

Carbapenems resistance among Gram-negative bacteria isolated from poultry samples in Gaza-Palestine

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Abstract

Background: Food is fundamental for everyone's life. Therefore, the safety of food is a high priority in public health. Certain Gram-negative bacteria are important and common cause of human infections and could be transmitted through food handling and consumption. Carbapenemase-producing Gram-negative (CRGN) bacteria are becoming a global threat. Infections caused by CRGN are difficult to cure because the carbapenems are last resort drugs for their treatment. The main objective of this research is to determine the occurrence of Carbapenem-resistance among Gram-negative bacteria isolated from poultry samples.

Methods: Two hundred twenty samples (chicken litters, water, chicken feed, and intestinal content) were collected from slaughterhouses, farms, and homes from different locations in Gaza strip. Samples were cultured onto MacConkey and Blood agar plates. Gram negative isolates were identified using conventional techniques. Disk diffusion method (based on CLSI recommendations) was used to determine the antimicrobial susceptibility against 14 antimicrobials including two carbapenems (Meropenem and imipenem). Carbapenemase production was detected by the Modified Hodge Test (MHT). The Multiple Antibiotic Resistance (MAR) index for each isolate was calculated.

Results: *Escherichia* species were the most frequent isolates (39.5%), followed by non-lactose fermenting *Enterobacteriaceae* (29.5%), and other lactose fermenting *Enterobacteriaceae* (29%). Non-fermenting Gram-negative bacilli were the lowest (4.8%). The majority of isolates were resistant to most tested antimicrobial agents. A prominent ex-

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ception was observed with meropenem, and amikacin with only 4% resistance. A total of 34.7% of isolates were resistance to imipenem. High level of intermediate results was detected for imipenem (45.2%). From a total of 124 isolates, 44 carbapenem-resistant (35.5%) were detected. None of the five meropenem resistant isolates and only five out of the 43 imipenem resistance isolates tested positive for carbapenemase production, suggesting different mechanism of actions. Most isolates showed resistance to three or more antibiotics and are regarded as multidrug resistant strains (MDR). These isolates were demonstrated in 117 (94.3%) with MAR index (higher than 0.3).

Conclusion: Resistance to carbapenems as well as to other antimicrobials was high among Gram-Negative bacteria isolates as indicated by the MAR index. Concerned public health authorities should consider these alarming finding and implement an immediate monitoring program for poultry and prevention measures should also be promoted and implemented.

Keywords

Antimicrobial Resistance, Zoonosis, Carbapenem-Resistance, Gaza-Palestine.

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Background

Methods of raising and slaughtering food-producing animals have been dramatically transformed since the first half of the twentieth century [1]. These unprecedented transformations include more specialized, intensified production and increasing flock/herd sizes. This includes the proliferation of large Animal Feeding Operations (AFOs) and Concentrated Animal Feeding Operations (CAFOs) (Environmental Protection Agency, 2014). These methods raise concerns of transmission of viral and bacterial pathogens and the spread of antibiotic resistance from the perspective of emerging infectious diseases [2]

By the time these intensified farming methods started to be implemented, transmission of infectious diseases amongst the densely confined ani-

mals caused problems for the agricultural industry. Carbapenems are a β -lactam group of drugs that were developed in the 1980s. They are often used as antibiotics of last resort for treating infection due to multidrug-resistant Gram-negative bacilli (GNB). At the beginning, nearly all *Enterobacteriaceae* were susceptible to carbapenems. However, this scenario has changed with the emergence of carbapenem resistant bacteria in the last years [3]. Carbapenems are not approved for use in livestock production anywhere in the world [4]; as a result, animal-feed use is assumed to be rare.

Despite of the lack of direct selection pressure, little is known about the prevalence of carbapenems resistant Gram negative bacilli, and more specifically carbapenems resistant *Enterobacteriaceae*, in poultry and livestock populations and their asso-

ciated environments. Even though there remains a low probability of direct selection, Carbapenem Resistant Gram Negative Bacteria (CRGNB) have been reported by investigators in few studies. *bla*NDM-1 was detected in *Acinetobacter* isolates from a chicken farm [5].

In 2006, a study in Gaza showed that all gram negative were susceptible to imipenem [6]. However, recent studies have reported high percentages of carbapenem resistance in clinical isolates [7]. There are no-published data concerning the prevalence of carbapenems resistance among poultry GNB isolates in the Gaza region. Unfortunately, the antimicrobial drug use in the veterinary sector is uncontrolled especially in poultry and animal husbandry [8].

