ANTIMICROBIAL DRUG RESISTANCE IN STRAINS OF *Escherichia coli* ISOLATED FROM FOOD SOURCES

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SUMMARY

A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in medicine and agriculture. Antibiotic resistance in *Escherichia coli* is of particular concern because it is the most common Gram-negative pathogen in humans. Hence this study was conducted to determine the antibiotic sensitivity pattern of *E. coli* isolated from different types of food items collected randomly from twelve localities of Hyderabad, India. A total of 150 samples comprising; vegetable salad, raw egg-surface, raw chicken, unpasteurized milk, and raw meat were processed microbiologically to isolate *E. coli* and to study their antibiotic susceptibility pattern by the Kirby-Bauer method. The highest percentages of drug resistance in isolates of *E. coli* were detected from raw chicken (23.3%) followed by vegetable salad (20%), raw meat (13.3%), raw egg-surface (10%) and unpasteurized milk (6.7%). The overall incidence of drug resistant *E. coli* was 14.7%. A total of six (4%) Extended Spectrum β-Lactamase (ESBL) producers were detected, two each from vegetable salads and raw chicken, and one each from raw egg-surface and raw meat. Multidrug resistant strains of *E. coli* are a matter of concern as resistance genes are easily transferable to other strains. Pathogen cycling through food is very common and might pose a potential health risk to the consumer. Therefore, in order to avoid this, good hygienic practices are necessary in the abattoirs to prevent contamination of cattle and poultry products with intestinal content as well as forbidding the use of untreated sewage in irrigating vegetables.

KEYWORDS: *E. coli;* Multidrug resistant; ESBL; Agriculture; Medicine.

BACKGROUND

Escherichia coli is the most prevalent facultative anaerobic species in the gastrointestinal tract of human and animals, usually a harmless microbe, but it is also a medically important bacteria causing a number of significant illnesses[14].

Vegetables may be contaminated through insufficiently-treated water and fertilizers or may be compromised by the use of biocides during cultivation[7]. Similarly, animals can also become infected from water or food contaminated with wastes of human or animal origin or with human carrier workers. One of the possible ways of entry of various microbes could be the handling of meat and meat products by adopting improper hygienic measures during handling and processing[20].

Raw meat and vegetables are particularly likely to carry large numbers of bacteria. The same *E. coli* and *Klebsiella* spp. serotypes have been found in food and in the patients who consumed it[11,12.] A sterile diet was shown to lower the number of *E. coli* serotypes found in the feces of test persons[6]. Bacteria escaping alive through the digestive tract to the colon are often transient[13,] the resident flora having a protective effect against intruders. The transfer of drug resistance within the gastrointestinal tract is still possible; thus, if our food contains substantial numbers of resistant bacteria, it could be an important source of resistance in fecal flora.

It has been suggested that resistance in bacterial populations may spread from one ecosystem to another[18]. The wild dissemination of antimicrobial resistance among bacterial populations is an increasing problem worldwide.

Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food producing animals. Many findings suggest that inadequate selection and abuse of antimicrobials may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult[21]. Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents[32]. Over the years, resistance to cephalosporins among members of enterobacteriaceae has increased mainly due to the spreading of Extended-spectrum β-Lactamases (ESBL)[40].

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As commensal bacteria constitute a reservoir of resistance genes for (potentially) pathogenic bacteria, their level of resistance is considered to be a good indicator for selection pressure by antibiotic use and for resistance problems to be expected in pathogens[24]. Hence the aim of this study was to determine the antibiotic sensitivity pattern of *E. coli* isolated from different types of food items collected from in and around the Hyderabad city of Andhra Pradesh, India. The result of this study demonstrated that organisms harboring Extended Spectrum β-Lactamase (ESBL) enzymes are multi-drug resistant showing resistant to 12 or more drugs tested and thus, could pose serious challenge to the public health.

METHODS

A total of 150 samples each comprising 30 numbers; vegetable salad (carrot, cucumber, cabbage, tomatoes, spinach, lettuce, beet root and radish), raw egg-surface, raw chicken, unpasteurized milk of buffalo, and fresh raw meat of sheep were collected randomly from twelve different localities of Hyderabad. All samples were aseptically collected and then packaged in sterile polythene zip bags and carried to the laboratory in aseptic conditions in a cold box within two hours from the time of purchase. Duplicate samples were obtained whenever possible. All samples were analyzed within 2-4 hours after their arrival to the laboratory. A sharp sterile knife was used to cut samples from surfaces in sterile trays.

