig. 4).

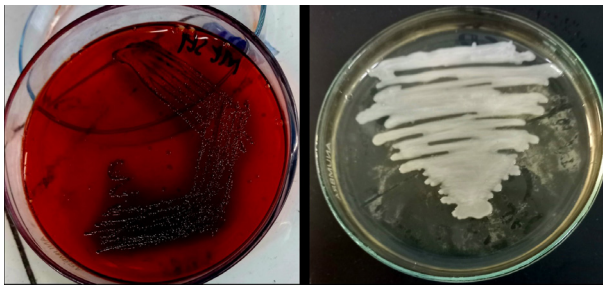

Fig. 4. Biofilm production by a bacterium (Left); Negative control (Right).

CONCLUSION

The study concludes that MDR and XDR isolates were prevalent in different food samples in a certain ratio. Also, there is an association between antibiotic resistance and metal resistance, but it is not a universal law. It was found that among many strains isolated, *Escherichia coli* was extremely drug resistant, but it was sensitive to 3 out of 5 metals used. When it comes to the prevalence of XDR isolates alone, 50% (2 out of 4) of XDR isolates were obtained from mutton; 50% (2 out of 4) of XDR isolates were collected from milk and capsicum. Talking about MDR prevalence alone, 50% of MDR isolates were obtained from fish, and 50% of MDR isolates were acquired from grapes. Collectively, 66% of isolates were XDR, with mutton being the source of XDR on the edge, whereas 33% of isolates were MDR. All isolates had the likelihood of producing biofilms except capsicum, although it was an XDR isolate. To wrap things out, this study concludes that there is a strong association between antimicrobial resistance, metal resistance, and biofilm production, but it is not true in all cases, as many of the bacteria have acquired different resistance mechanisms and many bacteria do not possess such mechanisms which render them sensitive to pressures applied.

DECLARATIONS

Authors' contribution

AR and DAB conceived the idea and edited the manuscript. IAN and EA contributed in the experiments and wrote the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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