The poultry sector in Gaza is vital since chicken meat is the major source of protein for over 2 million inhabitants. Given the unstable political and devastated economic situation, abuse and misuse of antimicrobials including those for human is increasingly observed. This may exert selective pressure on bacterial flora of chicken and can result in emerging resistant strains that might find its way to the population in Gaza.

Methods

Isolation Media and Regents

Commercially available culture media including MacConkey agar, Muller Hinton Agar (MHA), Brain Heart Infusion Broth (BHIB) and Triple sugar iron agar (TSIA), Sterile Normal saline, Gram stain, Oxidase test, Citrate test, urease test, Sulfide-indole-motility test (SIM media), Methyl Red (MR) and Voges-Proskauer (VP) (HiMedia, India) were prepared according to manufacturer's recommendations.

Sampling and Transportation

Sterile polyethylene cups were used to collect the fecal specimens, chicken intestine and sterile swabs used for cloacal specimens. Collected samples were

labeled with appropriate data, immediately placed in ice-box and sent within a maximum of two hours to the laboratory for investigation [9]. A total of 220 samples were obtained from four different localities in Gaza, over a period of 6 months (October 2016 to March 2017).

The study was approved by the Medical Laboratory Sciences Department of the Islamic University of Gaza. Verbal consent was obtained from the farm owners before sampling. The researcher did not handle live chicken. Fecal materials (droppings) from chicken were collected. Slaughtered chicken intestinal contents from slaughter houses were obtained.

Microbiological investigation

For the isolation of Gram-negative bacteria, each sample was streaked onto MacConkey agar and blood agar plates and incubated at 37 °C for 24 hours [4]. After the incubation period, positive cultures were subcultured onto MacConkey and isolates were identified based on colony color and morphology in addition to conventional biochemical reactions (e.g, Gram stain, indole, methyl red, Voges-Proskauer and citrate tests, and carbohydrate fermentation test). IdBact software (V1.1) was used in the identification of bacterial isolates based on API20E matrix.

Identified isolates were sub-cultured onto MacConkey agar and then incubated at 37 °C for a period of 24 hours. The next day, a single colony was taken using a sterile loop and inoculated into slant media (TSIA) and were stored at 2-8°C for short-term use. Single colony was taken inoculated into Brain Heart Infusion broth, 900 µL of the overnight culture was added to 100µL sterile glycerol in sterile plastic tubes and were stored at -20°C for long-term use and storage.

Antimicrobial susceptibility testing

The susceptibility of the isolates to carbapenems was determined using the disc diffusion method.

14 antimicrobial drugs were tested performed using Kirby-Bauer disk diffusion susceptibility test according to the procedures recommended by the Clinical Laboratory Standards Institute (CLSI, 2016) [10]. A lawn of standardized inoculum of the test organism was evenly distributed onto the surface of two Muller-Hinton Plates. Antimicrobial susceptibility disks were then placed onto inoculated plates, incubated at 37 °C for overnight. Sizes of the zones of inhibition were measured and interpreted as susceptible, intermediate, or resistant according to the interpretive chart of the CLSI, 2016.

The modified Hodge test

Meropenem and imipenem resistant isolates were tested for carbapenemase production by the Modified Hodge Test (MHT) according to the methods and interpretive criteria of the CLSI, 2016. In short, a lawn of *E. coli* was streaked onto the surface of Mueller Hinton agar plate and allowed to dry. Then a 10-µg meropenem or imipenem disk was placed in the center of the test area. The test organism was then streaked from the edge of the disk to the edge of the plate. After incubation, the plate will be examined for a clover leaf-like indentation [11].

Data analysis

Data collected was summarized, tabulated and analyzed using Statistical Package for Social Sciences (SPSS) software. The results were presented through histograms, tables and pie charts. Multiple Antibiotic Resistance index (MAR index) was calculated for each isolate.

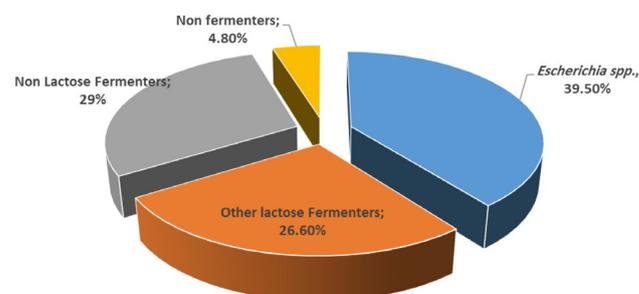
Results

Gram Negative isolates

Bacterial isolates were divided into four groups; *Escherichia* spp. (*E. coli* and *E. hermannii*), other Lactose Fermenter (*Enterobacter gergoviae*, *Citrobacter* spp., *Pantoea* spp., and *Klebsiella* spp.), non-Lactose

Fermenters (*Proteus* sp., *Yersinia enterocolitica*, and *Providencia rettgi*), non-fermenters (*Stenotrophomonas maltophilia*, and *Flavimonas oryihabitans*). *Escherichia* spp. were the most frequently isolated GNB (39.5%), followed by non-lactose Fermenter (29%), other lactose fermenter (26.6%) (Figure 1). The lowest frequency for non-fermenter (4.8%).

