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Class 1 Integron-Associated Multidrug Resistance In Some Food Borne Pathogens

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ABSTRACT

Spread of multidrug resistant (MDR) bacteria in meat products has great impact on human health. Twenty *E. coli*, 10 Salmonella and 25 *S. aureus* isolates, recovered from 250 retail meat samples purchased from various supermarkets in Zagazig, Egypt, were assayed for antimicrobial susceptibilities, antimicrobial resistance genes and the presence of class 1 integrons. Frequent resistance to amoxicillin- clavulanic acid and erythromycin was detected in *E. coli* and Salmonella. Meanwhile, *S. aureus* was frequently resistant to clindamycin, chloramphincol, erythromycin and sulfamethoxazole-trimethoprim. 10 *E. coli*, 6 Salmonella and 12 *S. aureus* exhibited multidrug resistance phenotypes (resistant to 3 drug or more). Eight out of 25 *S. aureus* isolates (32%) were MDR and vancomycin resistant (VRSA) with MIC ranged from 64 to 1024 µg/ml. *Van* A, *van* H, *van* S and *van* B were implicated in isolates of the area under study. Class 1 integron was detected in 9/16 (56%) MDR isolates of *E. coli* and Salmonella. Nevertheless, all VRSA contains no integron. These results highlighted the role of retail meat as a potential source for multidrug-resistant *E. coli*, Salmonella and VRSA.

INTRODUCTION

Meat and meat products are recognized as a major source of food borne pathogens causing food poisoning in humans. Currently, the most important pathogens associated with meat products are *Staphylococcus* species *E. coli* and *Salmonellae (1)*. Antimicrobial resistance, in particular multidrug resistance (MDR), is an increasing problem worldwide that is exacerbated by the diminishing number of new antimicrobial drugs in the pharmaceutical pipeline and considered as a serious danger to public health *(2)*. Surveys conducted by the National Antimicrobial Resistance Monitoring System (NARMS) indicate that retail meat is frequently contaminated with multidrug-resistant *Campylobacter*, *Salmonella*, *Enterococcus* species and *Escherichia coli (3)*.

Multidrug-resistant *S. aureus* is wide spread in the environment and has been recovered from foodstuffs, nasal mucosa and skin of healthy humans, clinical cases, food-producing animals, food-catering and aquatic environments *(4)*. Vancomycin resistance has been perceived as a fearsome threat to the already challenging therapy of MRSA and MDR-MRSA *(5)* where there are increasing numbers of reports indicating the emergence of vancomycin-resistant *S. aureus* (VRSA) strains in many intensive care units in different countries without full explanation for the sources of the dissemination of these strains.

The acquisition of an array of resistance genes by horizontal transfer, mediated by plasmids, via the food-chain is currently thought to play an increasing role in the development and dissemination of multidrug resistance *(6)*.

A substantial portion of the resistance genes present on plasmids is sometimes integrated into DNA elements called integrons *(7)*. These integrons, the fluid element of genetic transmission, have drawn great attention in mediating bacterial drug resistance, that constitute a site-specific recombination system capable of integrating and expressing the antimicrobial resistance genes in cassette structures (**6**). Integrons promote the capture of one or more gene cassettes within the same attachment site, thereby forming composite clusters of antibiotic resistance genes *(8)*.

To date, nine classes of integrons have been retrieved from GeneBank. Each class is distinguished by differences in the sequences of the integrase genes. However, only the first 4 classes have been confrimed *(9)*. Class 1, the best characterized integron, has been reported in food pathogens of several Gram-negative and Gram positive bacteria. Integrons of this class comprise 2 conserved segments flanking another, of variable length, within which are found antibiotic resistance gene cassettes. The 5′ conserved end (5′CS) encodes a DNA integrase (IntI1) that mobilizes and inserts gene cassettes through a site- specific recombinational mechanism at a specific site (attI) adjacent to the IntI gene (*10*). Also, it contains a promoter sequence needed for the expression of most of the genes carried on cassettes. The 3′ conserved end (3′CS) includes a truncated antiseptic resistance gene (*qac*EΔ1), a sulphonamide resistance gene (*sul*1) and an open reading frame (orf5) of unknown function (*11*).

