Antimicrobial resistance patterns of pathogenic bacteria isolated from chicken's liver

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Citation

Abstract
Meat and meat products are a main source of pathogenic bacteria in the human food chain. A total of 175 samples of chicken's liver (as raw meats) were examined for the presence of aeromonas, campylobacter, Escherichia coli, salmonella and yersinia. The samples were randomly collected from different butcheries during 10 sampling visits in the Tripoli city- Libya from June 2011 to July 2012. Samples were processed by using standard microbiological techniques. The majority (86.3%) of chicken samples (n = 151) were contaminated with different pathogenic bacteria. The observed contamination was followed the trend \[ E. coli \] \( \Rightarrow \) Aeromonas spp \( \Rightarrow \) Salmonella spp \( \Rightarrow \) Campylobacter spp \( \Rightarrow \) Yersinia spp \] which were 46.3%, 16.57%, 13.14%, 8.57% and 1.71%, respectively. In vitro activities of 12 antibiotic substances against the isolates were determined by disc diffusion test (Kirby Bauer method). The multiple resistances patterns to antibiotics were observed in all isolates. The highest rate of resistance was against Erythromycin and Cephalothin which was 100%. followed by Neomycin (93%) \( \Rightarrow \) Ampicillin (88.7%) \( \Rightarrow \) Sulfamethoxazole trimethoprim (85.7%) \( \Rightarrow \) Tetracycline (84.96%) \( \Rightarrow \) Doxycycline (78.19%) \( \Rightarrow \) Nitrofurantoin (76.69%) \( \Rightarrow \) Amoxicillin (74.4%) \( \Rightarrow \) Nalidixic acid (71.4%) \( \Rightarrow \) Augmentin (64.66%) \( \Rightarrow \) Chloramphenicol (58.64%). This study confirmed that the significant increase in the resistance incidence of the isolated strains from poultry. This was probably due to the excessive use of antibiotics as growth promotion (feed additives) to prevent disease, the human abuse of antibiotics, resistance transfer among different bacteria and possible cross resistance between antibiotics used in poultry, domestic animals and those used in human medicine.

1. Introduction

Meat, raw meat and meat products are very important source of pathogenic bacteria in the human food chain. Contamination in poultry will lead to human illness, but it usually stems from microorganisms that have infected the bird. Aeromonas spp. is Gram-negative, rod-shaped and facultative anaerobic bacteria. It has been widely reported that the Aeromonas spp was isolated from a wide range of mammals, surfaces water, and sewage, in fish, shell fish, birds (1-4). Pathogenicity of Aeromonas spp. are associated with the liberation of virulence factors and cell associated endotoxin (3). Moreover, Aeromonas spp. is purely isolated from liver, lungs, heart and spleen of rabbit with severe outbreak of hemorrhagic septicemia with highly mortality rate (4).
Campylobacter spp is motile, Gram negative, slender curved or spiral rods, appearing vibrioid, and microaerophilic. Although many animal species harbor Campylobacter in their intestinal tract, wild birds and domestic poultry are the most important reservoirs and considered as the largest potential source of Campylobacter for human (5). Normally, Campylobacter infections are self-limiting and the treatment with antibiotics is therefore most often not required. Nevertheless, under definite clinical circumstances, such as in severe cases with prolonged and serious symptoms or in immunocompromised hosts (6). Escherichia coli is the most important agent causing secondary bacterial infection in poultry and may also be a primary pathogen (7). E. coli is the common frequently reported disease in surveys of poultry diseases or condemnations at processing (8). Colibacillosis refers to any localized or systemic infection caused entirely or partially by Avian pathogenic E. coli (APEC). APEC is a very common bacterium which is responsible for significant economic losses in aviculture in many parts of the world (9). In the past few years, both the incidence and severity of E. coli have been speedily augmented and it is possible to maintain and grow to be greater crisis in the poultry industry. Antimicrobial therapy is an important tool in reducing both the incidence and mortality associated with APEC (10). Salmonella spp. is important zoonotic pathogens in humans and animals (11). Salmonella spp causes a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. Food-borne diseases caused by Salmonella spp. occur at high frequently in industrialized nations and developing countries and represent an important public health problem worldwide.12,13 Salmonella is among the major bacterial pathogens of poultry in the all world and most Salmonella infection in humans result from the ingestion of contaminated poultry (14).

