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Incidence of *Enterobacteriaceae* in Retail Meat Samples and Their Antibiotic Susceptibility

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Authors' contributions

This work was carried out in collaboration between all authors. Author PS designed the study and wrote the first draft of the manuscript. Authors SD, PS and DS managed the analyses of the study performed, the statistical analysis and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The study aims at accounting the prevalence of pathogenic bacteria within the family *Enterobacteriaceae* in retail meat samples. Eighty samples were collected from four different locations of Allahabad region. Isolation was done using selective plating according to ISO Standard; ISO 21528-1:2004. Differentiation and characterization of different isolates was based on their growth characteristics on specific culture media, their biochemical confirmatory tests and Gram-staining reactions. Total soluble proteins of the isolates were estimated by Biuret method. Antibiotic susceptibility of the isolates was tested against antibiotics including ampicillin, streptomycin and ciprofloxacin at different concentrations. A total of 62 isolates were obtained and identified as *Klebsiella planticola*, *Citrobacter youngae*, *Enterobacter sp, E. cloacae*, *K. ornithinolytica* and *K. pneumonia.* All the isolates were susceptible to ampicillin, streptomycin and ciprofloxacin at concentrations of 10, 50, 80 and 100 μg/ml. The virulent proteins were highest in *Enterobacter* (127.3 mg/ml) followed by *C. youngae* (119.4 mg/ml). Meat sold in the local markets of Allahabad showed presence of pathogenic bacteria belonging particularly to the family *Enterobacteriaceae*; indicating poor hygienic conditions as well as improper storage environment. The results revealed that *K. pneumonia* (29.9%) represented the major part of bacterial flora, in the samples followed by *C. youngae* (20%).

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1. INTRODUCTION

The most serious meat safety issues resulting in immediate consumer health problems are associated with microbial, especially bacterial pathogens. Several researchers have reported that the meat samples are highly susceptible to contamination with high level of *Klebsiella pneumoniae, Enterobacter* spp, *Pseudomonas aeruginosa, Escherichia coli, Salmonella* sp, *Serratia marcescens* and *Proteus vulgaris, Staphylococcus aureus* and *Bacillus* spp. The possible sources of these bacteria are likely to come from the skin of the animal from which the meat was obtained. Other potential sources of microbial contaminations are the equipment used for each operation that is performed until the final product is eaten, the clothing and hands of personnel and the physical facilities themselves are all implicated [1]. Retail cut could also result in greater microbial load because of the large amount of exposed surface area, more readily available water, nutrient and greater oxygen penetration available [2]. Hence retail cuts displayed are conducive for microbial growth and proliferation which leads to spoilage of the meat. The microbiological safety of meat and poultry products has assumed paramount importance for industry, consumers, and public health officials. Approximately 30,000 cases of foodborne illness are reported annually in Canada [3], although an estimated 2.2 million cases of foodborne disease occur each year [4]. There have been cases of numerous outbreak of food borne diseases and gastrointestinal illness such as dysentery, cholera, diarrhoea in many cities in Ghanan which have been associated with the consumption of meat products that are contaminated by microorganisms through unhygienic practices. From the farm to the consumer, the processing, transportation, and storage of meat products potentially provide growth conditions and nutrient content to support unwanted microbial growth. Surveillance data may be used to inform the development of food safety standards and define research priorities based on risk assessments. Also it may provide an indication of direct consumer exposure. A comprehensive understanding and study of the microbial ecology of meat produce sold in our markets and the factors that lead to microbial contamination and their multiplication is needed for effective management and maintenance of high quality and safe food. The main objective of this study therefore was to determine the level of pathogenic bacterial contamination specially those belonging to *Enterobacteriaceae* on meat products.

2. MATERIALS AND METHODS

2.1 Collection of Sample

The study was conducted on eighty meat samples collected from different locations of Allahabad during January to May 2016 (*Teliarganj, Katra, Allahapur, and Civil lines).* The samples were collected and transported to the laboratory in an ice-box immediately.

2.2 Media and Chemicals

All bacteriological media, chemicals and antibiotics were procured from Hi-Media Lab, India. Reference strains of *E.coli* (MTCC 3221), *Salmonella enterica* (MTCC 3224), *and Shigella flexenerii* (MTCC 1457) were procured from MTCC, IMTECH, Chandigarh, India.