Numerous studies have focused on the prevalence and characteristics of class 1 integrons among Gram negative bacteria, however, integrons have been found in Gram□positive bacteria, the role that integrons play in drug-resistant *Staphylococcus aureus* (SA) remains unclear (*12*). Hence, this study aimed to characterize class 1 integrons in multidrug-resistant *E. coli*, Salmonella and vancomycin resistant *Staphylococcus aureus* isolates from meat and meat products

MATERIAL AND METHODS

Sampling and bacterial identification

Two hundred and fifty samples of fresh meat and meat products (minced meat, sausage, burger and blobev), 50 for each, were collected from 14 supermarkets in Zagazig city, Sharkia Governorate. All samples were subjected to conventional identification using methods described by ISO, Oxoid, U.S. Food and Drug Administration Bacteriological Analytical Manual (*13*). *E. coli* and Salmonella were further identified with API identification kits (BioMerieux, Marcy, France) and serotyped using commercial antisera (Difco, Detroit, Michigan) used according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Disc diffusion

The antibiotic-resistance profile was determined by the disc agar diffusion technique (*14*) using 9 antibiotic discs. *S. aureus* isolates were tested for their susceptibility to the following antimicrobial agents: methicillin (ME), amoxicillin- clavulanic acid (AMC), vancomycin (VA), gentamicin (CN), clindamycin (DA), ciprofloxacin (CIP), chloramphenicol (C), erythromycin (E) and sulfamethoxazole-trimethoprim (SXT). Moreover, *E.coli* and Salmonella isolates were tested for their susceptibility to the following antimicrobials: gentamicin (CN), streptomycin (S), ciprofloxacin (CIP), amoxicillin- clavulanic acid (AMC), ceftriaxone (CRO), doxycyclin (DO), chloramphenicol(C), erythromycin (E) and sulfamethoxazole- trimethoprim (SXT).

Broth microdilution

The minimum inhibitory concentrations (MICs) of vancomycin against *S. aureus* isolates (Sigma, USA) were determined by reference broth microdilution method according to Clinical and Laboratory Standards Institute (*15*) guidelines using Muller Hinton broth medium (Difco, USA) and custom-designed 96-well panels (Corning, USA). The concentration of stock solution should be 1000 mg/L or greater and the inoculum size was approximately $5x10^5$ CFU/ml. A double fold serial dilution was made for each antimicrobial agent starting from a concentration of 1024µg/ml.

Isolation of plasmid DNAs

Plasmid DNAs of multidrug resistant isolates were isolated using QIAprep Spin Miniprep Kits.

PCR amplification

PCR amplification was performed with a PTC-100 programmable thermal cycler (Peltier-Effect cycling, MJ, Research, INC., UK) in a volume of 25 *µl* consisting of 12.5 µl of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 0.1µl of 100 pmole of each primer (Sigma, USA), 2 µl of template DNA and water nuclease-free up to 25 µl.

Primers sequences and PCR amplification cycles of oligonucleotide primers among MDR isolates are illustrated in Table (1).

