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Class 1 integron and associated gene cassettes mediating multiple-drug resistance in some food borne pathogens

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Abstract

The emergence of antibiotic resistance among food borne pathogens has become a serious problem worldwide. The present study reports antibiotic resistance profile of some food borne bacterial pathogens recovered from retail meat of bovine origin and their relevant resistance genes carried on class I integron in Egypt. Thirty-two *Escherichia coli*, 15 salmonella and 25 *Staphylococcus aureus* isolates were assayed for their antimicrobial susceptibilities. Frequent resistances to amoxicillin-clavulanic acid and erythromycin were observed in *E. coli* and *Salmonella* species. Moreover, all *S. aureus* isolates were methicillin-resistant and 52% of the isolates were resistant to each of clindamycin and amoxicillin-clavulanic acid. This is the first report of the comprehensive identification and confirmation of vancomycin resistant *S. aureus* (VRSA) from meat specimens in Egypt. Interestingly, 31.25, 40 and 48% of *E. coli, Salmonella* species and *S. aureus* exhibited features of MDR , respectively. Class 1 integrons were commonly found in 66.67 and 50% of MDR salmonella and *E. coli*, respectively; while, VRSA isolates were negative. Three different gene cassette arrays encoding resistance to aminoglycoside (*aad*A2), beta- lactames (*bla*PSE−1) and trimethoprim and aminoglycosides (*dfr*A15- *aad*A1) were characterized among the integrase-positive strains. These findings illustrated the role of retail meat as a potential source for the dissemination of MDR *E. coli*, salmonella and VRSA in Egypt.

Introduction

Food borne illnesses caused by non- typhoid salmonella, *S. aureus* and *E. coli* represents a significant public health problem with major economic and social effects (de Sousa, 2008). Such pathogens are responsible to impose a substantial encumbrance of infection in the developed countries, while the impact in case of developing countries is higher (Pires *et al*., 2012).

The prevalence of antimicrobial drug resistance among food borne pathogens is increased due to its use in human therapy and animal farming for therapeutic and prophylactic purposes. The emergence and dissemination of MDR Gram-negative bacteria is an established and growing global threat (Platell *et al*., 2011). Today, methicillin-resistant *S. aureus* (MRSA) strains are major pathogens worldwide and most are MDR (Appelbaum, 2006). Over the past decade, the changing pattern of resistance in *S. aureus* has underscored the need for new antimicrobial agents. Vancomycin is the drug of last resort for highly drug resistant *S. aureus* (Périchon and Courvalin, 2004). The first strain of *S. aureus* with reduced susceptibility to vancomycin was reported in Japan in 1997 (Hiramatsu *et al*., 1997) Presently, VRSA has been isolated in different countries; hence, the burden has become a global phenomenon. There has been no report indicating the emergence of VRSA strains from meat products in Egypt as yet.

In most instances, resistance genes often associated with integrons and/or transposons are clustered within antimicrobial resistance islands that can be horizontally transferred by conjugative or mobilization plasmids (Miriagou *et al*., 2006). An integron is a site-specific recombination system capable of integrating and expressing open reading frames (ORFs) contained in modular structures called gene cassettes (Mazel, 2006; Labbate *et al*., 2009). Integrons comprise three essential components: an integrase gene (*IntI*1), encodes a site-specific recombinase; an adjacent attachment site (*attI1*), acts as a receptor for gene cassettes; and a promoter region (Pc), which promotes the expression of any suitably integrated gene (Carattoli, 2001). To date, nine classes of integrons may be

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retrieved from GenBank; only the first four classes have been confirmed. Among them, class 1 integron is the most widely disseminated among the members of the family *Enterobacteriaceae* (Goldstein *et al*., 2001; Phongpaichit *et al*., 2008). The accumulation of multiple resistance gene cassettes (up to about six) has associated these elements with MDR (Leverstein-van Hall *et al*., 2002; Leverstein-van Hall *et al*., 2003). Recently, integrons have been found in Gram-positive bacteria, but their role in drug-resistant *S. aureus* remains unclear (Nield *et al*., 2004).

Herein, the present study was conducted to uncover class I integron among MDR *E. coli*, *Salmonella* species and VRSA isolates from meat and meat products of bovine origin in Egypt and further characterize the respective gene cassettes involved therein.

