

Full Length Research Paper

Isolation, characterization and antibiotics susceptibility of β -glucuronidase producing *Escherichia coli* and other enteric bacteria from ground beef

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The current work aimed to determine the prevalence of *Escherichia coli* in fresh ground beef purchased from butchers' shops in Suez Governorate, Egypt, and the antibiotics susceptibility pattern of the isolated bacteria. *E. coli* was isolated and detected on tryptone bile glucuronide agar (TBGA) plates as chromogenic selective medium for this species. The sensitivity and resistance of the isolated bacteria to antibiotics were performed according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS). A total of 299 bacterial isolates were recovered from 130 ground beef and *E. coli* had the highest frequency of occurrence (81.5%). The isolated enteric bacteria were identified phenotypically and genotypically as *Serratia marcescens*, *E. coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* and deposited in the GenBank nucleotide sequence database under accession numbers KU237235, KU237236, KU237237 and KU237238, respectively. Antibiotic susceptibility test showed that the four isolated species were susceptible to norfloxacin, pefloxacin, kanamycin and ceftriaxone, and resistant to clindamycin and the other tested antibiotics showed different susceptibility pattern with each tested species. Precautions and strict hygienic measures should be taken during the processing stages of ground beef in order to avoid contamination by enteric bacteria.

Key words: Contamination, Enterobacteriaceae, genotype, resistance, susceptibility, meat.

INTRODUCTION

Over the past decade, increase of bacterial contamination in meat intended for human consumption in many countries is one of different factors responsible for human illness. The trend of some researchers

nowadays is to establish a structured model for improving the food control systems for producing safer food by reducing the number of food-borne pathogens. Ground beef is a processed meat product that might be widely

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exposed and handled during the manufacture process and the probability of contamination of the processed meat is high. The different causes of ground beef contamination may be the use of raw meat, different processing steps during production and the large surface area due to reduction in particle size (Kang et al., 2001; Gundogan and Avci, 2013). The contamination of meat surface by members of the family Enterobacteriaceae will make the produced meat hazardous to human due to their potentiality in causing food poisoning and also they show the hygienic standard of the slaughterers' shops (Nossair et al., 2014).

Enterobacteriaceae are Gram negative bacteria used as indicator microorganisms and a measure of the hygienic status of food products (Jordan et al., 2007; Gundogan et al., 2011). The members of this family have been known to cause health hazard for human and lead to spoilage and deterioration of food products, particularly meat (Ayhan et al., 2000). Some bacterial species of Enterobacteriaceae including *Escherichia coli*, *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp. may be recognized using standard methods that detect their ability to ferment glucose or lactose quickly (usually within 24 and 48 h) generating acid and gas (Molina et al., 2015). These species are known as coliform bacteria and are often used as faecal indicator organisms by food and water, due to their normal habitat which is the gastrointestinal tract of human and animals. These bacterial species have a high spoilage potentiality for food under the favorable conditions for their growth and multiplication (Ray and Bhunia, 2008).

Enterobacteriaceae are the main cause of severe infections from contaminated food, and many commensal species of this family are widely exposed to an extensive use of the currently used antibiotics, consequently, they have developed resistance (Paterson, 2006). Because of self-medication, the suboptimal quality of bacteria can be transferred to pathogenic species (Doucet et al., 2001; Schoeder et al., 2004). Bacterial species of the genus, *Klebsiella* and *Enterobacter* spp. are among the most common Gram-negative bacteria next to *Escherichia coli* that cause nosocomial infections and often give rise to urinary and respiratory tract infections (Saidi et al., 2014).

The main goal of this study was to investigate the best detection method by using selective differential medium for isolation of *E. coli*, *Klebsiella*, *Enterobacter* and *Serratia* spp. from ground beef and to determine their susceptibility to some common antibiotics.

MATERIALS AND METHODS

Samples collection

A total of 130 fresh ground beef samples were collected from different fresh meat shops located in Suez Governorate, Egypt. All samples were collected aseptically in sterilized plastic bags, transported to the laboratory under chilled conditions and processed immediately for microbiological analysis.

Isolation of bacteria

Enrichment culture

Samples were enriched using tryptone soy broth (TSB) to enhance the appearance and growth of the different species belonging to Enterobacteriaceae. Twenty five grams from each food sample were weighed out and homogenized into 225 ml of sterile buffered peptone water in a stomach bag for 5 min (Hara-Kudo et al., 2008) and incubated at 37°C for 4 h. 1 ml was transferred to TSB broth tubes and incubated at 37°C for 18 h.