Table 1.Oligonucleotide primers used for detection of *van* genes among VRSA

Target gene	Sequence (5̇-3̇)	PCR amplification cycles	Product size (bp)	Reference
Van A	F:ATGAATAGAATAAAAGTTGC R:TCACCCCTTTAACGCTAATA	35 cycles: 98°C, 10s; 50°C, 1min and 72°C, 30s.	**474**	*16*
Van H	F:ATCGGCATTACTGTTTATGGAT R:TCCTTTCAAAATCCAAACAGTTT	40 cycles: 94°C, 30s; 60°C, 2min and 72°C, 55s.	**943**	*17*
Van X	F:ATGGAAATAGGATTTACTTT R:TTATTTAACGGGGAAATC	35 cycles: 98°C, 10s; 50°C, 1min and 72°C, 30s.	**609**	*18*
Van R	F:AGCGATAAAATACTTATTGTGGA R:CGGATTATCAATGGTGTCGTTGG	40 cycles: 94°C, 30s; 62°C, 2min and 72°C, 2min.	**645**	*17*
Van S	F:AACGACTATTCCAAACTAGAAC R:GCTGGAAGCTCTACCCTAAA	40 cycles: 94°C, 30s; 60°C, 2min and 72°C, 2min.	**1094**	*17*
Van B	F:GTGACAAACCG GAG GCGAGGA R:CCGCCATCCTCCTGCAAAAAA	30 cycles: 94°C, 30s; 50°C, 45s and 72°C, 30s.	**800**	*16*
*Intl**	F: CCT CCC GCA CGA TGA TC R: TCC ACG CAT CGT CAG GC	30 cycles: 94°C, 45s; 60°C, 45s and 72°C ,55s	**280**	*9*
*Intl***	F:GCCACTGCGCCGTTACCACC, R:GGCCGAGCAGATCCTGCACG	35cycles: 94°C, 30s; 60°C, 40s and 72°C, 60s.	**898**	*19*

*Intl**: Class 1 integron of *S. aureus*

*Intl***: Class 1 integron of Enterobacteriaceae

Table 4. Occurrence of *van* genes among VRSA isolates from meat and meat products with different minimal inhibitory concentration to vancomycin

Code No.	MIC	MBC	*Van* genes					
			Van A cluster					*Van* B
			Van A	*Van* H	*Van* X	*Van* S	*Van* R	
77M	1024	1024	+	-	-	+	-	+
83M	512	1024	+	-	-	-	-	-
88M	512	1024	-	+	-	-	-	+
90M	256	512	-	+	-	-	-	+
60M	128	256	-	-	-	-	-	-
196Sa	128	256	+	-	-	-	-	-
200Sa	64	128	-	-	-	-	-	+
94M	64	128	-	+	-	-	-	-

(+): Positive (-): Negative (M): Minced meat (Sa): Sausage (MIC): Minimum inhibition concentration. (MBC): Minimum bactericidal concentration

Genetic characteristics of the MDR isolates

Class 1 integrons were screened among 8 multidrug vancomycin resistant *S. aureus* and 16 multidrug resistant Enterobacteriaceae members (10 *E. coli* and 6 Salmonella isolates) using specific primers for integrase gene (intI1) and none of multidrug resistant *S. aureus* were integron-positive whereas 9 members of multidrug resistant Enterobacteriaceae (5 *E.coli* and 4 Salmonella) harboured these integrons (Fig 5. Table 5).

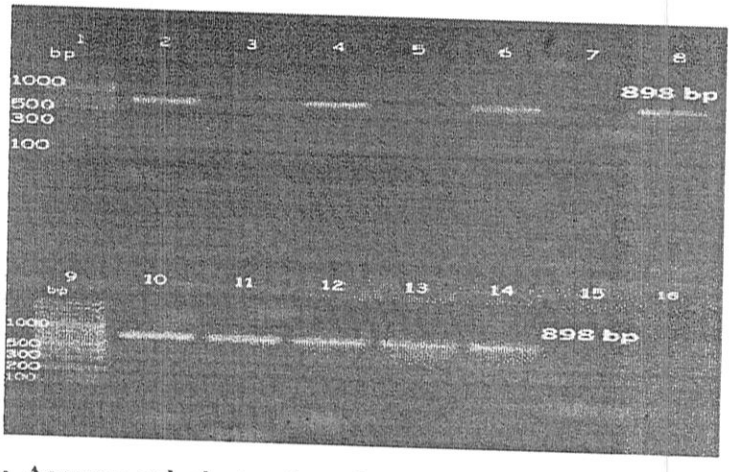

Fig.5.: Agarose gel electrophoresis of class1 integron among DNA products of multidrug resistant *E. coli* and Salmonella isolates. Nine isolates only representative class 1 integron positive. lane 1: 100 bp marker (ladder), lanes 2, 4, 6, 8, 10: *E. coli* isolates of code No. 183Sa, 91M, 57M, 66M and 82M respectively. Lanes 11, 12, 13, 14: Salmonella isolates of code No. 75M, 161Sa, 198Sa and 193Sa, respectively.

