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Complexity, Heterogeneity and Mutational Analysis of Antibiotic Inactivating Acetyl Transferases in MDR Conjugative Plasmids Conferring Multi-Resistance

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Research Article

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ABSTRACT

Drug acetylating transferases (*AAC*) are enzymes that inactivate aminoglycoside antibiotics by acetylating its O^- and N^- atom in the drug. *AAC* genes were detected in bacterial plasmids and integrons of many pathogenic bacteria rendering drug resistance. The unique CAT enzyme was discovered early that could acetylate chloramphenicol at 1' and 3' $^-$OH group and largely used as reporter gene in expression vectors. Aminoglycoside 6'-N-acetylating enzymes were mainly classified as acc6'-Ia to *aac*6'-If and genes were designated as *aac*A1 to *aac*A8 but *aac*A16 or *aac*A41 types isomers were also sequenced. The isomers *aac*A3, *aac*A4 and *aac*A8 are very identical contrary to other *aac*A1 isomers. Aminoglycosides 3'-acetylating enzymes were designated as *aac*3'-Ia to *aac*3'-Xa and genes were designated as *aac*C1 to *aac*C10. Sequence analysis suggested that *aac*2', *aac*4' and bifunctional enzymes (*aac*6'-*aph*2'') were different class of acetylating enzymes. But *aac*6'-1b-cr protein that was involved in ciprofloxacin resistance resembled to *aac*6'-1b with point mutations. Interestingly, cat gene has no similarity to *aac*A1 or *aac*C1 genes and so far was ignored as being non-clinical origin. But now *cat*B3 gene was reported in many MDR plasmids of pathogens like *Shigella flexneri* (pR100), *Yersinia pestis* (pIP1202), *Escherichia coli* (pNR1), *Pseudomonas aeruginosa* (pOZ176), *Klebsiella pneumoniae* (pNDM-MAR) and *Salmonella enterica* (pHXY0908). Such plasmids were also frequently associated with *acc*3' and *aac*6' enzymes including diverged β-lactamase genes (*bla*TEM, *bla*NDM, *bla*CTX-M etc) and drugs efflux genes (acrAB, mexAB/CD/XY, *tet*A/S) as well as AG adenyl transferases (*aad*) and AG phospho transferases (*aph*) genes. Surely, appearance of *cat*, *amp*, *tet* genes in conjugative plasmids of superbugs is frightening as those genes are randomly used in expression vectors for RDT work. Diversities among drug acetylating enzymes were found very high suggesting multiple mechanisms of their origin.

INTRODUCTION

The MDR is a unique phenomenon where bacteria acquire MDR genes in plasmids and chromosomes and can survive in stressful environment containing high concentration of antibiotics and other pollutants [1]. Such bacterial infections, on the other hand are hard to cure by antibiotics contributing huge life and wealth loss worldwide [2]. Aminoglycoside acetyl transferases modify the drug's structure by acetylating its N-atom or O-atom in the drug and as a result such acyl-drug could not bind the target site to inhibit bacterial replication, transcription or translation [3-8]. So patient could not get rid of bacterial infections by simply taking few tablets of ampicillin, streptomycin or gentamycin antibiotics because acetyl transferases were activated in plasmids that inactivate drugs.

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The AMR mechanisms broadly classified into six major categories: (1) activation of β-lactamase (*bla*) (2) activation of drug modifying enzymes (*aac*, *aad*, *aph*) (3) activation of drug efflux genes (acr, *tet*, cmr, mex) including ABC drug transporter (4) alteration of target sites by mutations (rRNA, PBPs) (5) neutralizing antibiotic after binding with drug like *tet*M and (6) lower expression of porins restricting drug entry like imipenem. Thus it is very complex to stop gene creation in nature under stressful environment and none could even imagine such changes how created an acute problem in medicine today [9].

The first acetylating enzyme was discovered as cat gene or chloramphenicol acetyl transferase. Chloramphenicol was first discovered in actinomycete isolated from soil by Ehrlich in 1947 followed by Tamura in 1971 from *Streptosporagium viridogriseum*. Chloramphenicol inhibits the bacterial 30S ribosome and is a very good antibiotic as it cures the common diseases caused by *S. aureus*, *E. coli*, *K. pneumoniae*, and more. However, soon cat gene was discovered in many bacterial pathogens those were found resistant to chloramphenicol [10]. Cat enzyme is different from "*amp*" or "*tet*" gene's mode of actions in that it acetylates the chloramphenicol drug (1' and 3' positions) in such a way that acetylated drug no longer able to bind the bacterial ribosome. Thus cat gene containing bacteria (in plasmid) easily grow containing MIC amount of chloramphenicol *in vitro* as well as *in vivo* in patient blood [11,12].