Plating on selective media

A loopful inoculum of each sample was streaked in triplicate onto tryptone bile glucuronide agar (TBGA) plates and incubated overnight at 37°C. The different colored colonies were selected on the chromogenic selective agar and the well separated pure colonies were picked up on nutrient agar slants as pure cultures and stored at 4°C.

Phenotypic characterization of the isolated bacteria

Morphologically typical colonies were verified by Gram staining, IMViC tests, fermentation of sugars like glucose and lactose, triple sugar iron agar test, oxidase test, urease test, catalase test, pigment production and motility test. Also, the selected colonies were streaked on nutrient agar (NA), Mac Conkey agar (MAC), xylose lysine deoxycholate agar (XLD agar) and Brilliant Green Agar (BGA) media to differentiate between the selected isolates according to the colonies colour.

Genotypic characterization of the selected isolated bacteria

The isolated strains were characterized genotypically with reference to virulence marker genes employing polymerase chain reaction.

DNA extraction

The isolates were grown in 2 ml of BHI broth overnight at 37°C. Cultures were harvested by centrifugation (8000 g for 10 min) and suspended in 400 µl of TE solution (10 mM Tris HCL; 1 mM EDTA, pH 8.0). Bacteria were lysed by addition of 10 µl (20 mg/ml) proteinase K and 100 µl of 10% SDS followed by incubation at 37°C for 1 h. The preparation of total genomic DNA was conducted according to the method of Abd-Alla et al. (2012).

PCR Amplification

The 16S rRNA encoding gene was amplified by the polymerase chain reaction (PCR) from purified genomic DNA using the bacterial universal PCR primers 16S F: 5'-GAGTTTGATCCTGGCTTAG-3' and 16S R: 5'-GGTTACCTTGTTACGACTT-3'. The reaction was performed in a thermal cycler (Eppendorf, Germany) with a pre-heated lid. The cycling conditions included an initial denaturation at 94°C for 5 min followed by 37 cycles each of 30 s denaturation at 94°C, 30 s annealing at 51°C and 30 s extension at 72°C. It was followed by final extension of 5 min at 72°C. After the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

PFGE agarose gel documentation

PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose TBE-gels (Tris-base Boric EDTA-gel) and the gels were

Table 1. Information of antibiotics discs used in disc diffusion assay

| Antibiotic | Abbreviation | Dosage (μg) | Mode of action |
|-------------------|--------------|--------------------------|-------------------------------|
| Norfloxacin | NOR | 10 | Blocking DNA replication |
| Pefloxacin | PEF | 5 | DNA gyrase inhibitor |
| Rifampicin | RD | 5 | RNA synthesis inhibitor |
| Kanamycin | K | 30 | 30S inhibitor |
| Streptomycin | S | 10 | 30S inhibitor |
| Clindamycin | DA | 2 | 50S inhibitor |
| Ceftriaxone | CRO | 30 | Cell wall synthesis inhibitor |
| Cephalothin | KF | 30 | Cell wall synthesis inhibitor |
| Cefuroxime sodium | CXM | 30 | Cell wall synthesis inhibitor |
| Cephalexin | CL | 30 | Cell wall synthesis inhibitor |

Table 2. Frequency and number of isolates recovered from 130 ground beef samples and their grouping according to the colonies colour on TBGA medium.

| Group | Colony colour | Frequency | Percent | Number of isolates |
|-------|---------------|-----------|---------|--------------------|
| A | Red | 7 | 5.4 | 14 |
| B | Green | 106 | 81.5 | 125 |
| C | White | 56 | 43 | 60 |
| D | Colourless | 87 | 66.9 | 100 |

visualized and pictured under UV light. Gels photos were captured using gel documentation system then analyzed by Gel Docu advanced ver.2 software. PCR products of about 1500 bp were purified from gel with the QIA quick PCR purification kit (Qiagen, Hilden, Germany).