Table 5. Characterization of integron positive isolates

Isolate code No.	Isolate serotype	Antimicrobial resistance pattern
75M	*S.* Typhimurium	CN,S,AMC,DO,C,E,SXT
161Sa	*S.* Typhimurium	CN,S,AMC,E
193Sa	*S.* Typhimurium	CN,S,AMC,CRO,DO,E,SXT
198Sa	*S.* Enteritidis	S,AMC,DO,C,E,SXT
66M	*E. coli* (O26:K58)	CN,S,AMC,E,SXT
82M	*E. coli* (O78:K80)	CN,S, CIP, AMC, CRO, Do, C, E, SXT
57M	*E. coli* (O26:K58)	CN,S, AMC, E
91M	*E. coli* (O127:K63)	S, AMC,CRO,C,E,SXT
183Sa	*E. coli* (O111:K58)	CN,S, AMC,E

(CN): Gentamicin, (SXT): Sulfamethoxazole -trimethoprim, (CRO): Ceftriaxone, (CIP): Ciprofloxacin, (DO): Doxycyclin, (S): Streptomycin, (C): Chloramphenicol, (AMC) : Amoxicillin-clavulanic acid, (E): Erythromycin, (M): Minced meat, (Sa):Sausage.

DISCUSSION

In various countries, it is noted that antimicrobials can be purchased without any prescription and indiscriminate use of antimicrobial agents by unskilled practitioners both in the veterinary and public health sectors become common. Furthermore, there is lack of quality compliance and monitoring drugs at all levels leading to presence of MDR pathogen in foods of animal origin (***20***).

In the present study, 69 staphylococci isolates were isolated from 250 examined samples (27%). The prevalence of staphylococci differed among countries such as 63% in Georgia (***21***), 16.4% in Iowa (***22***), 12% in Italy (***23***) and 7.6% in Spain (***24***). Phenotypic identification classified the staphylococci isolates into *S. aureus* and coagulase negative staphylococci. In this work, *S. aureus* was found in 25 meat samples (10%). Similar results were observed in a study carried out in Korea (***25***) but was lower than that in Netherlands (45.57%) (***26***) and higher than that in China (3.1%) (***27***).

Regarding to the incidence of *E. coli* and *Salmonella* spp in obtained meat samples, they were recorded with lower percentages (8% and 4%, respectively). This recovery rate was varied among many countries such as in Vietnam where the prevalence of *E. coli* and *Salmonella* Spp were 90% and 60%, respectively (***28***), 36% and 1%, respectively in Northwestern Greece (***29***) and 52% and 3.2%, respectively in Iran (***30***). The variation in the results obtained by different investigators may be due to difference in manufacturing practices, handling, environmental conditions or time of exposure.

E. coli O111 was the most prevalent serotype among *E. coli* isolates with a percentage of 40% while *Salmonella* Typhimurium serotype was predominated with a higher percentage (50%). Similar findings were obtained with several authors (***31, 32, 33***).