Figure 1: Isolated organisms categorized into *Escherichia* species, other lactose fermenting *Enterobacteriaceae*, non-lactose fermenting *Enterobacteriaceae* and the Non-fermenters group.



E. coli (26%) was the most frequently isolated species, followed by *Proteus* spp. (23.4%) and *E. hermannii* (12.9%). Only two genera from the non-fermenter group were isolated; *Stenotrophomonas maltophilia* (3.2%) and *Flavimonas oryihabitans* (0.8%) (Table 1)

Antimicrobial susceptibility testing

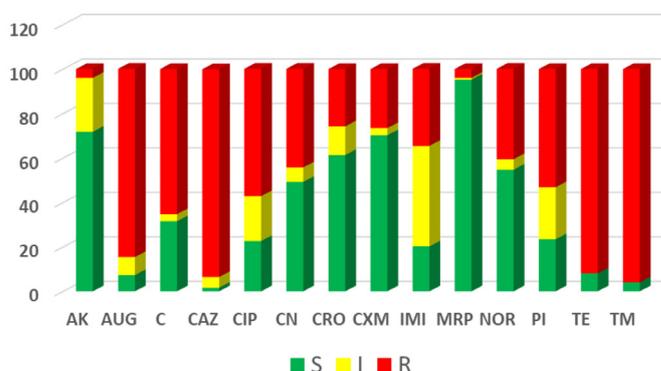
The isolates were obtained from different locations (Shejayya, Joher EL-Deek, Homes, and SAWAFERI), each 67, 14, 12, and 31 isolates, respectively. All isolates were tested for their susceptibility patterns to 15 antimicrobial agents. The percentage of isolates susceptible, intermediate and resistant to each antimicrobial agent is depicted in (Figure 2).

All isolates in general, exhibited resistance to at least one of the tested antimicrobial agent. Ceftazidime (1.6%), trimethoprim (4%), amoxicillin/clavulanic acid (7.3%) and tetracycline (8.1) were shown to be least effective antimicrobials as evident by the

Table 1. Frequency of Gram-negative bacilli isolates recovered from chicken samples .

Isolate	Frequency	Percent
<i>E. coli</i>	33	26.6
<i>Proteus ssp.</i>	29	23.4
<i>Escherichia hermannii</i>	16	12.9
<i>Citrobacter ssp.</i>	15	12.1
<i>Enterobacter gergoviae</i>	12	9.7
<i>Klebsiella ornithinolytica</i>	6	4.8
<i>Stenotrophomonas maltophilia</i>	4	3.2
<i>Pantoea ssp.</i>	4	3.2
<i>Yersinia enterocolitica</i>	2	1.6
<i>Providencia rettgi</i>	2	1.6
<i>Flavimonas oryihabitans</i>	1	0.8
Total	124	100.0

Figure 2: Antimicrobial susceptibility of 124 Gram negative isolates.



AK: Amikacin, AUG: Amoxicillin/clavulanic acid, C: Chloramphenicol, CAZ: Ceftazidime, CIP: Ciprofloxacin, CN: Gentamicin, CRO: Ceftriaxone, CXM: Cefuroxime, IMI: Imipenem, MRP: Meropenem, NOR: Norfloxacin, PI: Piperacillin, TE: Tetracycline, TM: Trimethoprim

Table 2. Antibiotic resistance profiles of the isolated organisms.