Materials and Methods

Sampling and isolates characterization

Two hundred and fifty meat samples of bovine origin comprised of fresh meat (50) and meat products (minced meat, sausage, burger and blobev), 50 each, were collected randomly from 14 local supermarkets in Zagazig, Sharkia province, Egypt. All samples were subjected to conventional methods for isolation and identification of *E. coli, Salmonella* species and *S. aureus* (FDA, 1998). *E. coli* and *Salmonella* species were further identified with API20E identification kits (BioMérieux, Mary l'Etoile, France) and serotyped in the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt using commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Antibiotic susceptibilities were determined by the standard disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2011). *E. coli* and *Salmonella* species isolates were tested using gentamicin, streptomycin, ciprofloxacin, amoxicillin-clavulanic acid, ceftriaxone, doxycyclin, chloramphenicol, erythromycin and sulfamethoxazoletrimethoprim, while *S. aureus* isolates were tested using methicillin, sulfamethoxazoletrimethoprim, amoxicillin-clavulanic acid, vancomycin, gentamicin, chloramphenicol, clindamycin, ciprofloxacin and erythromycin.

Minimum inhibitory concentration (MIC) of vancomycin (Sigma, USA) was also determined against *S. aureus* isolates by a broth microdilution method as recommended by CLSI (CLSI, 2011).

PCR screening of van genes and class 1 integron

Plasmid DNAs of bacterial isolates were extracted using QIAprep Spin Miniprep Kits (Qiagen, UK). To assign *S. aureus* as VRSA isolates, PCR amplification of *van*A gene cluster (*van*R, *van*S, *van*H, *van*A, *van*X) and *van*B gene were carried out using previously published oligonucleotide primers and cycling conditions (Miele *et al*., 1995; Saha *et al*., 2008; Chakraborty *et al*., 2011).

Based on antimicrobial resistance profiles, MDR *E. coli*, *Salmonella* species and VRSA isolates (resistant to three or more classes of antimicrobial agents) were screened for the presence of class 1 integron and their associated gene cassettes using specific oligonucleotide primers and reaction conditions as described before (Levesque *et al*., 1995; Kerrn *et al*., 2002; Ren *et al*., 2013). Appropriate positive and negative controls were included in all PCR assays.

Characterization of inserted gene cassettes by DNA sequencing

The template amplicons were purified with QIAquick PCR purification kit (QIAGEN, Valencia, CA) following the manufacturer's instructions; each purified amplicon was sequenced in both forward and reverse directions using the amplification primers. The genetic materials inserted within the integron variable regions were identified by direct sequencing in an automated sequencer (Macrogen Inc., Korea ABI 3730XL DNA analyzer). Sequence homology was performed using the BLAST program available at the website of the National Center of Biotechnology Information *(www.ncbi.nlm.nih.gov)*. Alignment of the nucleotide sequences was performed by the use of Molecular Evolutionary Genetics Analysis version 5 (MEGA5) program (Tamura *et al*., 2011) available at *http://www.megasoftware.net*, then translation to amino acid sequences was performed using the ExPASy (Expert Protein Analysis System) Translate Tool (*http://us.expasy.org/*, Swiss Institute of Bioinformatics SIB, Geneva, Switzerland).

Statistical analysis

Differences in occurrence of *E. coli,* salmonella and *S. aureus* among examined meat and meat products were tested using Freeman-Halton extension of Fisher's exact test (2×3 Contingency Table) through crosstabs procedure of the Statistical Package for Social Sciences version 21.0 (SPSS for Windows 21.0, Inc., Chicago, IL, USA). Results were expressed as number of isolates and percent in brackets (%). A p-value <0.05 was considered statistically significant.

Table 1. Occurrence of *E. coli, Salmonella* and *S. aureus* in examined meat and meat products

Specimens	*E. coli* [a]	*Salmonella* species [a]	*S. aureus* [a]	P-value
Fresh meat	1	0	3	0.324
Minced meat	6	1	9	0.023 [b]
Burger	9	1	4	0.024 [b]
Sausage	12	13	5	0.099
Blobev	4	0	4	0.129
Total (250)	32 (12.8)	15 (6)	25 (10)	-

[a]: Number of isolates (%)
[b]: Bacterial pathogens were significantly associated with minced meat and burger samples compared with other meat samples ($p< 0.03$).