In connection with antimicrobial resistance pattern of *S. aureus* isolates, all *S. aureus* isolates were resistant to methicillin (MRSA) as reported by other investigators (***34, 35***) who attributed these resistance to the methicillin inhibition of penicillin-binding proteins *(PBPs)* that are involved in the synthesis of peptidoglycan, an essential mesh-like polymer that surrounds the cell, through the expression of a foreign PBP *(PBP2a)*. The incidence of 8 out of 25 *S. aureus* isolates which were resistant to vancomycin and

multidrug resistant represents the great hazard on the public health. Several investigators recorded the emergence of multidrug resistant VRSA isolates in many intensive cares in different countries as in Egypt 10 VRSA were obtained isolates from 220 *S. aureus* isolates (*36*). Two vancomycin resistant *S. aureus* among 783 isolates were reported in India (*35*). Meanwhile, one isolate of VRSA was recorded in Pakistan and in Iran *(37, 38)*.

The MIC of vancomycin resistant *S. aureus* isolates showed a wide range between 64-1024 µg/ml. These were all MRSA and resistant to majority of the other antibiotics tested. Higher MIC values were also recorded in Iran *(38)* where only one VRSA isolate was recovered with MIC value of 512 µg/ml. Meanwhile, somewhat lower MIC values were obtained in India (16-64 µg/ml) *(39)* and in Iraq (32-128 µg/ml) *(40)*.

Overall, MIC/MBC confirm those of previous studies as (*41, 42*) which show higher vancomycin MICs and MBCs for VRSA strains. Thus, bactericidal activity, which determined by MBC/MIC ratio, shows that vancomycin is considerably loss it's bactericidal activity and many sights are directed for selection of another bactericidal antibiotic.

Well coincidence between genotype and phenotype for 7 vancomycin resistant *S. aureus* was found as the results confirmed the possession of *van* genes by the plasmids of VRSA isolates. Via these seven isolates, three exhibited part of *van* A cluster, one harboured *van* B only and three isolates accommodated part of *van* A cluster and *van* B together (one of them of the highest MIC value (MIC ≥ 1024 µg/ml) harboured three genes (*vanA*, *vanS* and *van*B)).

Dezfulian *et al.* and Woodford *et al.* (*38, 43*) attributed the difficulties with amplification of other genes in *van*A cluster to disruptions of these regions with insertion sequences (multiple mutations that occurred during replication of bacterial genes having species specificity and species diversity or during inter-species mobilization of resistance genes or gene acquisition).

Alongside, only one VRSA isolate was negative for *van* genes by PCR, although it showed vancomycin MIC value equal 128 µg/ml. Therefore, the absence of *van*A/B genes in this strain does not rule out that this strain is not VRSA. **Cui *et al.* and Palazzo *et al.*** (*41, 44*) hypotheses that cell wall thickening is responsible for development of vancomycin resistance as they suggested that dense accumulation of vancomycin molecules within the thickened cell-wall significantly delays the timing of complete inhibition of cell-wall synthesis by not allowing efficient penetration of vancomycin molecules through the thickened cell-wall layers.

The majority of reports recorded the emergence of VRSA and VISA (vancomycin intermediate *S. aureus*) in clinical sample without harboring of any *van* genes (*45, 46, 59*).

Regarding to antibiogram of *E. coli*, similar findings have been reported in previous studies in Malaysia and United States where there were high frequencies of *E. coli* antimicrobial resistances to erythromycin and amoxicillin clavulanic (*48, 49*). Furthermore, multidrug resistance was obtained in 50% of isolates which was higher than that recorded in Japan by percentage 40.6% (**51**) and lower than that obtained in China by percentage 85% (*50*).

As regards antibiotic resistance of Salmonella, it is in conformity with that recorded in previous study (*52*). Also, it was noted an incidence of multidrug resistance among 60% of Salmonella isolates which was higher than that obtained in many reports (*51, 47*) with percentages 28.5% and 14.4%, respectively.

PCR assay washout to detect class 1 integrons among MDR *S. aureus* isolates. These results go hand in hand with many researchers (*9, 53, 54*).