Antimicrobial	<i>Proteus ssp.</i>	<i>Yersinia enterocolitica</i>	<i>Enterobacter gergoviae</i>	<i>Providencia rettgi</i>	<i>E. coli</i>	<i>Flavimonas oryihabitans</i>	<i>Stenotrophomonas maltophilia</i>	<i>Citrobacter ssp.</i>	<i>Escherichia hermannii</i>	<i>Klebsiella ornithinolytica</i>	<i>Pantoea ssp</i>
N	29	2	12	2	33	1	4	15	16	6	4
Resistance	%										
Amikacin	6.9	0	8.3	0	6.1	0	0	0	0	0	0
Amoxicillin/ clavulanic acid	79.3	50	66.7	100	90.9	100	100	100	81.3	83.3	75
Chloramphenicol	58.6	50	50	50	69.7	100	75	53.3	81.3	66.7	100
Ceftazidime	89.7	50	100	100	97	100	100	100	81.3	100	100
Ciprofloxacin	58.6	50	50	50	54.5	100	100	40	75	33.3	75
Gentamicin	34.5	100	50	50	48.5	100	75	26.7	43.8	66.7	25
Ceftriaxone	17.2	0	25	0	36.4	100	25	26.7	31.3	16.7	0
Cefuroxime	10.3	50	50	0	30.3	0	75	20	18.8	33.3	50
Imipenem	37.9	50	25	50	36.4	100	50	46.7	31.3	0	0
Meropenem	0	0	16.7	0	9.1	0	0	0	0	0	0
Piperacillin	37.9	50	50	0	72.7	100	50	40	50	50	100
Tetracycline	89.7	50	83.3	100	93.9	100	75	100	100	83.3	100
Trimethoprim	93.1	100	91.7	100	97	100	75	100	100	100	100
Norfloxacin	46.2	0	71.4	50	38.9	100	33.3	16.7	60	0	0

high resistance percentages. While meropenem was the most effective with 95.2% activity followed by amikacin (71.8). High percentage of intermediate results were detected for imipenem (45.2%) and amikacin (24.2%). (Figure 2).

The percentage of antimicrobial susceptibility of all isolates are shown in Table 2. In general, almost all isolates showed high percentage of resistance to trimethoprim, tetracycline, amoxicillin/clavulanic acid and ceftazidime. *Enterobacter gergoviae* and *E. coli* were the only species that showed resistance to meropenem (16.7%) and (9.1%), respectively.

Table 3. Carbapenems resistance according Location.

Location	Carbapenems Resistance		Non Carbapenems Resistance		Total	
	N	%	N	%	N	%
Shejaya	17	25.4	50	74.6	67	100.0
Joher EL-Deek	7	50.0	7	50.0	14	100.0
Home	6	50.0	6	50.0	12	100.0
Sawaferi	14	45.2	17	54.8	31	100.0
Total	44	35.5	80	64.5	124	100.0

P value= 0.08

Figure 3: Modified Hodge test plate for carbapenemase production (isolates number 196 and 203 are positive while isolates 137 and 175 are negative).



Carbapenems resistance

An isolate was considered as carbapenems resistant if it exhibited resistance to either or both of the tested carbapenems (meropenem and imipenem). The percentage detected in this study was 35.5% with most resistance against imipenem and few cases only were resistant to meropenem.

No significant differences in carbapenems resistance among the isolated organisms with regard to the source (P value >0.05), although, isolates from Shejaya area had lower resistance rates (Table 3).

Carbapenemase activity

MHT was used to check for the production of carbapenemase by IMI and MRP resistant isolates (Figure 3). None of the meropenem resistant isolates and only five out of the 41 imipenem resistant isolates tested positive for carbapenemase production. MHT using meropenem 10 µg (MRP10) and imipenem (IMI 10) discs on a Muller-Hinton agar plate.

Multiple antibiotic resistance (MAR) index

The majority of isolates were resistant to three or more antibiotics which are considered as multidrug resistant strains (MDR). MDR isolates were present in 117 of the isolates (94.3%). The frequency of MDR to 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 of total 14 antimicrobial agents was 5 (4%), 7 (5.6%), 12 (9.6%), 14 (11.2%), 19 (15.3%), 15 (12.0%), 21 (16.9%), 9 (7.25%), 9 (7.25%), and 6 (4.8%), respectively.

Discussion

The increasing prevalence of antimicrobial resistance created serious problems for treatment of bacterial infections and continue to be a major challenge for public health worldwide. The high emerging of multidrug resistance is aggravated by the use of antibiotics without restrictions in agriculture, especially in food animals. The spread of antibiotic resistant bacteria from chicken or animal samples in general

poses public health risk and increases the likelihood to spread in the human population.

In this study, we aimed to investigate carbapenems resistance among GNB isolated from chicken. The overall percentage of CR among GNB was 35.5%. This is not comparable to the prevalence rate of 59% obtained from Spain [12, 13] but much higher than that obtained from Qatar (2.2%) [14]. A study by Gregg *et al.*, (2018), showed greater than 50% CRE in any of the isolates obtained from poultry meat [15], while Gernot *et al.*, (2014) showed no CRE in any of the isolates obtained from Chicken Meat in Austria [16].

