



First Characterization of Class 1 Integron in *Corynebacterium bovis* Isolated from Subclinical Bovine Mastitis

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Abstract | Integrons play major roles in spreading and expression of antibiotic resistance genes among bacteria especially Gram-negative bacteria. However, the incidences of class 1 integrons in Gram-positive bacteria remained elusive. The present study was conducted to monitor the prevalence of class 1 integrons in *Corynebacterium bovis* (*C. bovis*) isolated from cases of bovine mastitis in Egypt. A total of 35 *Corynebacteria* spp., isolated from milk samples of subclinical mastitis, were examined using PCR analysis with 16S rRNA specific primers. The results revealed a prevalence of 8 (22.8%) *C. bovis* strains in the examined samples. All isolates of *C. bovis* showed an elevated level of resistance, mostly against spectinomycin, streptomycin followed by tetracycline, ampicillin, kanamycin, chloramphenicol and sulfamethoxazole-trimethoprim. Using PCR and DNA sequencing, two isolates of *C. bovis* were identified containing class 1 integrons that encoded aminoglycoside adenyltransferase type A (*aadA1*) as a gene cassette. The *aadA1* confers resistance to spectinomycin/streptomycin. Additionally, class 1 integron resistance genes in *C. bovis* isolates were described utilizing PCR and DNA sequencing. These resistance genes conferred resistance to several antimicrobial agents frequently used in dairy farming. This study reports, for the first time, the detection and identification of class 1 integron containing *aadA1* gene in *C. bovis* of animal origin.

Keywords | PCR, DNA sequencing, *aadA1*, Gene cassette, *C. bovis*

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INTRODUCTION

Corynebacteria spp. are one of the most isolated pathogens responsible for subclinical mastitis in dairy cows. However, quick, reliable and simple techniques for the identification of species of the genus *Corynebacterium* are not currently available (Juliano *et al.*, 2014).

The wide utilization or misuse of antibiotics in veterinary medicine initiates the development of resistance and increase incidence of multidrug resistance (MDR) in bacteria. This leads to implication of substantial public

health impacts (Pyorala, 2009; Richet, 2012; Saini *et al.*, 2012; Chen *et al.*, 2017).

Integrons are portable hereditary components having a system of site-specific recombination, which permits capture the genes responsible for antibiotic resistance (Partridge *et al.*, 2009; Chen *et al.*, 2011). Integrons play important roles in spreading and expression of antibiotic resistance genes among bacterial populations through transposons or plasmids (Mazel, 2006). The integron gene sequence arrays are formed when multiple gene cassettes are integrated at one site on a plasmid or on the

chromosome (Recchia and Hall, 1995, 1997).

According to the similarities of integrase amino acid sequence, integrons are classified into several classes; the most common one is class 1 integrons which are detected in Gram-negative bacterial isolates from various sources (Mazel, 2006; Chen et al., 2011; Mokracka et al., 2012). The class 1 integron contains two conserved segments as following; a 5' conserved segment (5'-CS), a 3' conserved segment (3'-CS) and in between them a recombination site at which the gene cassettes have been integrated (Collis et al., 2002; Correia et al., 2003). The 5'-CS made out of integration site (*attI*), an integrase gene (*intI*), promoters responsible for gene cassettes and the integrase expression (Ploy et al., 2000; Mazel, 2006). The 3'-CS involved *sulI* genes and the truncated *gacEΔ1*, which confer resistance to sulfonamides and disinfectants, respectively (Paulsen et al., 1993).

The class 1 integrons genes are mostly detected in Gram-negative bacteria (Martinez-Freijo et al., 1998; Partridge et al., 2001). However, the incidence of class 1 integrons in Gram-positive bacteria remained least explored (Nesvera et al., 1998; Clark et al., 1999; Tauch et al., 2002).

The present study aim to detect *C. bovis* strains isolated from subclinical cases of bovine mastitis using PCR techniques and to genetically characterize class 1 integron resistance genes using advanced genomic approaches.

MATERIALS AND METHODS

SAMPLING AND ISOLATION

A total of 150 mastitis milk samples, obtained from cases of subclinical mastitis (positive for California mastitis test), were gathered from different farms of dairy cattle at Giza governorate in the Egypt. At these farms, cows suffered from decreased milk production with no clinical signs. A total of 35 isolates were identified as *Corynebacteria* species through microbiological cultures according to the method described by Oliver et al. (2004).