On other hand, class 1 integrons were detected in 9 out of the 16 Enterobacterial isolates (5 *E.coli* and 4 Salmonella) with

percentage of 56%. Other reports have also revealed the prevalence of class 1 integrons in Gram-negative isolates from food samples as 12% in Norway (*55*), 38% in Thailand (*10*), 41.3% in United States (*56*), 45% in China (*50*) and 99% in Portugal (*57*) indicating that class 1 integrons are widespread among Gram-negative isolates.

The other multidrug resistant isolates had other antibiotic resistance genes not encoded by class 1 integron. These resistances may have resulted from chromosomal mutation, plasmid acquisition or presence of other integrons besides class 1 types (*58*).

Although our study shows that integrons contribute to the occurrence and transmission MDR in Enterobacteriaceae, further studies need to be conducted to determine gene cassettes involved in transmission of linked resistance genes carried by these integrons.

Conclusion

This is believed to be the first report at least in Egypt for isolation of VRSA (8 isolates) harboring *van* genes from food products where meat serves as a vehicle for the acquisition of vancomycin resistance to human and may lead to a high fatality rate especially among immunocomprised individuals. Besides, no incidence was detected for class 1 integron among all MDR *S. aureus* isolates whereas 9 members (56%) of MDR Enterobacteriaceae (5 *E.coli* and 4 Salmonella) harboured these integrons which were located on plasmids and act as important contributors and possible mechanism to the development and dissemination of multidrug resistances to human. Finally, ciprofloxacin is a drug of choice for treatment of all food borne microorganisms obtained in this study.

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الملخص العربي

الانتجرون فئة ١ والمرتبط بالميكروبات متعددة المقاومة للمضادات الحيوية والمعزولة من الاغذية

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انتشار البكتيريا ذات المقاومة للأدوية المتعددة في منتجات اللحوم له تأثير كبير على صحة الإنسان. تم تقييم ومعايرة عشرين عترة من الميكروب القولونى , ١٠من السالمونيلا و ٢٥ من المكور العنقودى الذهبي - المعزولين من ٢٥٠ عينة لحوم تم شراؤها من محلات تجارية مختلفة بمدينة الزقازيق، مصر- لقابليتهم للمضادات الحيوية المختلفة, وجود جينات مقاومة للمضادات الحيوية ووجود الفئة الاولى من الانتجرون. تم الكشف عن المقاومة المتكررة إلى حمض الكلافولانيك – أموكسيسيلين و الاريثروميسين في عترات الميكروب القولونى و السالمونيلا . وفي الوقت ذاته كانت عترات المكور العنقودى الذهبى مقاومة للكليندامايسين ، الكلورامفينيكول ، الاريثروميسين و السلفاميثوكسازول - ميثوبريم في كثير من الأحيان. اظهرت ١٠من عترات الميكروب القولونى و ٦ من السالمونيلا و ١٢ من المكور العنقودي الذهبي مقاومة للأدوية المتعددة (مقاومة لـ٣ مضادات حيوية أو أكثر). علاوة على ذلك ثمانية عترات من المكور العنقودي الذهبي (٣٢ ٪) كانت مقاومة للأدوية المتعددة و مقاومة ايضا للفانكومايسين حيث تراوح الحد الأدنى المثبط للفانكومايسين بين٦٤ -١٠٢٤ ميكروجرام لكل مل.وقد تم الحصول علي جينات الفان ا، الفان ح ، الفان س والفان ب من عترات المكورالعنقودي الذهبي المقاومة للفانكوميسين الموجودة قيد الدراسة . تم الكشف عن الفئة الاولى من الانتجرون في ١٦/٩ (٥٦ ٪) من عترات الميكروب القولونى و السالمونيلا المقاومة لادوية متعددة بينما عترات المكور العنقودي الذهبي المقاومة لفانكوميسين جميعها لايحمل الانتجرون. فقد أبرزت هذه النتائج دور منتجات اللحوم كمصدر محتمل لنقل البكتريا القولونية, السالمونيلا والمكورة العنقودية الذهبية المقاومة للفانكوميسين المقاومين لمضادات حيوية متعددة.