Yersinia Spp. are Gram-negative, rod-shaped bacteria which are responsible for disease syndromes ranging from gastroenteritis to plague. These organisms belong to the family Enterobacteriaceae. It includes 11 species; three of which are pathogenic in human: Yersinia pestis (bubonic plague), Yersinia pseudo tuberculosis (mesenteric adenitis), and Yersinia enterocolitica. The latter is an oxidase-negative, non- lactose-fermenting, Gram-negative Coccobacillus that is motile at 22°C but is not motile at 37°C (15, 16). After ingestion of the organism, the terminal ileum is the site of mucosal adherence and penetration, followed by reproduction of the organism in Peyer patches. Nonspecific ileocolitis is often noted, with an inflammatory infiltrate in the lamina propria. Although Yenterocolitica does produce a heat-stable enterotoxin similar to that of E. coli, the enterotoxin does not contribute to the pathogenicity of the organism (15,16). Poultry is one of the commodities most commonly associated with foodborne disease outbreaks in the preceding years. The aim of present study was determine the antimicrobial resistance patterns of various pathogenic bacteria isolated from chicken's liver collected from different butcheries located in Tripoli - Libya.

2. Material and Methods

2.1. Preparation of Samples

The 175 chickens' livers were collected from different butcheries located in Tripoli city, Libya. The samples were then placed in sterile bags, closed tightly, labeled appropriately and immediately transferred to the laboratory, where they processed. All the materials used for this study including culture media, reference antibiotics, and reagents were purchased from Oxoid, Basingstoke, United Kingdom unless otherwise stated.

2.2. Isolation and Identification of Aeromonas spp

The frozen liver samples collected were thawed at room temperature for 3 hours. one gram of each sample were excised using a sterile scalpel and homogenized in 9ml of Tripticase Soy broth (TSB) supplemented with ampicillin (20 μg mL⁻¹). The TSB was then incubated for 24 hour at 37°C. The primary isolation of the organism was obtained by culturing the broth on Rimler-Shotts medium and incubated at 37°C for 18-24 hours. Using a sterile platinum loop, each culture was separately streaked onto the surface of Starch Ampicillin Agar (SAA) plates, labeled and incubated at 37°C for 24 hours, and observed for signs of growth and colony appearance.

Suspected colonies were then transferred onto 5% sheep blood agar and trypticase soy agar (TSA) plates and triple sugar iron (TSI) slant and incubated at 37°C for 24 hour. The isolated bacterial was identified by culture morphology, Gram-stain and biochemical test. The colonies that showed typical reaction in TSI and were positive for cytochrom oxidase and catalase tests were confirmed by using Analytical Profile Index 20E (API 20E) (17, 18).

2.3. Isolation and Identification of Campylobacter spp

The chicken's liver samples were examined for the presence of Campylobacter according to validate method for isolation of Campylobacter from foods specified recommended by the UK Ministry of Agriculture, Fisheries and Food (19). Briefly, frozen samples were thawed at room temperature for about 3 hours. Twenty five grams of each sample were homogenized for 1 min in a stomacher (Stomacher 400 Lab Blender; Seward Medical, London, UK) with 225 ml of Campylobacter selective enrichment broth, Bolton Oxoid, Basingstoke, UK) supplemented with Bolton Broth (SR0183, Oxoid) and Lakedod SR48, Oxoid). The homogenates were then transferred to screw-capped sterile bottles and additional broth was added leaving very little headspaces above the liquid (1.5-2 cm) to ensure microaerophilic conditions. The bottles were then incubated aerobically at 37°C for 4hr followed by 42°C for 48hr (20). Following the enrichment, 0.1-0.2 ml of each enriched samples was streaked onto Blood Free Campylobacter...
Selective Agar Base (CM0739, Oxoid) supplemented with Charcoal Cefoperazone Desoxycholate Agar Supplement (CCDA Selective Supplement) (SR0155, Oxoid). Plates were incubated for 24-48 hours at 42°C under a microaerophilic atmosphere achieved by using Campylobacter Gas-generating Kits (Oxoid, BR56) in conjunction with their catalyst containing jars. Presumptive identification of Campylobacter colonies was based on the colonial appearance and Gram-staining. Microscopic examination revealed Gram-negative with curved or spiral-shaped rods. One to three presumptive Campylobacter colonies from each selective agar plate were sub-cultured and were confirmed to the species level by the traditional biochemical tests, such as motility, catalase and oxidase tests, oxidation (21).