DNA sequencing

Purified PCR products were sequenced by cycle sequencing with dideoxy mediated chain-termination (Sanger et al., 1977). DNA sequencing was applied by 3500 Genetic Analyzer, Applied Biosystems (Biotechnology Research Center, Suez Canal University, Ismailia, Egypt). For sequencing of the purified PCR products, the same primers were used. Sequences of the 16S rRNA of isolates were first analyzed using the advanced BLAST search program at the NCBI website: <http://www.ncbi.nlm.nih.gov/BLAST/> in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny was evaluated using CLUSTALW program (<http://clustalw.ddbj.nig.ac.jp/top-eh.html>). The phylogenetic tree was displayed using the TREE VIEW program. Phylogenetic tree derived from 16S rRNA gene sequence was generated in comparison with 16S rRNA gene sequences from different standard bacteria strains obtained from GenBank.

Antibiotic sensitivity testing

Antibiotics sensitivity of the selected bacterial isolates was studied against 10 different antibiotics according to methods of Bauer et al. (1966). Bacterial inocula of the tested bacteria were prepared by growing the bacteria in nutrient broth medium for 18 h at 37°C. One hundred microliter of the bacterial inoculum was seeded individually into sterilized plates and the melted nutrient agar medium was poured and left for solidification. The discs impregnated with

various commercially available antibiotics with known dosage were placed on the surface of the nutrient agar plates, incubated at 37°C for 18 to 24 h and the formed inhibition zones were measured and recorded. Larger zones of inhibition indicate a higher level of antibiotic effectiveness and the tested bacterial strain is designated as sensitive to the antibiotic. The absence of apparent zone of inhibition indicates that the bacterial strain is resistant to this particular antibiotic drug. The specific information of each antibiotic including its abbreviation, name, dosage and inhibition mechanism are listed in Table 1.

RESULTS AND DISCUSSION

A total of 299 bacterial isolates were recovered from 130 ground beef samples on TBGA medium as selective differential medium for isolation of *E. coli* (Table 2). The isolation of *E. coli* from different food products and clinical samples on the chromogenic selective TBGA medium was performed by different researchers (Kodaka et al., 1995; Popovic et al., 2010). Tryptone bile glucuronide agar (TBGA) is a selective differential medium recommended for detection of β -glucuronidase-positive *E. coli* in food products prepared for human consumption. The medium contains bile salts that are selective against Gram-positive bacteria and X-glucuronide (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) as a chromogen for the detection of β -D-glucuronidase enzyme which is present in *E. coli* only of coliforms. *E. coli* absorb the chromogenic substrate, 5-bromo-4-indolyl-D-glucuronide, X-glucuronide. The intracellular



Figure 1. Colonies morphology of the coliform bacteria isolated from ground beef samples on TBGA medium.

enzyme β -D-glucuronidase of *E. coli* is able to break the bond between the substrate and the chromophore which dimerises in the presence of oxygen to form an insoluble coloured compound within the cell. The released chromophore is colored and accumulates inside the cells (Feng and Hartmann, 1982). The accumulation of the coloured chromophore results in blue colonies on the agar. The chromogenic substrate is concentrated within the colony facilitating their enumeration visually among the other organisms on the plate without UV lamp.

In the current study, it was observed that there are three others colonies colour on this medium besides *E. coli* colonies. The recovered bacterial isolates were divided into four different groups: A, B, C and D on the basis of their colonies colour on TBGA plates. The bacterial groups: A, B, C and D were characterized by colony colour as red, green, white and colourless, respectively (Figure 1). The appearance of these different coloured colonies besides the *E. coli* colonies might be due to different intracellular enzymes by these bacteria or different reactions between the components of the medium and intracellular metabolites. Generally, this chromogenic medium may be suitable for better detection of these four groups of isolated bacteria. Isolation and enumeration of *E. coli* is used as reliable indicator of fecal contamination and probability of toxigenic microorganisms' presence in this food. Group B which represents *E. coli* had the highest frequency of occurrence and represented by 81.5% recovered from 106 samples out of 130 samples, while groups A, C and D is represented by 5.4, 43 and 66.9%, respectively (Table 1). The results are almost in line with that of Greeson et al. (2013) who studied the prevalence of Enterobacteriaceae in 36 samples of meat and reported that *E. coli* was the most frequent contaminant and its prevalence was 72.2%. *E. coli* is known as a fecal

contamination indicator in foods due to its presence in the intestinal tract. The gastrointestinal tract and the hands of personnel were recorded as major transferors of *Klebsiella* spp. and *E. coli* (Gundogan and Yakar, 2007). On the other hand, the current study results are in contrast with those of Mohammed et al. (2014) who recorded low frequency (15.89%) of *E. coli* isolated from 384 meat samples. Also, a similar study on isolation of *E. coli* from retail meat was performed by Nossair et al. (2014) who isolated different members of Enterobacteriaceae from 50 samples of retail meat collected from buffaloes and it was found that different species of bacteria were isolated at different rates, where *E. coli* is the highest isolated bacterium (40%) followed by *K. pneumoniae*. The obtained results showed that the examined ground beef of cow origin were highly contaminated with *E. coli* and other enteric bacteria which showed fecal contamination potential for severe hazard (Mohammed et al., 2014).