To isolate bacteria, a 25-g portion of samples (in case of eggs, each egg separately) was placed into sterile 225 mL Tryptic Soy Broth (TSB) for 6-8 h at 37 ºC.

Culture in TSB was streaked onto MacConkey's agar (MAC) plates and incubated for 18-24 hours at 35+2 °C. Lactose fermenting colonies were picked and identified[8] by gram stain, motility and standard biochemical tests, *viz*., catalase, oxidase, fermentation of lactose and glucose using triple sugar iron agar, production of indole, methyl red test, voges proskauer test, urease test and utilization of citrate.

Samples were also processed to isolate other medically important food borne pathogens like *Salmonella* spp, *Staphylococcus aureus* and *Bacillus cereus*. Biochemically confirmed isolates of *E. coli* were subjected to antimicrobial sensitivity testing.

Antimicrobial Susceptibility Testing: Susceptibility tests were performed using the Kirby-Bauer method on Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (NCCLS 2002)[26] and using 19 antibacterial agents: Ampicillin (10 mcg), Amoxycillin (25 mcg), Amoxyclav (20/10 mcg (30 mcg)), Aztreonam (30 mcg), Cefotaxime (30 mcg), Ceftazidime (30 mcg), Ceftriaxone (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Colistin (10 mcg), Co-trimoxazole (1.25/23.75 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), Ofloxacin (5 mcg), Piperacillin+tazobactum (100/10 mcg), Streptomycin (10 mcg), Tetracycline (30mcg), and Tigecycline (15 mcg).

The *E. coli* isolates were inoculated in nutrient broth and incubated at 35+2 °C for five h. The broth was diluted in normal saline solution to a density of 0.5 McFarland turbidity standard. Cotton swabs were used for streaking the diluted broth onto Mueller-Hinton agar plates. After air drying, antibiotic discs were placed 30 mm apart and 10 mm away from the edge of the plate. Plates were inverted and incubated aerobically at 35+2 °C for 16 to 18 hours. The zone of inhibition and resistance was measured, recorded, and interpreted according to the recommendation of the CLSI (NCCLS 2002). The ATCC strain of *E. coli* 25922 was used as a control strain. All the bacteriological media and antimicrobial disks were purchased from HiMedia Laboratories, Mumbai, India. Isolates with resistance or with decreased susceptibility to any of the 3rd Generation Cephalosporin (3GC) were selected for further study.

Extended Spectrum β*-Lactamase (ESBL) Confirmatory Tests*

Double Disc Synergy Test (DDST)[17]: The isolated colonies were inoculated in nutrient broth at 35+2 °C for five h. The turbidity was adjusted to 0.5 McFarland standard and lawn culture was made on Mueller-Hinton agar using sterile swab. An Augmentin disc (20/10 mcg) was placed in the center of plate. Both sides of the Augmentin disc, a disc of cefotaxime (30 mcg) and ceftazidime (30 mcg), were placed with center to center distance of 15 mm to the centrally placed disc. The plate was incubated at 35+2 °C overnight. ESBL production was interpreted as the 3rd-generation cephalosporin disc, inhibition was increased towards the Augmentin disc or if neither discs were inhibitory alone but bacterial growth was inhibited where the two antibiotics were diffused together.

Phenotypic Confirmatory Disc Diffusion Test (PCDDT) for ESBL[25]: ESBL production was confirmed among potential ESBL-producing isolates by phenotypic tests. Lawn culture of the organism was made and a 3rd-generation cephalosporins ceftazidime (30 mcg) disc and ceftazidime + clavulanic acid (30 mcg + 10 mcg) disc was placed with 25 mm apart. An increase of ≥ 5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime was confirmed as ESBL producers.

95% confidence interval (CI) was calculated for incidence, drug resistance and ESBL production in *E. coli* strains. The difference in resistant and susceptibility pattern between ESBL and non-ESBL producers of *E. coli* strains results was analyzed statistically using X^2 testing and *p* value of ≤ 0.05 was regarded as significant.