Results

Bacterial incidence in examined meat samples

Microbial analysis of meat homogenates revealed the occurrence of three major food borne pathogens; *E. coli, S. aureus* and *Salmonella* species. Of all the meat samples tested, 32 (12.8%) were positive for *E. coli*, 25 (10%) were found to carry *S. aureus* and only 15 (6%) were infected by *Salmonella* species. The proportions of bacteria varied among different meat and meat products of bovine origin. Our data showed a significant trend ($P<0.05$) in the overall percentage distribution of contaminating pathogens across minced meat and burger samples only (Table 1). Serotyping of *E. coli* isolates revealed that O111 was the most prevalent serotype (40.62%) followed by O26 (12.5%), O128, O124 and O127 (9.37% each) and O55, O78, and O119 (6.25% each). With regard to salmonella serotypes, *S.* Typhimurium predominated (50%) over *S.* Enteritidis (30%) and *S.* Anatum (20%).

Drug resistance analysis of bacterial isolates

Among 32 tested *E. coli* isolates, drug resistance was mostly observed for erythromycin and amoxicillin-clavulanic acid (93.75% each). Besides, antibiogram of 15 salmonella isolates revealed 100% resistance against erythromycin, followed by amoxicillin-clavulanic acid (86.67%). Consistently, all *S. aureus* isolates (n=25) showed resistance to methicillin and more than half of the isolates were resistant to clindamycin and amoxicillin-clavulanic acid (52% each) (Figure 1). Moreover, MDR was observed in 31.25%, 40% and 48% of *E. coli,* salmonella and *S. aureus* isolates, respectively. Unfortunately, 32% of *S. aureus* isolates (mostly from minced meat) were resistant to vancomycin. All these isolates showed resistance to seven antibiotics including vancomycin which had MIC values ranged from 64-1024 µg/mL (Table 2).

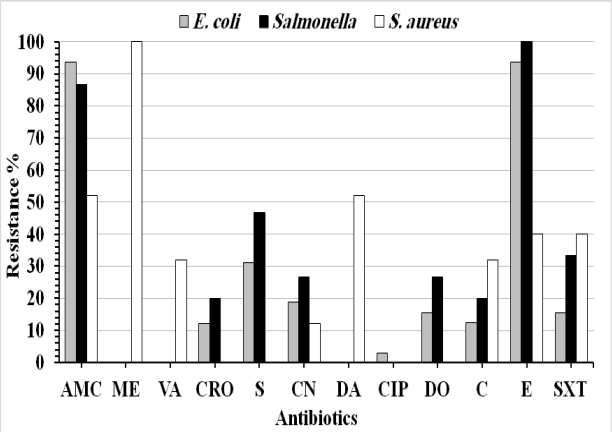

Figure 1. Frequency of antimicrobial resistance of bacterial isolates from retail meat. AMC: Amoxicillin-clavulanic acid, ME: Methicillin, VA: Vancomycin, CRO: Ceftriaxone, S: Streptomycin, CN: Gentamicin, DA: Clindamycin, CIP: Ciprofloxacin, DO: Doxycyclin, C: Chloramphenicol, E: Erythromycin, SXT: Sulfamethoxazole-trimethoprim

Characterization of MDR VRSA genotypes

Based on the resistance of eight MDR *S. aureus* to vancomycin, PCR amplification confirmed the possession of *van* genes among 7 isolates; however, one VRSA isolate was negative. Hence, the absence of *van* genes does not necessarily rule out that strains are not VRSA. Among six *van* genes, only PCR products of *van*B, *van*A, *van*H and *van*S were amplified from extracted plasmid DNAs (data not shown) with high predominance of *van*B (50%) and MIC≥64 µg/mL. However, neither *van*X nor *van*R gene was detected among the tested isolates. In addition, an isolate gave positive results for *van*A and *van*B genes together with a high MIC level (1024 µg/mL). Interestingly, the prevalence of MDR VRSA isolates harboring *van* genes from meat products is believed to be the first report at least in Egypt.