The isolated bacteria were members of the intestinal flora of human and animals and many of them might lead to food deterioration and toxicities (Gundogan and Yakar, 2007; Haryani et al., 2007). These results emphasized the role played by meat in transmission of *E. coli* that could constitute public hazard and food poisoning outbreaks (Reuben and Gyar, 2015; Kabiru et al., 2015). Contamination of both carcasses and the environment by *E. coli* from the intestinal contents of cattle during slaughter is one of the most significant risk factors in transmission to humans (Koohmaraie et al., 2005; Bosilevac et al., 2009). Moreover, Enterobacteriaceae contaminating ground beef in butchers' shops may originate from human carriers (workers) who handle and prepare the meat during cutting and grinding. Also, infected rodents that may be present in the butchers' shops or slaughterhouse could represent a neglected

Table 3. Phenotypic characterization of the isolated coliform bacteria.

| Test | Group A | Group B | Group C | Group D |
|------------------|------------|--------------------------|--------------------------|--------------------------|
| Gram stain | -ve | -ve | -ve | -ve |
| Cell shape | Short rods | Short rods | Short rods | Short rods |
| Oxidase | - | - | - | - |
| Methyl red | - | + | - | + |
| VP | + | - | + | - |
| Citrate test | + | - | + | + |
| Glucose ferm. | + | + and gas | + and gas | + |
| Lactose ferm. | - | + and gas | + | + |
| TSI | + | + and gas | + and gas | + |
| H ₂ S | - | - | - | - |
| Urease | - | - | - | - |
| Motility | + | + | - | - |
| Indole | - | + | - | - |
| Pigment | Red | Negative | Negative | Negative |
| Nutrient agar | Red | Colourless | Colourless | Colourless |
| MAC agar | Red | Pink | Pink | Pink |
| XLD agar | Red | Pink with yellowish zone | Pink with yellowish zone | Pink with yellowish zone |
| BG agar | Red | Colourless | Colourless | Colourless |

nsorce of contamination by *E. coli* and other coliforms bacteria (Okonko et al., 2010).

The four isolated coloured bacterial groups were characterized morphologically, biochemically and genotypically. The morphological and biochemical characterization of the total recovered bacterial isolates belonging to the different four groups: A, B, C and D were performed by conventional methods. All the tested isolates belonging to the four groups were negative for Gram staining and have short rods shape. Also, the four groups were negative for oxidase and positive for glucose fermentation. On the other hand, the four groups differed in their result for lactose fermentation as the test was positive for the three groups: B, C and D, and was negative for group A. The results of the other biochemical tests showed more differences between the four bacterial groups in this investigation. Group A was characterized by red pigment production and was positive for VP, citrate and motility, whereas, was negative for methyl red, H₂S, urease and indole. Group B was positive for methyl red, motility and indole but negative for VP, citrate, H₂S and urease. Group C was positive for VP and citrate but was negative for methyl red, H₂S, urease, motility and indole. The fourth group showed positive result for each methyl red and citrate and gave result for VP, H₂S, urease, motility and indole. The colonies colour differed on different culture media as presented in Table 3. Based on the morphological and biochemical characterizations, the isolated strains belonging to the four groups: A, B, C and D were related to *Serratia marcescens*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, respectively, according to the Bergey's Manual of Determinative Bacteriology (Holt, 1994).