2.4. Isolation and Identification of E. coli

The various liver samples collected were thawed. 10 grams of each sample were weighed and then separately inoculated into 90 ml of Buffer peptone Water (BPW) (CM509, Oxoid, Basingstoke, UK) as enrichment medium, incubated at 37°C for 24 hrs. Subsequently, a loopful of BPW was streaked onto McConkeyagar (MA) and Eosin methylene Blue Agar plates (EMB)(CM 07, CM 69, Oxoid, Basingstoke, UK). The plates were then incubated at 37°C for 24 hours. Isolated black-colored colonies with metallic sheen were again fished out into nutrient broths and incubated at 37°C for 24 hours. The E. coli isolates were screened through the various microscopic examination and biochemical reactions to confirm their identities (22). The various subcultures were streaked onto nutrient agar slants, incubated at 37°C for 48 hours, and then kept in the refrigerator at −20°C for further identification and antibiotic sensitivity studies.

To detect the presence of E. coli 0157:H7 in chicken's liver samples; Isolated strains of E. coli re-streaked onto MacConkey sorbitol Agar(Merck-Germany) and incubated at 42°C for 24 hours. At the end of the incubation, five colonies of sorbitol negative (-ve) were picked up to the agglutination test to determine the strains of E. coli 0157:H7. Cultures identified as E. coli 0157:H7 latex agglutination test (Oxoid, UK) as described by the manufacturer. All the tests were performed on reference-typed culture of E. coli (ATCC 25922).

2.5. Isolation and Identification of Salmonella sp

Isolation of Salmonella were aseptically processed as a following; after thawing the Frozen samples at room temperature for about 3 hours, 25 grams of each sample was excised using a sterile scalpel. Each sample was put into a sterile stomacher bag containing 225 ml of BPW (CM509, Oxoid, Basingstoke, UK) as enrichment medium. The samples were then mixed for 2 minutes using a stomacher ND and incubated at 37°C for 24 hours. After incubation, 1 ml of BPW was transferred into 9 ml of selenite F broth (Merck-Germany) and incubated at 37°C for 18-24 hrs. Following incubation, a loopful of selenite F broth was streaked on to Salmonella Shigella agar (Merck-Germany) which was incubated at 37°C for 24 hours. Non lactose fermenting, with black center colonies and urease negative bacteria had selected and picked up to identify by using API 20E system (bio-Merieux, France) (23). The isolates, which tested positive for Salmonella, were subcultured and then kept in the refrigerator at −20°C for antibiotic sensitivity studies.

2.6. Isolation and Identification of Yersinia enterocolitica

The samples were examined for the presence of Yersinia enterocolitica according to the International Organization for Standardization protocol ISO/DIS 10773). In brief, the samples were thawed and aseptically 25 g of chicken's liver sample was weighed and treated as mentioned before in 225 ml of phosphate-buffered saline supplemented with 1 % mannitol and 0.15% bile salts. In order to improve the chances of recovering pathogenic Y. enterocolitica, enrichment broth was added to each tube containing homogenate supernatant of liver samples and incubated at 4°C for 7 days. After incubation, a loopful of cold enrichment broth was streaked onto Yersinia-selective agar base (YSA) (Oxoid, Basingstoke, United Kingdom) and incubated at 30°C for 18 to 20 hours. Up to five whole small diameter (<1 mm) colonies with typical a bull’s eye appearance (deep red centers surrounded by outer transparent zones) on each YSA plate were taken and streaked again onto blood agar plates MERCK-Germany) for pure culture. One colony from the blood agar was picked up and inoculated onto a urea agar slant (Difco, Detroit, Mich.) then incubated for 24 hours at 30°C. The colonies that were urease positive were selected for further identified by using the API 20E test (Biomerieux, Marcy l'Etoile, France) (24).