Class 1 integrons and associated cassette arrays

In this approach, 24 MDR bacterial isolates (10 *E. coli*, 6 *Salmonella* species and 8 VRSA) were examined for the presence of class 1 integrons harbouring resistance gene cassettes. Overall, class 1 integrase gene, *int*I1, was commonly found in 9 isolates [4 salmonella (66.67%) and 5 *E. coli* (50%)]; while, none of VRSA investigated in this study harboured class 1 integrons. PCR amplification of the variable regions of class 1 integron positive isolates revealed three different fragment sizes of approximately1 kb, 1.2 kb and 1.4 kb in two *E. coli* and four *Salmonella* species isolates. One *E. coli* isolate possessed 1000 and 1200 bp amplicons and

Table 2. MICs and vancomycin genes in VRSA isolates

Code Number	Vancomycin MIC *	*Van* A	*Van* H	*Van* X	*Van* S	*Van* R	*Van* B
1	128	-	-	-	-	-	-
2	1024	+	-	-	+	-	+
3	512	+	-	-	-	-	-
4	512	-	+	-	-	-	+
5	256	-	+	-	-	-	+
6	64	-	+	-	-	-	-
7	128	+	-	-	-	-	-
8	64	-	-	-	-	-	+

Code numbers 1-6: *S. aureus* isolates from minced meat; Code numbers 7, 8: *S. aureus* isolates from sausage; MIC: minimum inhibition concentration.
* MIC values for vancimycin are expressed in terms of µg/mL.

the other one harboured 1000 and 1400 bp. Moreover, two salmonella class 1 integron variable regions contained 1000 bp amplimer and the remainder yielded 1000 bp and 1200 bp amplicons (Figure 2). DNA sequence analysis revealed that 1 kb gene cassette fragment gave 100% homology with *aad*A2 gene (accession number KJ813805) conferring resistance to aminoglycoside antibiotics; whereas 1.2 kb fragment was 100% identical to *bla*PSE−1 gene (accession number KJ874349) accounting the resistance to beta- lactames. A class 1 integron carrying *dfr*A15- *aad*A1 cassette array (amplicon size 1.4 kb) was exclusively found in one MDR *E. coli* isolate conferring resistance to trimethoprim and aminoglycosides (accession number KJ874350) with two silent mutations at position 1450 (CAT→CTT) and 1463 (GTA→GTT); none of which resulted in amino acid substitutions. In particular, PCR results and DNA sequence analysis showed consistence with the antimicrobial susceptibility phenotypes as explained in Table 3.

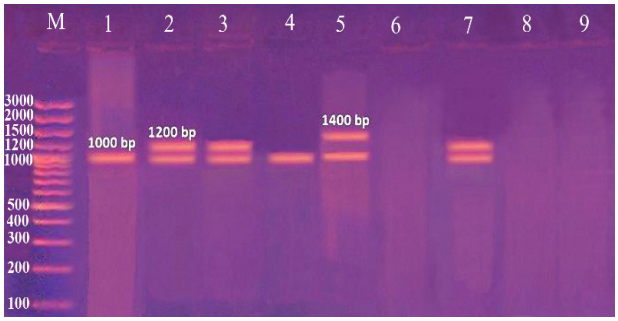

Figure 2. PCR amplification of the integron-variable regions generated with 5`CS and 3`CS primers. Six representative integron-positive strains carrying different gene cassettes are shown. Lane M, 100-bp ladder; lanes 1-4, amplicons from *Salmonella* isolates (amplicon sizes in parentheses); lanes 1 and 4, aadA2 (1000 bp); lanes 2 and 3, *aad*A2, *bla*PSE−1 (1000, 1200); lanes 5-9, amplicons from *E. coli* isolates; lane 5, *aad*A2, *dfr*A15-*aad*A1 (1000, 1400); lane 7, *aad*A2, *bla*PSE−1 (1000, 1200)

Discussion

Meat products are recognized as major sources of food borne pathogens that raised serious concern to public health worldwide causing food poisoning in humans. Currently, the most important pathogens associated with meat products are *S. aureus , E. coli* and *Salmonella* species. Hence, these pathogens were therefore selected for our study. Additionally, bacterial resistance to multiple antimicrobials is adding to the problem of meat contamination in the food environment. Therefore, the current work was to study antibiotic resistance profile of some food borne pathogens in retail meats sold commercially in Egypt in addition to molecular analysis of class 1 integrons among MDR isolates.