The phenotypic-based identification was confirmed by genotypic identification. One isolate was selected from each of the four groups and their DNA were extracted, and a fragment of about 1500 bp from each one was amplified using the universal primers 16S F and 16S R (Figure 2). Comparison between 16S rRNA gene sequences of the tested isolates Ras1, Ras2, Ras3 and Ras4 belonging to groups A, B, C and D, respectively and 16S rRNA gene sequences on GenBank database as determined using Blast search analysis, were done. Sequencing of 16S rRNA genes of the tested isolates Ras1, Ras2, Ras3 and Ras4 had 16S rRNA gene with 99% nucleotides identity to that of *S. marcescens* NR102509.1, *E. coli* NR 114042.1, *E. cloacae* NR 118011.1 and *K. pneumoniae* NR 112009.1, respectively, available in Genbank database (Figure 3). The phylogenetic tree was drawn from 16S rRNA sequence data by the neighbor-joining method. The tested isolates Ras1, Ras2, Ras3 and Ras4 were identified as *S. marcescens*, *E. coli*, *E. cloacae* and *K. pneumoniae*, respectively. The nucleotide sequences of *S. marcescens* Ras1, *E. coli* Ras2, *E. cloacae* Ras3 and *K. pneumoniae* Ras4 were deposited in the GenBank nucleotide sequence database under accession numbers KU237235, KU237236, KU237237 and KU237238, respectively.

Five isolates of each of *S. marcescens*, *E. coli*, *E. cloacae* and *K. pneumoniae* recovered in this investigation were selected and tested for their susceptibility to ten antibiotics (Figure 4). The analysis of the strain resistance to antimicrobial agents showed that norfloxacin, pefloxacin, kanamycin and ceftriaxone inhibited the growth of all tested isolates belonging to *S.*

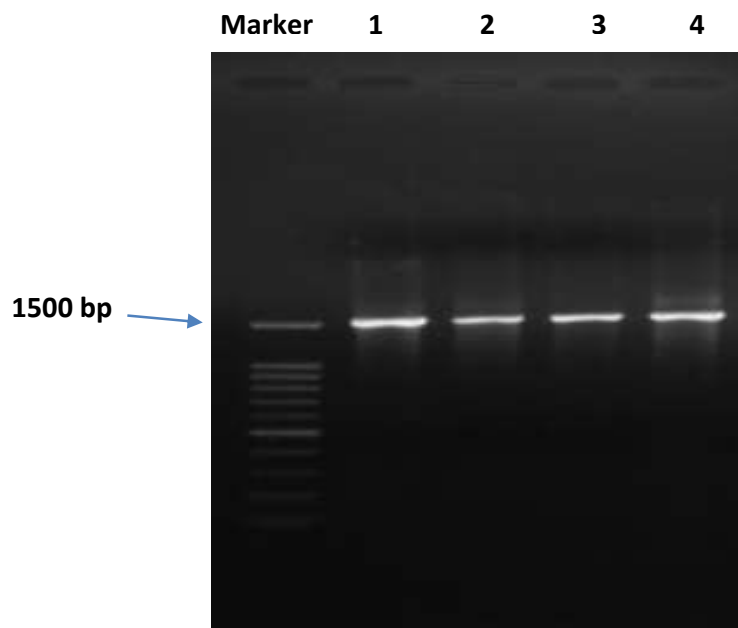


Figure 2. PCR gel electrophoresis of 16S rRNA gene of the four isolated bacterial species showing bands at 1500 bp.

marcescens, *E. coli*, *E. cloacae* and *K. pneumoniae*. Other tested antibiotics showed differential susceptibility. All the tested isolates of the four tested species were resistant to clindamycin. The tested isolates of *S. marcescens* were also resistant to cefuroxime sodium, cephalixin, cephalothin, streptomycin and rifampicin, whereas, were highly sensitive to norfloxacin, pefloxacin, kanamycin and ceftriaxone. The tested isolates of *E. coli* were highly sensitive to norfloxacin, pefloxacin, ceftriaxone and cephalothin but with moderate sensitivity to cefuroxime sodium, cephalixin and streptomycin and had low sensitivity to the others antibiotics. *E. cloacae* isolates were resistant for cephalixin, cephalothin and clindamycin, however, were highly sensitive to norfloxacin, kanamycin and ceftriaxone. On the other hand, *K. pneumoniae* was resistant to cephalothin, rifampicin in addition to clindamycin and was highly sensitive to norfloxacin, pefloxacin and ceftriaxone. Shiga toxin-producing *E. coli* isolated from samples of meat were multi-resistant, exhibiting resistance to ampicillin, ciprofloxacin, tetracycline, sulfamethoxazole-trimethoprim, gentamycin and streptomycin (Li et al., 2011). In a study carried out by Kalmus et al. (2011) to evaluate antibiotic resistance of *E. coli*, ampicillin, streptomycin and tetracycline resistance were observed in 24.3, 15.6 and 13.5%, respectively, among the *E. coli* isolates. While examining the hygienic and sanitary quality of pasteurized cow's milk, *E. coli* was identified in 77.05% of the samples and the highest rates of resistance to antimicrobial agents were obtained for ampicillin (19.2%), cephalothin (18.9%) and tetracycline