2.3 Isolation of *Enterobacteriaceae*

The isolation of enteric pathogens from the samples was done according to ISO Standard; ISO 21528-1:2004. For isolation of *Enterobacteriaceae* the sample is serially diluted and pour plated using VRBGA as a medium for 24 hrs at 37ºC. The typical characteristic colonies were counted and purified using streaking on nutrient agar. A total of 62 isolates were selected for further studies.

2.4 Biochemical Characterization of Isolates

All of the isolated colonies after being sub-cultured on Nutrient Agar were further confirmed by performing different sets of biochemical tests. These tests are: Catalase test, l-Lysine Decarboxylation Test, Indole Test, Nitrate Reduction Test, Methyl red test, Voges-Prauskauer test, Citrate Utilization Test, Motility test, Gelatine liquefaction test, Urease Test, TSI (Triple sugar iron agar) test, and Malonate utilization test [5].The tests were done as per the procedures of Bergy's Manual of detection [6].

2.5 Carbohydrate Fermentation Pattern

All the selected isolates were tested for sugar fermentation. The identification of isolates was

done on the basis of carbohydrate fermentation pattern utilising sugars such as glucose, lactose, sucrose, raffinose, mannitol, sorbitol and xylose [6,7].

2.6 Protein Profiling

The protein profiling of the isolated colonies was done by Biuret method [8] with some modifications. All of the isolates were grown overnight in luria broth, at 37°C for 24 hrs and then centrifuged at 8,000 g for 10 mins. From the supernatant 0.5 ml were taken in a test tube and 0.2 ml of biuret reagent was added to it. The mixture was homogenised and incubated at 27°C for 30 mins. A blank was prepared by adding 0.5 ml distilled water to 0.2 ml of biuret reagent, and incubated at room temperature for 30 mins. After completion of the incubation, the absorbance of the sample was taken at 570 nm, against the blank. A control tube was prepared adding 0.5 ml of Luria broth to the 0.2 ml biuret reagent, and the reading was taken at 570 nm. A standard curve of Bovine Serum Albumin (BSA) standard, were plotted with different known concentrations and the protein concentrations of the samples (mg/ml) were determined from the standard curve.

2.7Antibiotic Susceptibility Test

This test was performed to determine the susceptibility of the isolates to most common antibiotics. The antibiotic susceptibility was analysed as per Kirby Bauer method [9]. Antibiotic susceptibility test was done by the disk diffusion method. Isolates were grown overnight in nutrient broth at 37°C and standardized to 0.5 McFarland standards (1.5 x 10^8 CFU/ml). Whatman filter paper was used to prepare discs and antibiotic solutions of ampicillin, streptomycin and ciprofloxacin at different concentrations of 10 μg/ml, 50 μg/ml, 80 μg/ml and 100 μg/ml each, were selected for the study. The nutrient agar plates were prepared and swabbed with the isolates grown in nutrient broth. The filter paper discs were placed on the plates already swabbed with the isolates and the antibiotic solutions were spread on the discs. The plates containing different antibiotic discs were kept in incubator at 37°C for 24 hours. After incubation the zones of inhibition were measured in triplicate.

3. RESULTS AND DISCUSSION

3.1 Isolation of *Enterobacteriaceae* Isolates

In the present study isolation of *Enterobacteriaceae* was done from eighty meat samples obtained from four different locations (Katra, Civil lines, Teliyarganj, Allahpur) (Table 1). Presence of a large number of isolates was observed among all the locations. A total of 62 isolates i.e. 76.25% of prevalence was found in the samples. The isolation of these groups of organisms indicated faecal and environmental pollution. In this study isolation of Enterobacteriacaeae strains as 76.25% from collected samples indicated public health hazards and concern. Similar studies have shown that 68% of supermarket poultry products were contaminated by *E. coli* in a study in Spain [10] and about 76% of ground meat retailed in Monterrey, Mexico is contaminated with the presence of *Enterobacteriaceae* [11].