DNA EXTRACTION AND PCR

The DNA extraction was carried out by utilizing multi-genomic DNA extraction kit (Qiagen, Germany), according to manufacturer's instructions. All isolates of *Corynebacteria* spp. were subjected to PCR amplification (Table 2) using thermal cycler (Peltier Thermal Cycler - MG 960T enzyme® - USA) with the primer pairs (Sigma, USA) F-5' GCGAACGGGTGAGTAACACG 3' and R-5' TCTGCGATTACTAGCGACTCCG 3' (Huxley et al., 2004), as shown in Table 1.

ANTIMICROBIAL SENSITIVITY TEST

The phenotypes of antimicrobial sensitivity of *C. bovis* isolates were tested on Mueller-Hinton agar (Oxoid,

Basingstoke, Hampshire, United Kingdom) plates using a disk diffusion method according to the guidelines and standards showed by Clinical and Laboratory Standards Institute (CLSI, 2018). Antibiotic susceptibility of tested organisms were determined for tetracycline (TET), 30 µg; spectinomycin (SPX), 10µg; kanamycin (KAN), 30 µg; streptomycin (STR), 10 µg; sulfamethoxazole-trimethoprim (SXT), 23.75/1.25 µg and ampicillin (AMP), 10 µg. The sensitivity disks were punched from (Oxoid, UK). The recorded results were depending on the zone-size in chart of interpretation according to manufacturer's instructions.

PCR AND SEQUENCING OF CLASS 1 INTEGRON FROM *C. BOVIS*

All PCR reactions were adjusted to 25 µl of total volume; 12.5 µl of 2xMaster Mix (Intron Biotechnology, Inc), 5 µl DNA of *C. bovis*, 1.25 µl of 20 pmol/µl of each primers (Sigma, USA) of class 1 integron and finally, water nuclease-free up to 25 µL. All PCR reactions of *C. bovis* isolates were submitted to PCR amplification with thermal cycler (Peltier Thermal Cycler - MG 960T enzyme® - USA) with 5'-CS and 3'-CS primers specific to class 1 integron, as described in (Table 2) (Lin et al., 2016). This primer pair target the region between the 5'-conserved segment (5'-CS) and 3'-CS of class 1 integrons as previously described (Table 1).

Analysis of PCR products was carried out through gel electrophoresis (SCIE-PLAS, UK) on a 1% agarose gel prepared with Tris/Acetate/EDTA (TAE) (Intron Biotechnology, Inc) buffer plus agarose powder (Biotechnology grade AGA001.100 - Bioshop® Canada Inc) stained with ethidium bromide (0.5 µg/ml). 10-µL aliquot of a molecular marker containing a DNA Ladder (Intron Biotechnology, Inc) was added to the first well of each gel, control negative in the second, control positive (*K. pneumoniae* KPB 127 contain two integrons (*dfra12* - *aadA2*; and *aadA2*) isolated by (Hazim et al., 2016) in the third well and 10-µL of samples in the other wells (Figure 2) at 80 V for 1 h, after that single DNA bands were visualized by ultraviolet trans-illumination (UV) according to target size of gene as showed in Table 2. A QIA quick Gel Extraction Kit (Qiagen, Germany) was used for purification of PCR fragments from the agarose gel according to the manufacturer's instructions. Finally, the sequencing for PCR product with both DNA strands was performed by using an ABI automatic DNA sequencer (Model 373; Perkin-Elmer).

COMPUTER ANALYSIS OF THE SEQUENCE DATA

The data of DNA sequenced data of amplified gene was identified by the database of GenBank using the BLAST program available at the NCBI BLAST homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Table 1: Primers used for PCR reactions and DNA sequencing.

Target	Primer name	Oligonucleotide sequence (5' - 3')	Amplicon size (bp)	Reference
C. bovis	F	GCGAACGGGTGAGTAACACG	1250 bp	Huxley <i>et al.</i> (2004)
	R	TCTGCGATTACTAGCGACTCCG		
Class I integron	5'-CS	GGCATCCAAGCAGCAAG	Variable	Lin <i>et al.</i> (2016)
	3'-CS	AAGCAGACTTGACCTGA		

Table 2: General PCR conditions of different primers.