2.7. Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method (17) was used for determining the susceptibility of the identified pathogenic bacteria to Amoxicillin (25µg), Ampicillin 10µg, Chloramphenicol (30µg), Neomycin (30µg), Nalidixic acid (30µg), Tetracycline (30µg), Erythromycin (15µg), Augmentin (30µg), Doxycycline (30µg), Nitrofurantoin (30µg), Sulfamethoxazole-trimethoprim (25µg) and Cephalothin (30µg). The antibiotics used were all obtained from Oxoid, UK. The bacterial suspension turbidity adjusted to McFarland standard number 0.5, in Mueller Hinton broth (Merck-Germany) and cultured fluently over the entire surface of Muller Hinton agar (Merck-Germany) with sterile cotton swab. Commercial antibiotic disks containing single concentrations of each antibiotic were then placed onto the inoculated plate surface. The plates were then incubated at 37°C for overnight. The zone of growth inhibition around each disk was measured in millimeters. The method was triplicated and the mean zones of inhibition were interpreted using a zone size interpretation chart provided by the Clinical and Laboratories Standards Institute (17).
3. Results and Discussion

Table 1. Percentage of distribution and prevalence of different bacterial species isolated from chicken’s liver samples.

<table>
<thead>
<tr>
<th>Sr. NO</th>
<th>Bacteria Species</th>
<th>No of Isolates</th>
<th>% of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aeromonas spp.</td>
<td>29</td>
<td>16.57</td>
</tr>
<tr>
<td>2</td>
<td>Campylobacter spp.</td>
<td>15</td>
<td>8.57</td>
</tr>
<tr>
<td>3</td>
<td>Salmonella spp.</td>
<td>23</td>
<td>13.14</td>
</tr>
<tr>
<td>4</td>
<td>E. Coli</td>
<td>81</td>
<td>46.30</td>
</tr>
<tr>
<td>5</td>
<td>Yersinia spp.</td>
<td>3</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td><strong>Total Isolates</strong></td>
<td><strong>151</strong></td>
<td><strong>86.29</strong></td>
</tr>
</tbody>
</table>

The significance of poultry meat as a vehicle for the transmission a variety of diseases has been well known, especially in countries where standards of hygiene are not strictly compulsory. Chicken liver is a specific food type with respect to bacteria risk evaluation. Presently, information is inadequate regarding the prevalence of various pathogenic bacteria in foods in Libya. Therefore, the present study was undertaken to determine the prevalence rate of pathogenic bacteria isolated from chicken’s liver in city of Tripoli- Libya, by using culturing methods and antibiotic sensitivity test. The results of our study showed that bacterial growth was found in 151 out of 175 samples. A total of 151 isolates recovered on MacConkey agar (Oxoid, Basingstoke, UK) were identified to be E. coli, Aeromonas spp., Salmonella spp., Campylobacter spp., Yersinia spp. These were screened through the various microscopic examination and biochemical reactions. The observed contamination was followed the trend E. coli 〉 Aeromonas spp. 〉 Salmonella spp. 〉 Campylobacter spp. 〉 Yersinia spp. (Table 1). To clarify our finding the results were presented as a diagram showed in figure 1.

From figure 1, it can be seen that the isolation of *Escherichia coli* was much higher percentage 81(46.3%) than Aeromonas spp 29 (16.57%), Salmonella spp 23 (13.14%), Campylobacter spp 15 (8.57%) and Yersinia spp. 3 (1.71 %), respectively. Moreover, 14 (17.3%) strains of *E. coli* 0157: H7 was identified out of 81 strains *E. coli* and that confirmed by using antisera (latex agglutination test). *E. coli* is recommended to be totally absent from poultry meat before such can be considered fit for human consumption. Levels obtained from our study are reflections of the high rates obtained from markets. 46.3% (81/175) obtained from work is very high in comparison to other studies (7, 25).