(17.1%) (Zanella et al., 2010). *E. coli* and other coliforms recovered from humans and animals had antibiotic resistance and several species were resistant to many antimicrobial agents commonly used in human and veterinary medicine (Greeson et al., 2013). The treatment of cattle with common antibiotics leads to increase in resistance and transfer of these resistant strains to human hosts (Rinsky et al., 2013; Barnett and Linder 2014; Cordoba et al., 2015).

Conclusion

The contamination of food products by bacteria exclusively, Enterobacteriaceae, represents a major problem in food production and leads to food spoilage and human illness. Ground beef processing passes through different steps which may be the route to contamination by *E. coli* and other enteric bacteria. Based on the results obtained in the current investigation, it was observed that ground beef may act as a vector for transmission of *E. coli* and other members of Enterobacteriaceae to man. So, more precautions and strict hygienic measures should be taken to clean and disinfect meat during the processing stages of production in order to avoid contamination by these bacteria. Also, the treatment of cattle with therapeutic antibiotics should be reduced or replaced by natural therapeutic herbs in order to reduce the appearance of resistant enteric bacteria in these animals and the produced food, and reduce the spread of antibiotic resistance among normal

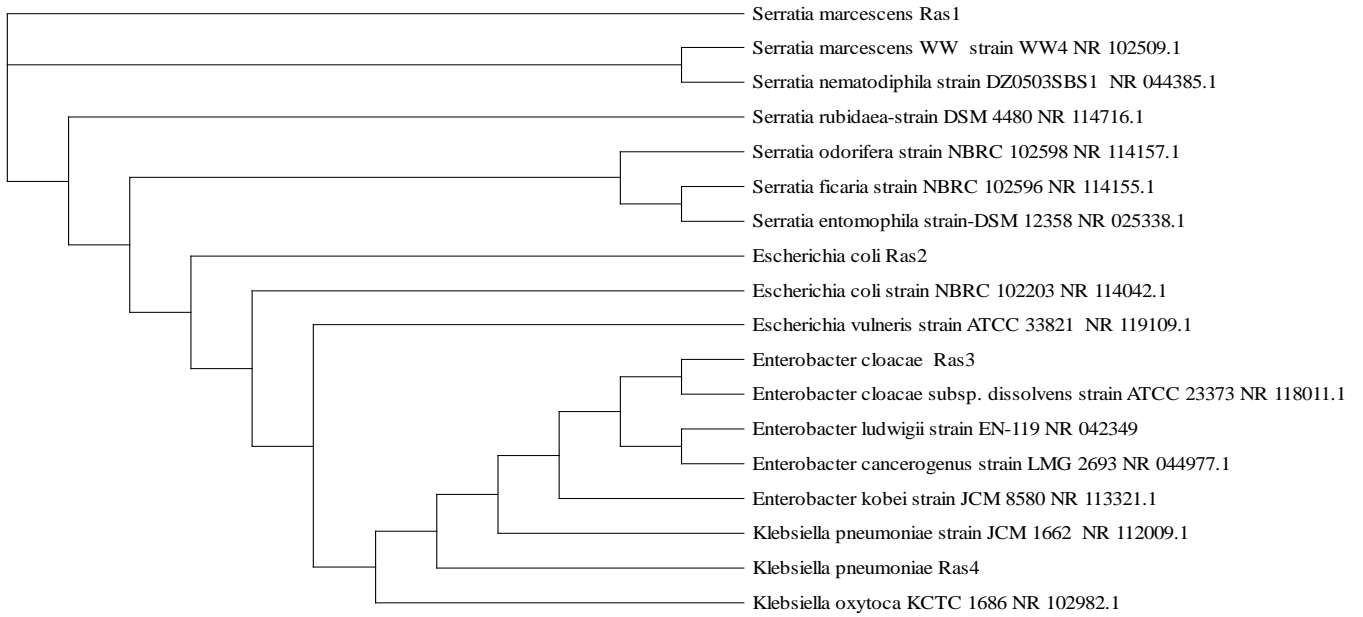


Figure 3. Neighbor-joining tree based on 16S rRNA gene sequences showing positions of the isolated bacteria and related strains.