Gene	Pre denaturation	Denaturation	Annealing	Extension.	Cy.	Final extension	Target
C. bovis: C-F C-R	94°C/2 min	94°C/30 sec	58°C/30 sec	72°C/1 min	30	72°C/1 min	1250 bp
Class I integron 5'-CS 3'-CS	94°C/10 min	94°C/1 min	55°C/1 min	72°C/3 min	30	72°C/10 min	Variable

Table 3: Incidence of C. bovis and class 1 integron.

Total no. of bacteriologically examined <i>Corynebacteria</i> spp. isolates	C. bovis isolated by PCR		Class 1 integron isolates by PCR	
	No.	%	No.	%
35	8	22.8	2	5.7

Percent according to total no. of *Corynebacteria* spp.

Table 4: Results of antimicrobial susceptibility patterns of C. bovis isolates and interpretation according to (CLSI. 2018).

No of isolate	AMP	STR	SPX	KAN	SXT	CHL	TET
C2	R	S	R	S	S	S	R
C3	R	R	R	R	S	S	R
C6	I	R	R	R	R	R	R
C8	R	R	R	S	R	I	S
C11	R	R	R	R	S	S	R
C16	S	R	S	R	R	R	R
C23	R	I	R	S	I	I	R
C31	S	R	R	S	S	S	R
Incidence of resistance	5 (62.5%)	75%	7 (87.5%)	3 (37.5%)	3 (37.5%)	2 (25%)	5 (62.5%)

S: sensitive; R: resistant; I: intermediate; AMP, ampicillin; CHL, chloramphenicol; KAN, kanamycin; SPX; spectinomycin STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.

RESULTS

Bacteriological examination of collected 150 subclinical mastitis milk samples (positive for CMT), revealed 35 (23.3%) isolates of *Corynebacteria* spp. The antimicrobial sensitivity of the *C. bovis* to antimicrobial agents is shown in Table 2. The *C. bovis* isolates showed resistance against streptomycin (87.5%), spectinomycin (75%), tetracycline (62.5%), ampicillin (62.5%), kanamycin (37.5%), sulfamethoxazole-trimethoprim (37.5%) and chloramphenicol (25%). Class 1 integron resistance gene was found in 2 (5.7%) isolates, as shown in Table 4.

In the present study, 35 isolates of *Corynebacteria* spp. were inspected using PCR with 16S rRNA gene, which is a primer specific for *C. bovis*. The result showed 8 positive strains of *C. bovis* out of 35 (22.8%) tested samples (Table 3) with a target PCR amplicon size of 1250 bp (Figure 1, Table 1).



Figure 1: Amplification products of *C. bovis* was analyzed by electrophoresis on a 1.5% agarose gel. Lane M: 100-bp DNA ladder, lane (1-2-3-4-5-6-7-12): positive at size 1250 bp. Lane (8-9-10-11): negative.

Further, PCR and DNA sequencing of 8 positive *C. bovis* fragments using primers specific to class 1 integrons revealed two isolates (5.7%) of *C. bovis* which have one

type of class 1 integron with middle size (974 bp), as shown in (Figure 2) and contains a single gene cassette of *aadA1* (aminoglycoside adenytransferase type A), which confers resistance to streptomycin/spectinomycin. Up to date the *aadA1* gene has not been previously identified in *C. bovis*.