The reasons for these differences may be due the time each country started using carbapenems in clinical practice and regulations and restrictions imposed on antibiotic use in animal farm. Other factors may contribute to variable spread rates of resistance (sample size, sample sources, time of the study took place, type of isolated bacteria, average cleaning farmer, ventilation inside farms and the use of machines in farms).

In this study, *Escherichia* spp. were the most frequently isolated bacterial species (39.5%). This result is close to the results obtained by Chika *et al.* who reported *E. coli* (30.3%) as the most prevalent organisms isolated from cloacal swabs of poultry birds [17]. However, it differs greatly from our results in that they isolated from *Pseudomonas aeruginosa*, *Klebsiella* species in abundance (32.7%, 34.8%, respectively). Chika *et al.* (2017), reported in their study the isolation of *E. coli*. as the most prevalent organisms isolated from the Poultry samples imipenem (31.0%), meropenem (58.6%) and ertapenem (75.9%) [4, 18]. Akinduti *et al.*, (2012) reported in their study that *E. coli*, *Klebsiella* species and *P. aeruginosa* were the most prevalent organisms isolated from environmental samples including samples from poultry farms [19]. Dandachi *et al.* reported in their study that *E. coli* (92%) and *K. pneumoniae* (3%) were the most prevalent organisms isolated from fecal swabs of 49 examined poultry farms [20].

Most of the 117 isolates (94.3%) obtained in this study had resistance to three or more antibiotics and are regarded as MDR. The prevalence is almost close to the one previously reported in broiler chickens 42%-83.3% in Egypt [21]. However, our result is relatively similar to studies reported from Romania (69%) [22], and Ecuador (60%) [23], but it is much higher than the ones described in Germany (44%) [24], Japan (23%) [25], and Vietnam (3.2%) [26].

The occurrence of CRE in livestock and seafood has been also reported in African, American, Asian, and European countries. Two studies investigated the transmission of CRE between animals and exposed humans [27].

A study that investigated the susceptibility of the bacterial isolates to the carbapenems including imipenem, meropenem and ertapenem, showed that out of the 168 *E. coli* isolates recovered, a total of 87 isolates (51.8%), 93 isolates (55.4%), and 146 (86.9%) were resistant to imipenem, meropenem and ertapenem, respectively [17]. Out of the 33 *E. coli* isolates recovered in our study, 36.4% and 9.1% were resistant to imipenem, and meropenem, respectively. Based on the results described by the study by Webb and others, CRE appear to be lower than the previous ratio (34%, 8.3%) among poultry and turkey in Algeria, respectively [28, 29]. Ayandiran *et al.* (2018), reported that *Enterobacteriaceae* isolates from poultry farms samples in Nigeria were highly resistant to imipenem and meropenem [30].

The overall CRE detected in this study was 35.5% and mostly were resistance to imipenem and only few percentages were resistant to meropenem. In Southwest Nigeria, Ogunleye *et al.* (2008) reported that *E. coli* isolates from poultry were highly resistant to imipenem and meropenem [31].

Effective monitoring of the emerging antimicrobial resistance in zoonotic pathogens is important issue to the containment of any disease outbreak due to these microbes. Proper sanitization, improved hygienic practices and animal immunization

may reduce the need for antimicrobials in production of food-producing animals [17].

In our study, the dominant resistance was to tetracycline in 114 (91.9%) of the isolates, due to its low cost, efficacy, and lack of side effects. The tetracycline family is one of the most commonly used antibiotics in the poultry industry in certain countries. It has been widely used in the prevention and treatment of poultry colibacillosis [32].

A recent study from Zimbabwe reported that the highest antimicrobial susceptibility was observed for ciprofloxacin (100%) and gentamycin (97.1%), these results are much better than those obtained in our study (22.6%), (49.2%) respectively [33].

Conclusions

Resistance to carbapenems as well as to other antimicrobials among poultry isolates was high as indicated by the MAR index. Veterinary health authority in Gaza should consider these alarming findings and implement an immediate antimicrobial resistance monitoring program and regulate antibiotics use in poultry farms.

Acknowledgements

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Ethics approval and consent to participate

The study was approved by the Medical Laboratory Sciences Department of the Islamic University of Gaza. Verbal consent was obtained from the farm owners before sampling. The researcher did not handle live chicken.

Authors' contributions

AAM was involved in the literature search, study design, statistical analysis, and data interpretation; MRA was involved in sample processing and analysis and in writing the manuscript; MAS and HIH

were involved in sample collection, analysis and literature search.

All authors read and approved the final manuscript.

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