RESULTS

A total of 99 (66%) biochemically confirmed isolates of *E. coli* were isolated from a total of 150 different food items as listed in Table 1. All 99 isolates of *E. coli* tested for their antibiotic profile against 19 different antimicrobial agents.

Resistance to one or more antimicrobial agents was found in 22 (14.7%) isolates of *E. coli* detected from the total of 150 samples and a pattern of multiple drug resistance was observed (Table 2). The dominant type of resistance was to ampicillin and amoxicillin identically detected in 20 (13.3%) isolates, followed by tetracycline in 19 (12.6%), co-trimoxazole in 17 (11.3%), streptomycin in 12 (8%), ciprofloxacin and ofloxacin in 10 (6.6%) each, cefotaxime in 8 (5.3%), and gentamicin, chloramphenicol, and amoxyclave in 7 (4.6%) of each isolates. Twenty two *E. coli* isolates elicited 18 different patterns of antibiotic resistance to the agents used in this study (Table 2). None of the isolate was found resistant to imipenem, tigecycline and colisitin.

Of the 22 isolates of *E.coli*, eight were screened according to CLSI guidelines and selected for conformational tests of ESBL, namely, DDST

Table 1
Incidence of drug resistant *E. coli* from food items

S. No.	Type of food	No. of samples	Incidence of *E. coli* (%), 95%CI	Incidence of drug resistant *E. coli* (%), 95%CI	Incidence of ESBL Producers-*E. coli* (%), 95%CI
1	Vegetables salad	30	23 (76.7) 57.71-90.06	6 (20) 7.71-38.56	2 (6.7) 0.81-22.07
2	Raw egg - surface	30	18 (60) 40.60-77.34	3 (10) 2.11-26.52	1 (3.3) 0.08-17.21
3	Raw chicken	30	25 (83.3) 65.27-94.35	7 (23.3) 9.93-42.28	2 (6.7) 0.81-22.07
4	Unpasteurized milk	30	13 (43.3) 25.46-62.57	2 (6.7) 0.81-22.07	0 (0)
5	Raw meat	30	20 (66.7) 47.18-82.71	4 (13.3) 3.75-30.72	1 (3.3) 0.08-17.21
Total No. of Samples		150	99 (66) 57.82-73.52	22 (14.7) 9.42-21.35	6 (4) 1.48-8.50

Table 2
Antibiotic resistance profile of isolates of *E. coli*

Non-ESBL Producers	No. of isolates	Source of food
T	1	V
Co	1	RM
A, Amx, T	1	V
A, Amx, Co, T	4	C *2n*, UM, RM
A, Amx, Co, S	1	E
A, Amx C, G,S	1	V
A, Amx, Co, S,T	2	C, RM
A, Amx, Cip, Co, G, S, T,	1	V
A, Amx, AC, Ctx, Cip, Ofx, T	1	E
A, Amx, Cip, Co, G, Ofx, S, T	1	C
A, Amx, C, Cip, Co, G, Ofx, S, T	1	C
A, Amx, Ac, Ctx, C, Cip, Ofx, S, T	1	UM
ESBL Producers		
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, Ofx, T	1	C
A, Amx, Ac, At, Ctx, Caz, Ctr, Cip, Co, G, Ofx, S, T	1	RM
A, Amx, At, Ctx, Caz, Ctr, C, Cip, Co, G, Ofx, S, T	1	E
A, Amx, Ac, At, Ctx, Caz, Ctr, Co, G, Ofx, Pit, S, T	1	V
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, Mrp, Ofx, T	1	V
A, Amx, Ac, At, Ctx, Caz, Ctr, C, Cip, Co, G, Ofx, S, T	1	C

V: Vegetables salad, E: Raw egg - surface, C: Raw chicken, UM: Unpasteurized milk, RM: Raw meat. A: Ampicillin, Amx: Amoxycillin, AC: Amoxyclav, At: Aztreonam, Ctx: Cefotaxime, Caz: Ceftazidime, Ctr: Ceftriaxone, C: Chloramphenicol, Cip: Ciprofloxacin, Cl: Colistin, Co: Co-trimoxazole, G: Gentamicin, Ipm: Imipenem, Mrp: Meropenem, Ofx: ofloxacin, Pit: Piperacillin-tazobactum, S: Streptomycin, T: Tetracycline, Tgc: Tigecycline.

and PCDDT. Of these eight isolates, six isolates found ESBL-positive by the DDST were also ESBL-positive by the PCDDT and the remaining two isolates found ESBL-negative by both the techniques. Thus, a 100 per cent concurrence was noted in the results obtained by the DDST and PCDDT for the eight isolates tested retrospectively. All the six strains of ESBL-producing *E. coli*, showed enhanced susceptibility to ceftazidime and/or cefotaxime in the presence of clavulanic acid, a typical finding for an ESBL producer.