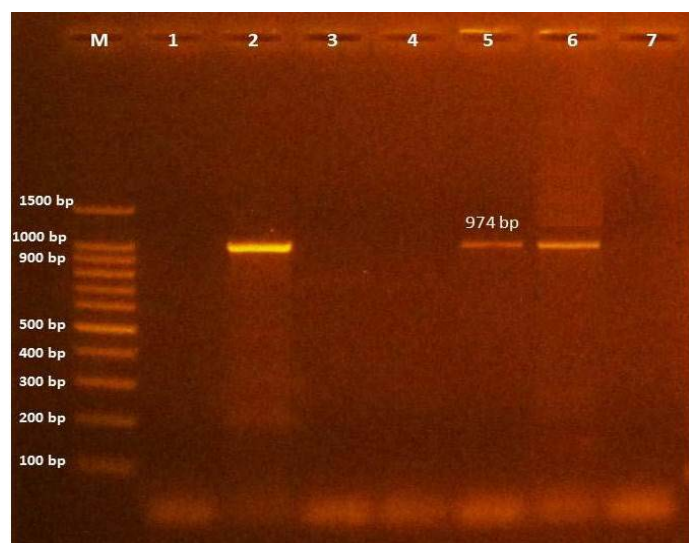


Figure 2: Amplification products of class 1 integron in *C. bovis* was analyzed by electrophoresis on a 1.5% agarose gel. Lane M: 100-bp DNA ladder, Lane (1): Control negative, lane (2): control positive (*K.pneumoniae* KPB 127 contain two integrons (*dfrA12 - aadA2*; and *aadA2*), lane (3-4-7): negative, lane (5-6): positive at size 974 bp (*aadA1*).

DISCUSSION

In this study, 8 out of 35 isolates were identified as *C. bovis* using PCR with a fair percentage (22.8%). This rate is lower than 54.0% strains as *C. bovis* reported by Watts et al. (2000) and also Theel et al. (2012) which detects *C. bovis* with a percent 88.5%. Higher incidence of *C. bovis* was reported from sub-clinical mastitis (96.1% and 97.2%) by Juliano et al., 2014 and Huxely et al., 2004, respectively.

The class 1 integrons were first reported in 1989 (Stokes and Hall, 1989) and were described as reservoirs and an essential source of antimicrobial resistance genes within microbial populations (Fujimura et al., 2004; Xu et al., 2007, 2009). However, these studies have been conducted in Gram-negative bacteria with few exceptions (Tauch et al., 2002; Nandi et al., 2004). While in recent studies, class 1 integron had been observed as antibiotic resistance gene in Gram-positive bacteria, particularly in methicillin-resistant coagulase negative staphylococci (MRCNS) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Xu et al., 2007, 2008, 2010).

Class 1 integron have been found mainly in Gram-negative bacteria (Mokracka et al., 2012; Malek et al., 2015). Up-to-date, only few reports on class 1 integron in Gram-

positive bacteria are available. The previously published papers of class 1 integron in Gram-positive bacteria with gene cassettes are as following; (1) Class 1 integron containing *aadA* gene was first report in *E. faecalis* strain and confers resistance to streptomycin/spectinomycin (Clark et al., 1999). (2) The genome of some Gram-positive bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci, *Staphylococcus warneri*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* carries class 1 integrons containing gene cassettes of *dhfrXII-orfF-aadA2* which confers resistance to trimethoprim, unknown function protein and aminoglycoside adenytransferase type A, respectively (Lei et al., 2006). (3) The *aadA2* gene cassette on a 29-kb plasmid pCG4 in *C. glutamicum*, which confers resistance to streptomycin/spectinomycin (Nesivera et al., 1998). (4) Class 1 integrons containing *aadA9* detected previously in *C. glutamicum* on a 27.8-kb R-plasmid pTET3, which confers resistance to spectinomycin, streptomycin and tetracycline (Tauch et al., 2002). (5) The *aadA2* gene cassette in *S. aureus*, which conferred resistance to aminoglycoside (Xu et al., 2007). (6) Class 1 integron of different gene cassettes with 2 *att* sites and 3 arrays of cassettes were detected in methicillin-resistant *S. aureus* (MRSA) (Shengjie et al., 2011).

CONCLUSION

In the present study, class 1 integron was identified with gene cassette (*aadA1*) in two isolates of *C. bovis* (5.7 %) obtained from milk samples of subclinical mastitis cows using PCR and DNA sequencing. These resistance genes confer resistance to several antimicrobial agents frequently used in dairy farming. This study is the first report for detection and identification of class 1 integron containing *aadA1* gene in *C. bovis* of animal origin.

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STUDY LIMITATION

Findings of this study were limited to a small sample size, also class 1 integron incidence is little in Gram positive bacteria. In addition, few Egyptian studies were accessed in this area of research.

Moreover, there were some difficulties related to the expense of chemicals and kits which restricted the application of PCR and DNA sequencing primers of multi drug resistance.

All authors contributed equally.

CONFLICT OF INTEREST

No conflict of interest declared.

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