It is well known that *Salmonella* in more than 25 g of poultry meat is considered unsafe for human consumption. From figure 1 it can be detected that 13.4% (33) of chicken liver from the 175 samples obtained were contaminated with *Salmonella*. This percentage was very high in comparison with the 2% from Osogbo (7) but interestingly it was comparable with those reported in some other African countries (25).

On the same figure, it showed that the incidences of a *yersinia spp*. were too low when compared with other enteric pathogenic bacteria although, Y. enterocolitica is a common foodborne pathogen and is implicated in such a wide range of gastrointestinal diseases (26) A study conducted in Argentina (15) showed that 38.65% of meat samples were contaminated with Y. enterocolitica, which was much higher than our results (1.71%).

![Figure 1](image1.png) 
Figure 1. Percentage of selected pathogenic bacteria isolated from chicken’s liver collected from various butcheries located in Tripoli, Libya. Results show that the isolation of *Escherichia coli* was much higher percentage than other isolates. Experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.

![Figure 2](image2.png) 
Figure 2. In vitro antimicrobial resistance patterns of isolated bacterial against different antibiotics. results show the isolates were resistant to a variety of antibiotics tested and the 100% of resistance was observed with both Erythromycin and Cephalothin. Experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.
The antimicrobial susceptibility patterns of the isolated strains were also conducted on isolated pathogenic bacteria. Thirteen different antibiotics were used against identified bacterial species from chicken's liver. The results of this study indicated that a large proportion of the isolates were resistant to a variety of the antibiotics tested. The highest rate of resistance was against Erythromycin and Cephalothin which was 100% followed by Neomycin (93%) > Ampicillin (88.7%) > Sulfamethoxazole trimethoprim (85.7%) > Tetracycline (84.96%) > Doxycycline (78.19%) > Nitrofurantoin (76.69%) > Amoxicillin (74.4%) > Nalidixic acid (71.4%) > Augmentin (64.66%) > Chloramphenicol (58.64%) (Figure 2). The percentages of resistance obtained with these antibiotics are comparable with those reported in other studies in Saudi Arabia (27) and in Ethiopia (28). The FDA has approved a competitive exclusion product designed to prevent the colonization of chicken intestines by pathogenic bacteria, such as Salmonella Spp., Campylobacter Spp., and E. coli, and also to reduce the use of antimicrobials and the spread of antimicrobial-resistance genes. Unfortunately, most of the medication used in the country is available without professional advice due to abnormal and illegal use of antibiotics that may affect public health. Total ban of antibiotics in food animals is not the answer to the problem of antibiotic resistance; it would have serious consequences for the economics of the food industry and animal welfare. Agriculture and the use of antibiotics in livestock have often been singled out as being responsible for the appearance of antibiotic resistance in human pathogens. Yes, it plays a role in the selection of antibiotic-resistant bacteria in animals (29). And even if evidence for the crossover of resistance from animal bacteria to human strains is scarce, we cannot pretend that the problem does not exist. Antibiotic usage needs rationalization, but not to the point of being detrimental to birds’ health and at the expense of poultry farmers.

4. Conclusion

A few number of studies have considered the occurrence and characterization of various pathogenic bacteria in Libya. These findings support the hypothesis that chicken meat is a possible source of many gastric diseases caused by enteric pathogens and may be a reservoir of infection in Libya. We conclude that control of such pathogenic bacteria in chicken meat is crucial for managing infection. The indiscriminate use of antibiotics should be concerned because antibiotics will soon completely lose their effectiveness against microorganisms, finally appropriate cold-preservation of chicken meat will avoid multiplication of contaminants. However, meeting the massive challenge of food safety in the 21st Century will require the application of new methods to identify, monitor and access foodborne hazard.

References