Observations of the present study showed heavy bacteriological load carried by the collected meat samples with low prevalence of three major food borne pathogens; *E. coli* (12.8%), *S. aureus* (10%) and *Salmonella* species (6%). This is inconsistent with a previous study in Thailand reporting the high occurrence of these pathogens in beef meat samples (56, 28 and 52%, respectively) (Angkititrakul *et al*., 2013). However, contamination rates of salmonella and *S. aureus* in this study were relatively higher than those recorded in Saudi Arabia (4%) (Al-Mutairi, 2011) and Pakistan (7%) (Hassan Ali *et al.*, 2010). The differences in prevalence rates among studies are mainly related to samples and sampling (type, source/ location and initial bacterial load), environmental and seasonal factors and the detection methodology

Table 3. Characterization of class 1 integrons in MDR *E. coli* and *Salmonella* serotypes from meat samples

Serotype	Antimicrobial resistance phenotype	Integron amplicon sizes (bp)	Inserted gene cassette arrays
E. coli O78:K80	CN, S, CIP, AMC, CRO, DO, C, E, SXT	1000, 1400	*aadA2*, *dfr*A15-*aadA1*
E. coli O26:K58	CN, S, AMC, E, SXT	1000, 1200	*aadA2*, *bla*PSE- 1
S. Typhimurium	CN, S, AMC, DO, C, E, SXT	1000	*aadA2*
S. Typhimurium	CN, S, AMC, E	1000, 1200	*aadA2*, *bla*PSE- 1
S. Typhimurium	CN, S, AMC, CRO, DO, E, SXT	1000, 1200	*aadA2*, *bla*PSE- 1
S. Enteritidis	S, AMC, DO, C, E, SXT	1000	aadA2

CN, gentamicin; S, streptomycin; CIP, ciprofloxacin; AMC, amoxicillin-clavulanic acid; CRO, ceftriaxone; DO, doxycyclin; C, chloramphenicol; E, erythromycin; SXT, sulfamethoxazole-trimethoprim.

used. From the obtained results, it was revealed that *E. coli* O111 and *Salmonella* Typhimurium were the most prevalent serotypes as previously stimulated (Abbassi-Ghozzi *et al*., 2012; Perelle *et al*., 2007).

A phenotypic resistance study is the first step of the antimicrobial resistance investigation. Our data demonstrated the widespread resistance of *E. coli* and *Salmonella* species to erythromycin, and amoxicillin-clavulanic acid as reported previously (Al-Sultan *et al.,* 2012), which is most likely resulted from the long-term and widespread abuse of these antimicrobials in animal farms. In addition, all *S. aureus* isolates were resistant to methicillin (MRSA) which is higher than those described in previous studies in Korea (1%) (Lim *et al*., 2010) and USA (1.3%) (Bhargava *et al*., 2011), however, different geographic locations and cold sampling seasons in these areas might have caused the variations. Furthermore, 31.25, 40 and 48% of *E. coli,* salmonella and *S. aureus* isolates exhibited MDR, respectively. Indeed, published data for these isolates from retail meats indicated a tendency toward lower resistance rates of *E. coli* and *S. aureus* to multiple antimicrobial agents than in the previous years (Li *et al*., 2011; Waters *et al*., 2011), while MDR salmonella isolates showed a higher resistance frequency than described in another investigation (Shen *et al*., 2008). Hence, functional surveillance of antimicrobial resistance and appropriate effective measures geared towards curbing indiscriminate and unregulated use of antibiotics are urgently needed to prevent outbreaks of MDR bacteria in Egypt.