3.2 Colony Morphology and Gram Reaction of the Isolates

The isolates were selected on the basis of their colony morphology and Gram-reaction. The

Table 1. Isolates in meat samples collected from different locations

Locations	No. of samples	No. of isolates	Coding of the isolates	Percentage	Growth on NA*
Katra	20	18	1,2,3,4,5,6,7,8,9,10,11,15,19 12,13,14,16,17	90%	++
Teliarganj	20	13	18,20,21,22,23,27,28,30,31,32, 34,40,41	65%	+++
Allahpur	20	16	24,25,26,29,33,35,36,37,38,39 42,43,44,45,46,47	80%	++
Civil Lines	20	15	48,52,53,49,50,51,54,55,56,57, 58,59,60,61,62	75%	+++
Total	80	62		76.25%	

**NA= Nutrient Agar, ++ = Moderate growth +++ = High growth*

isolates appearing as pink rods after Gram-staining were proceeded for further analysis. All the isolates were gram-negative. The colonies were selected by considering Gram-reaction, colonial morphology and the specific biochemical tests for *Enterobacteriaceae*. The pure cultures were grown in Luria broth and then stored in glycerol for further analysis (Table 2). Dubey *et al* [12] also isolated Enterobateriaceae spp. from raw fish specimens on the basis of morphology and Gram staining.

3.3 Biochemical Characterization of Isolates

The isolates after Gram-staining reactions were subjected to the specific biochemical tests. All the isolates showed a positive test for urease, except isolate 1,6 and 19. All the isolates except 10, 11 and 28 showed a positive catalase and lysine test. Citrate utilization test was also positive for all the selected isolates except 42, 43, 56-58. Other tests results were in accordance with the characteristic tests of *Enterobacteriaceae* family (Table 3). Studies of several researchers [13,14,15] are in agreement with the results of present study.

3.4 Sugar Fermentation Test

The carbohydrate utilization pattern of isolates was studied on the basis of sugar fermentation test. A total of 7 sugars were used for conformational identification of *Entero-bacteriaceae* i.e. Mannitol, Sorbitol, Raffinose, Xylose, Lactose, Sucrose, Glucose. Different isolates showed different sugar utilization pattern. All the isolates fermented sugars such as Mannitol, Sorbitol, Raffinose, Xylose changing the colour of the sugar broth and producing gas bubbles. Only 12 isolates (Table 4) showed no fermentation of lactose and sucrose. The results of sugar fermentation patterns were compared with those given for *Enterobacteriaceae* species in the Bergy's manual of determinative bacteriology [6]. The results for species identification including morphology, physiology, biochemical test and carbohydrate fermentation pattern were also subjected to a software called PIBwin [16]. The isolates identified were *K. planticola* (18%), *K. ornithinolytica* (11.6%), *Enterobacter cloacae* (5%), *Enterobacter* sp. (11.6%), *Klebsiella pneumonia* (29.9%), *C. youngae* (20%). The prevalence of various *Enterobacteriaceae* isolates identified is given in Fig. 1.

3.5 Antibiotic Susceptibility Test

All 62 isolates recovered from meat samples were checked for susceptibility to antibiotics. Antibiotic susceptibility test was determined by Kirby Bauer disc diffusion method using different antibiotics such as ampicillin, streptomycin and ciprofloxacin. The test was performed at different concentrations (10 μg/ml, 50 μg/ml, 80 μg/ml, 100 μg/ml, each) of antibiotics to find out the minimum and maximum resistance level of antibiotics. Table 4 shows that different zone of inhibition were produced by isolates at different concentration of antibiotics. 88-90% of isolates showed zone of inhibition in>20 mm range in presence of ciprofloxacin at 100 μg/ml which indicates the isolates are more susceptible to this

Table 2. Colony characteristics and gram reaction of isolates

Isolates	Percen-tage of isolates	Selective media	Colony morphology		
			Gram reaction	Color	Shape
1,3,5,15,17,18,24,26,27, 34,46,49,50,54,55	24.19	MCA	-ve, rods	Pink colony	Mucoid
35,36,37,40,41,42,51, 52,57,59,60,61	19.35	XLDA	-ve, rods	Red with black centered colony	Round
2,7,8,9,10,11,12,13,1, 16,19,20,30,31,32,33, 38,39,43,44,58	33.87	XLDA	-ve, rods	Yellow color colony	Round
6,21,22,28,29,45,53	11.29	DCA	-ve, rods	Black color colony	Flat
23,25,47,48,62	0.08	MCA	-ve, rods	Colorless colony	Flat

Table 3. Biochemical characterization of the isolates

Isolate no.	Tryptophan	Peptone	MR	VP	CU	Motility	Lysine	NR	Urease	Catalase
1,6,19	+	+	+	+	+	+	+	+	-	+
2,3,4,5,7,27,60	+	+	+	-	+	+	+	+	+	+
8,9,29,30,48,53,54,55	+	+	-	+	+	+	+	+	+	+
10,11,28	-	-	+	-	+	+	-	+	+	-
12,13,14,41	-	-	+	+	+	+	+	+	+	+
15,16,17,18,45,46, 47,49	-	+	+	-	+	+	+	-	+	+
20,21,22,23,26	-	-	-	-	+	+	+	-	+	+
24,25,31,32,33,34, 35,36,37,38,39,40	+	+	-		+	+	+	+	+	+
44,52,59	-	-	-		+	+	+	+	+	+
42,43,56,57,58	+	-	+		-	+	+	+	+	+