A significant difference in resistant and susceptibility pattern was found between ESBL and non-ESBL producers of *E. coli* strains (Table 2). In all the cases p value was < 0.05. Among non-ESBL producers only two isolates showed resistant to 3rd generation cephalosporin, cefotaxime. Whereas all six ESBL producers were resistant to all 3GC tested and also to aztreonam. Moreover, only ESBL producers have exhibited resistance to meropenem and piperacillin+tazobactum. Among six ESBL producers one strain was resistant to meropenem and another to piperacillin+tazobactam. All the six ESBLs were sensitive only to imipenem, meropenem, piperacillin + tazaobactum, tigecycline, colistin, chloramphenicol, gentamicin, streptomycin, ciprofloxacin and amoxyclav in different patterns.

The number of antibiotics against which each isolate showed resistance ranged between one and 14. Among the non-ESBL producers, two were exhibited resistant to two different single antibiotics (Tetracycline and Co-trimoxazole respectively), six were found to be resistant to less than five antibiotics, five showed resistance to 5-7 antibiotics and three showed resistance to 8-9 antibiotics. In case of ESBL producers, one isolate was resistant to 12 antibiotics, four to 13 antibiotics and one to 14 antibiotics.

DISCUSSION

Antimicrobial resistance has been recognized as an emerging worldwide problem in human and veterinary medicine[2,10] both in developed and developing countries. It is also well documented that widespread use of antibiotics in agriculture and medicine is accepted as a major selective force in the high incidence of antibiotic resistance among gram-negative bacteria[23]. A variety of foods and environmental sources

harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production[3,5].

Several studies have documented the drug resistant *E. coli* and other coliforms in vegetables[29], poultry[18], egg[4], milk[9] and raw meat[35].
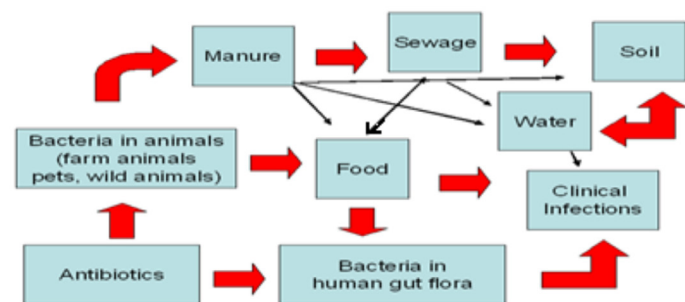

Fig. 1 - Antibiotic resistance flow chart in bacteria and the environment[36].

In this study, the highest percentages of drug resistance in isolates of *E.coli* were detected from raw chicken (23.3%) followed by vegetable salad (20%), raw meat (13.3%), raw egg-surface (10%) and unpasteurized milk (6.7%). The overall incidence of drug resistant *E. coli* was 14.7%.

Antibiotic resistance in *E. coli* is of particular concern because it is the most common Gram-negative pathogen in humans, the most common cause of urinary tract infections, a common cause of both community and hospital-acquired bacteraemia[33] as well as a cause of diarrhea[19]. In addition, resistant *E. coli* strains have the ability to transfer antibiotic resistance determinants not only to other strains of *E.coli*, but also to other bacteria within the gastrointestinal tract and to acquire resistance from other organisms[28].

Different use patterns of antimicrobial agents are expected to have some impact on the distribution of antimicrobial resistance phenotypes[1,22] and possibly of resistant determinants. The result of the antibiotic resistance analysis revealed that among 16 non-ESBLs, only four and two isolates had similar antibiotic patterns to four and five drugs respectively. All the six ESBLs had different patterns of drug resistance.