Vancomycin is the main antimicrobial agent available to treat serious infections with MRSA but unfortunately, vancomycin resistance in *S. aureus* has emerged over the last ten years. In the present study, the most striking finding was the relatively high prevalence rate of VRSA isolates (32%) which was surprising and of a serious concern. To the best of our knowledge, little literatures are available on the prevalence of VRSA isolated from meat samples at least in Egypt. Osman *et al*., 2015. recorded intermediate resistant *S. aureus* in raw meat (51%) which might indicate the dissemination of vancomycin resistance in the community and imply food safety hazards. This can be attributed to the high rate of indiscriminate abuse of vancomycin as a growth promoter in food-producing animals or to treat farm animals, which might be a potential reservoir for vancomycin resistance determinants. The findings of VRSA in meats are a motive of concern, as they may suggest the spread of such microorganisms or their genetic material outside clinical boundaries. These VRSA isolates were submitted to broth microdilution assay and they presented MIC values ranged from 64-1024µg/mL. Similarly, the VRSA strains isolated from chicken meat in Brazil exhibited high resistance to vancomycin (MIC, 512 µg/mL) (Martins *et al*., 2013). The limitation of our study is the absence of van genes in one VRSA isolate by PCR even in the presence of phenotypic resistance to vancomycin. This is in accordance with another study published in Nigeria. Hence, the absence of *van* genes does not necessarily rule out that strains are not VRSA (Alo *et al*., 2013). As proposed previously, cell wall thickening is the essential contributor for development of vancomycin resistance. As a result, more vancomycin molecules are trapped in the peptidoglycan synthesis occurs (Cui *et al*., 2003). In our study, *van*A gene was detected in 3 isolates which were resistant to vancomycin with MIC≥128 µg/mL, whereas, four isolates (50%) carried *van*B gene with MIC≥64 µg/mL. These results confirmed the criteria reporting that *van*A-type strains display high levels of inducible resistance to vancomycin, whereas *van*B-type strains have variable levels of resistance (Arthur *et al*., 1996). Moreover, it should be regarded that

*van*H and *van*S genes were detected in the current study as was previously observed in other studies in South Asia and Iran, respectively (Saha *et al*., 2008; Dezfulian *et al*., 2012).

In this study, class 1 integrons were commonly found in 66.67% and 50% of MDR salmonella and *E. coli* isolates, respectively; while, none of VRSA isolates harboured class 1 integrons. Thus, class 1 integrons contribute significantly to antibiotic resistance in Gram-negative organisms (Toleman *et al*., 2006). Comparable with other reports, lower detection rates of class 1 integrons among salmonella (54%) and *E. coli* isolates (12%) from retail meats were documented (Chen *et al*., 2004; Sunde, 2005), meanwhile, integron-bearing *S. aureus* was previously recorded (Ren *et al*., 2013; Xu *et al*., 2008).

Among class 1 integrase-positive isolates, only 66.67% possessed gene cassettes which was probably related to the absence of the 3`-conserved region in a large number of integrase positive isolates and/or attributable to attenuation of the promoters (Martinez-Freijo *et al*., 1999; Singh *et al*., 2005). DNA sequence analysis of variable regions of class 1 integrons revealed *aad*A2 and *bla*PSE−1genes conferring resistance to aminoglycoside and beta-lactames, respectively in both analyzed MDR *E. coli* and salmonella isolates. Furthermore, a class 1 integron carrying *dfr*A15- *aad*A1 cassette array was exclusively found in an *E. coli* isolate accounting for the resistance to trimethoprim and aminoglycosides. Such data are consistent with other previous investigations (Molla *et al*., 2007; Copur-Cicek *et al*., 2014).

The rapid and unabated spread of class 1 integrons-associated MDR bacteria may greatly hamper successful treatment of infections caused by such strains. This necessitates an establishment of functional antimicrobial resistance surveillance programs in Egypt.

Conclusion

In light of the results, our study has focused on the emergence of VRSA harboring *van* genes from meat samples in Egypt. In addition, class 1 integrons carrying gene cassettes, conferring resistance to aminoglycosides, ß-lactams and trimethoprim were widespread among MDR *E. coli* and salmonella isolates. Disturbingly, the location of integrons with their gene cassettes on the plasmid may share in the widespread dissemination of MDR among bacterial species. Hence, functional surveillance of antimicrobial resistance and appropriate, effective measures geared towards curbing indiscriminate and unregulated use of antibiotics are urgently needed to prevent outbreaks of MDR bacteria in Egypt.

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