Lysine= Lysine decarboxylase test, MR= Methyl red test, VP= Voges-Prauskaur test, CU= Citrate utilization test, NR= Nitrate reduction test

Table 4. Carbohydrate utilization pattern of the isolates

Isolate no.	Glucose	Lactose	Sucrose	Mannitol	Sorbitol	Xylose	Raffinose	Probability of organism
1,6,8,9,19,29, 30,48,53,54,55	+	+	+	+	+	+	+	*Klebsiella planticola* (18%)
2,3,4,5,7,27,60	+	+	+	+	+	+	+	*K. ornithinolytica*(11.6%)
10,11,28	+	+	+	+	+	+	+	*Enterobacter cloacae* (5%)
12,13,14,41,44,52,59	+	+	+	+	+	+	+	*Enterobacter* (11.6%)
15,16,17,18,20,21,22,23,26, 42,43,56,57,58,45,46,47,49	+	+	+	+	+	+	+	*K. pneumonia* (29.9%)
24,25,31,32,33,34,35,36,37,38,39,40	+	-	-	+	+	+	+	*Citrobacter youngae* (20%)

antibiotic and could be inhibited by its use (Table 5). According to reported studies, Salmonella spp. isolates in retail meats were commonly resistant to multiple antibiotics, including tetracycline, ampicillin, sulfonamides and streptomycin [17-23]. Other studies also found chicken and meat isolates were susceptible to ciprofloxacin [24,18,25,21, 20,26,27].

3.6 Protein Profiling of Isolates

Total soluble Protein profiling of the 62 isolates is depicted in Fig. 2. The results showed that in meat samples the virulent protein were *K. planticola* (118.1 mg/ml), *Citrobacter youngae* (119.4 mg/ml), *K. ornithinolytica* (103.8 mg/ml), *Enterobacter* sp. (127.3 mg/ml), *Enterobacter cloacae* (112.1 mg/ml) and *K. pneumonia* showed a protein content of (105.7 mg/ml). The total soluble protein of *Klebsiella* isolates from fishes has been studied by Dubey et al. [12]. The results indicated that in fishes virulent proteins were 86.3 mg/ml in *K. planticola*, 103 mg/ml in *K. ornithinolytica* and *K. pneumonia*. Virulent proteins are considered to be responsible for the pathogenicity and include hemolysins, enterotoxins, proteases, lipases, ribonucleases etc. [28]. These virulence factors are associated with structural components of pathogenic bacteria and toxins that are produced during its metabolism [29].

Table 5. Antibiotic susceptibility test results of the isolates

Antibiotics*	% of isolates in different ranges of diameter of inhibition zones (mm)				
	< 5 mm	5- 10 mm	10-15 mm	15-20 mm	> 20 mm
Amp (10 μg/ml)	-	17.8 %	77.1 %	5.0%	-
Amp (50 μg/ml)	-	14.2 %	81.0 %	4.7%	-
Amp (80 μg/ml)	-	-	78.5 %	21.4 %	-
Amp (100 μg/ml)	1.7 %	33.9 %	35.7 %	-	-
Strepto (10 μg/ml)	-	10.2%	82.6 %	7.2 %	-
Strepto (50 μg/ml)	-	-	85.8%	5.3 %	8.9 %
strepto (80 μg/ml)	-	-	10.7 %	69.7 %	19.6 %
Strepto (100 μg/ml)	-	-	10.2 %	80.9 %	8.9 %
Cipro (10 μg/ml)	-	-	8.4 %	91.6 %	-
Cipro (50 μg/ml)	-	-	2.5%	74.5 %	23 %
Cipro (80 μg/ml)	-	-	3.3%	6.4%%	90.3 %
Cipro (100 μg/ml)	-	-	-	12 %	88 %