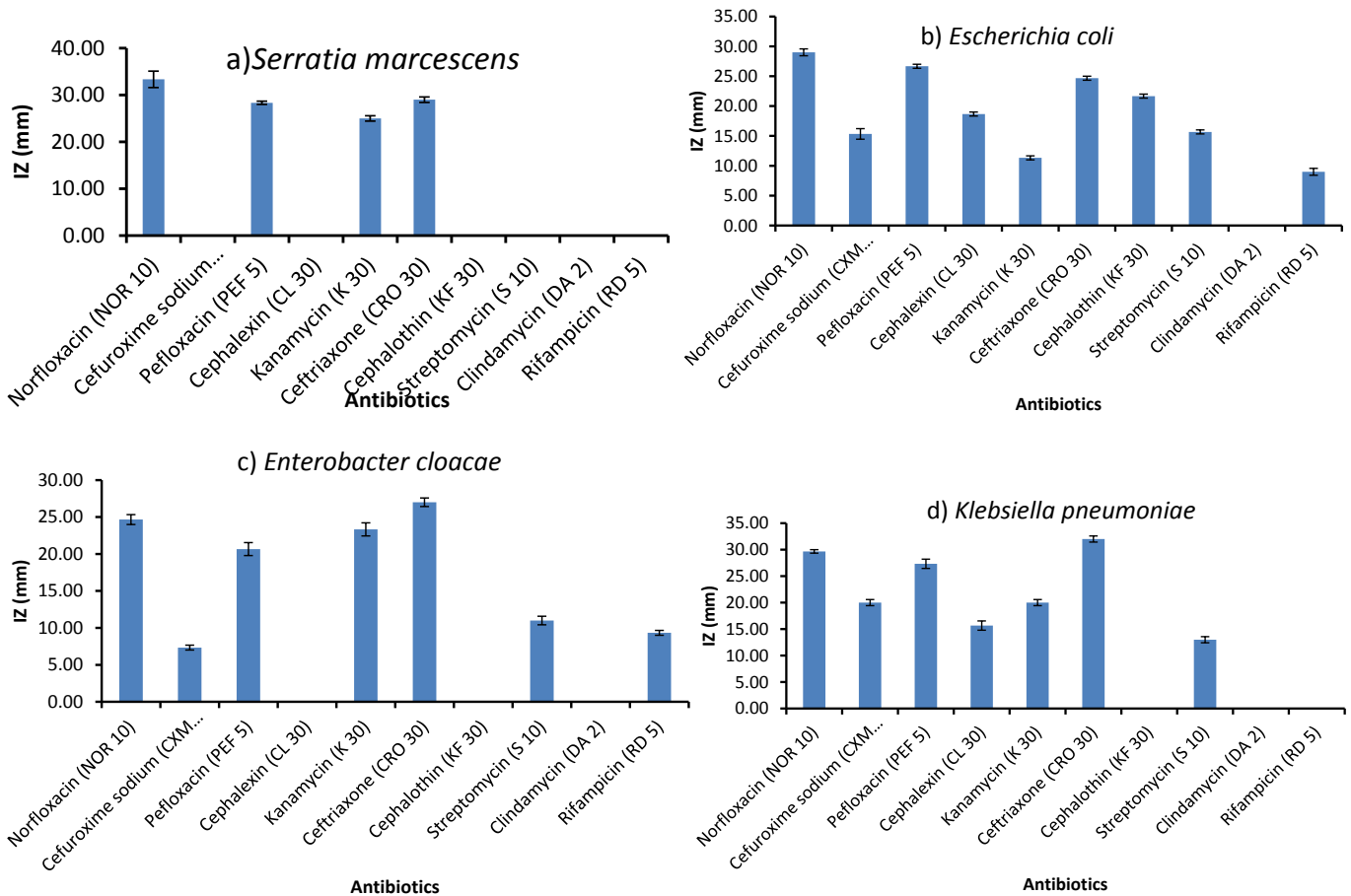


Figure 4. Antibiotic susceptibility indicated by inhibition zones (mm) formed against; a) *S. marcescens*, b) *E. coli*, c) *E. cloacae* and d) *K. pneumoniae*.

flora and pathogenic bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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