Current work revealed that all the co-trimoxazole resistant isolates except one were multi-drug resistant. In *E. coli*, trimethoprim-sulfamethoxazole resistance often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in integrons[15,39]. Multiple antibiotic resistance may be acquired through mobile genetic elements such as plasmids, transposons, and class 1 integrons[34].

The number of studies describing the prevalence of ESBL-producing Enterobacteriaceae has increased rapidly around the world[36]. A total of six (4%) ESBL producers were detected in this study, two each from vegetable salads and raw chicken, and one each from raw egg-surface and raw meat. No ESBL producer was detected in the unpasteurized milk.

The result of this investigation shows that organisms harboring Extended Spectrum β-Lactamase enzymes are multi-drug resistant showing resistant to 12 or more drugs tested and thus, could pose serious challenge to the public health. ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents[31].

In recent years, ESBL-producing Enterobacteriaceae isolates have shifted from the hospital to the community and the environment[27]. ESBL-producing Enterobacteriaceae have been recovered from different sources in the community, including cattle, chickens, pigs, raw milk, and lettuce[16,30,37], and a recent study from India reported that a substantial number of tap water samples were contaminated with carbapenemase bla_{NDM-1} producing organisms[38]. Most of the studies on this subject have been conducted in developed countries, but the major epicenters of ESBL-expressing bacteria are located in Asia, Africa, and the Middle East[36].

CONCLUSIONS

Even though the incidence of multidrug resistant and ESBL producers were not high in our study but still it is a matter of concern, since there is a reservoir of antibiotic resistant genes within the community, and that the resistance genes and plasmid-encoded virulent genes are easily transferable to other strains. Pathogen cycling through food is very common and might pose a potential health risk to the consumer.

Therefore, cautions are necessary to decrease the incidence of multi-drug resistant strains of *E. coli* in animals and people. In order to achieve this, good hygienic practices are necessary from the farm to the family table especially in the abattoirs to prevent contamination of cattle and poultry products and abattoir environment with intestinal content. Health authorities should focus on implementing the legislation that forbids irrigation with untreated sewage water of both root and leafy vegetables.

Furthermore, there is a need to emphasize the rational use of antimicrobials and strictly adhere to the concept of "reserve drugs" to minimize the misuse of available antimicrobials in agriculture and medicine. In addition, regular antimicrobial susceptibility surveillance is essential.

RESUMO

Resistência microbiana a drogas em linhagens de *Escherichia coli* isoladas de fontes alimentares

Variedade de alimentos e fontes ambientais contem bactérias resistentes a uma ou mais drogas antimicrobianas usadas em medicina e agricultura. Resistência antibiótica pela *Escherichia coli* é particularmente preocupante porque ela é o patógeno mais comum Gram negativo em humanos. Portanto este estudo foi conduzido para determinar o aspecto de sensibilidade antibiótica da *E. coli* isolados de diferentes tipos de alimentos obtidos ao acaso de 12 localidades de Hyderabad, India. Um total de 150 amostras compreendendo saladas, vegetais, superfícies de ovos crus, galinhas cruas, leite não pasteurizado e carne crua foram processados microbiologicamente para isolar *E. coli* e estudar o quadro de sensibilidade antibiótica pelo método de Kirby-Bauer. A maior percentagem de resistência à droga foi isolada de *E. coli* obtidos de galinha crua (23,3%) seguido de saladas e vegetais (20%), carne crua (13,3%), superfície do ovo cru (10%) e leite não pasteurizado (6,7%). Incidência total de *E. coli* resistente foi de 14,7%. Um total de seis (4%) Extended Spectrum β-Lactamase (ESBL) produtores foram detectados, dois cada de salada de vegetais e galinha crua e um cada de superfície de ovo cru e carne crua. Espécies resistentes a múltiplas

drogas de *E. coli* são matéria de preocupação uma vez que os genes de resistência podem facilmente ser transferidos para outras linhagens. O ciclo do patógeno é muito comum nos alimentos e pode ser risco potencial para a saúde do consumidor. Portanto, para evitar isto boas práticas de higiene são necessárias nos abatedouros para prevenir a contaminação de gado e aves com conteúdo intestinal assim como proibir o uso de águas de esgoto não tratadas para irrigar vegetais.

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