**Amp =Amphicillin, Strepto = Streptomycin, Cipro = Ciprofloxacin, - No isolates inhibited in the given inhibition zone*

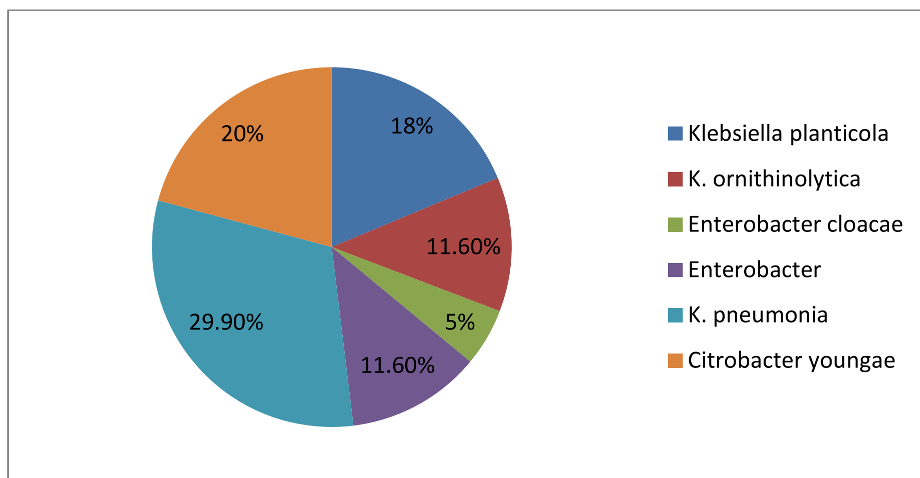

Fig. 1. Prevalence of Identified *Enterobacteriaceae* isolates

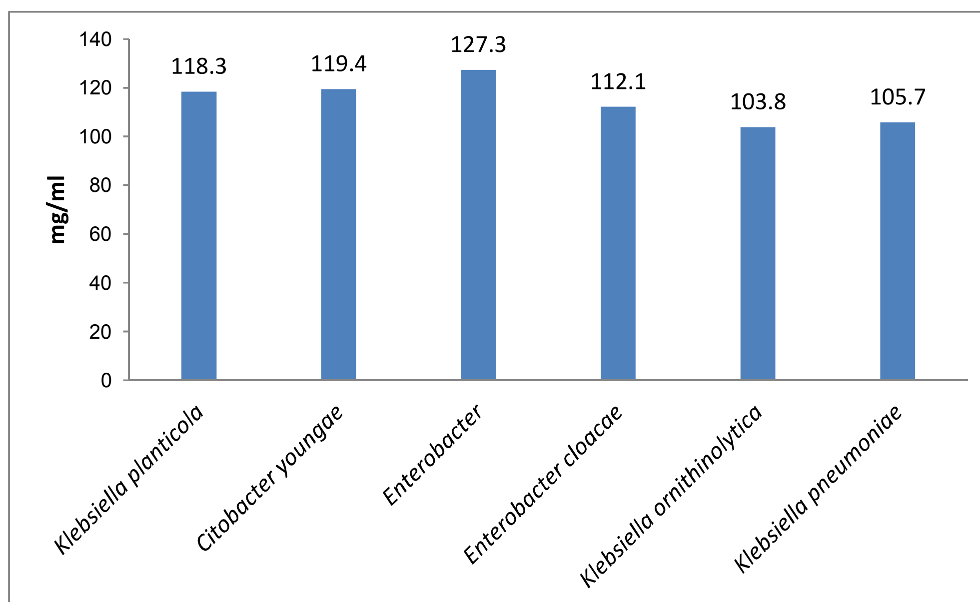

Fig. 2. Total soluble protein concentration of *Enterobacteriaceae* species isolated from meat samples

4. CONCLUSION

The wide presence of enterobacter isolates shows a high level of contamination in all the retail meat samples. Overall microbiological quality of the samples at different locations was unacceptable. Presence of *Klebsiellsa* spp., *Enterobacter* and *Citrobacter* spp. were confirmed in meat samples and it was found that *K. pneumonia* (29.9%), represented the major part of bacterial flora followed by *C. youngae* (20%). Although relatively few samples were tested, the study provides an indication of the occurrence of pathogens in retail meat available to consumers in the marketplace. Future surveys are required to be conducted for a longer period of time and with a larger number of samples to determine the prevalence of these pathogens. Careful handling of raw products is essential for prevention and control of emerging pathogens. In particular, for meat and poultry production, it is essential that hygiene be maintained during slaughter operations according to HACCP principles and codes of good manufacturing practices to reduce the risk of carcass contamination.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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