Sequence analysis suggested that cat gene was a different enzyme in amino acid sequence than *aac*A1or *aac*C1 and other isomers. Why cat gene was not considered as *aac* enzyme was not sure to say but *aac*A1 enzymes are linked to clinical origin found in MDR plasmids with other MDR genes like *bla*TEM, *str*A/B, *sul*1/2, *tet*A/S type genes including *Tnp* and *Tra* genes [13-15]. So cat gene (designated as catB3 and was very famous for CAT *in vitro* transcription technology) was isolated from small R-plasmids in bacteria that were not clinically relevant [10]. However, it was detected now in many large conjugative plasmids of common pathogenic bacteria [16].

Similarly, *aac*(6') enzymes was symbolized as *aac*A1 to *aac*A9 but other isomers like *aac*(3') designated as *aac*C1-*aac*C9 [17-20]. *aac*2'' and *aac*4' etc were kept aside and their divergence was labelled as *aac*(2'')-Ia/b/c or *aac*(2')-I/II etc [21-23]. Similarly, there are catB2/4/8 or catA_1/2 isomers in the literature. Mutations of cat gene as well as *aac*C1 and *aac*A1 genes were never investigated as compared to huge citations of β-lactamases mutations (*bla*TEM, *bla*OXA, *bla*CTX-M etc) that conferred PDR or XDR type resistance in bacteria [10]. The origin of this review however, lies on the facts that GenBank data analysis of *aac*A1/C1 genes contradicted highly for consensus primers that to be used to study the contamination of superbugs in Kolkata water bodies, particularly Ganga River water.

RESULTS

Similarities among cat gene isomers

GenBank data analysis clearly suggested that cat genes were widespread in bacterial plasmids and integrons **(Tables 1-4)**. Class I integron mediated catB3 genes of many bacteria like 2177bp IntI1 integron of *Pseudomonas aeguginosa* (AN:EF660562); 2731bp Class I integron of *Escherichia coli* (AN:ABP35557), 5857bp class I integron of *Klebsiella oxytoca*, 2297bp Class I integron of *Acinetobacter baumannii* (AN:ADF59078), 1738bp Class I integron of *Aeromonas veronii* (AN:ALB07153), 4632bp In846 integron of *Enterobacter cloacae* (AN:AGJ70489) and that of Proteobacteria (AN:WP_000186237) were identical in protein sequence [11]. Interestingly, such integrons were also linked to other type's aminoglycoside acetyl transferases like *aac*A1, *aac*A4 and *aac*A7 [12].

We observed that cat genes were now associated in conjugative plasmids which were hard to rescue in absence in drugs and also could deliver MDR genes into household bacteria. So it seems AMR is a ubiquitous phenomenon in modern days. As for example, in conjugative MDR plasmid (pI1-34TF; 167198bp) of *Escherichia coli* (AN: LN850163) four acetylating enzymes had been accumulated in four positions of plasmid spreading all across same distance. Two cat genes (cat_1 and cat_2; protein Ids. CRK62767 and CRK62815) were different where cat_2 was catB3 type but cat_1 was related to xenobiotic acetylating enzyme. Moreover two other acetylating enzyme, N-6 hydroxylysine 0-acetyl transferase (protein id. CRK62680; antibiotic_NAT-like) and SPBe2 prophage derived acetyl N-3'-transferase (yokD; protein Id. CRK62756) had roles in inactivating diverse drugs. Such plasmid did contain other mdr genes like *bla*OXA-1, *bla*TEM-1, macB (macrolide exporter), MFS, *aph* (phosphotransferase), *aad*3' (streptomycin adenyl transferase), *tet*A (*tet*racycline transporter) and tmrB (tunicamycin resistant protein). Further, such plasmid also accumulated HTH-type cmtR and envR, *tet*R transcription factors in close association with IS-elements and transposons. Multi-align and seq-2 sequence analysis were presented in **Figures 1 and 2** where catB3 was found similar but catB8 had many mutations and frequent carboxy-terminal deletions and substitutions [14].

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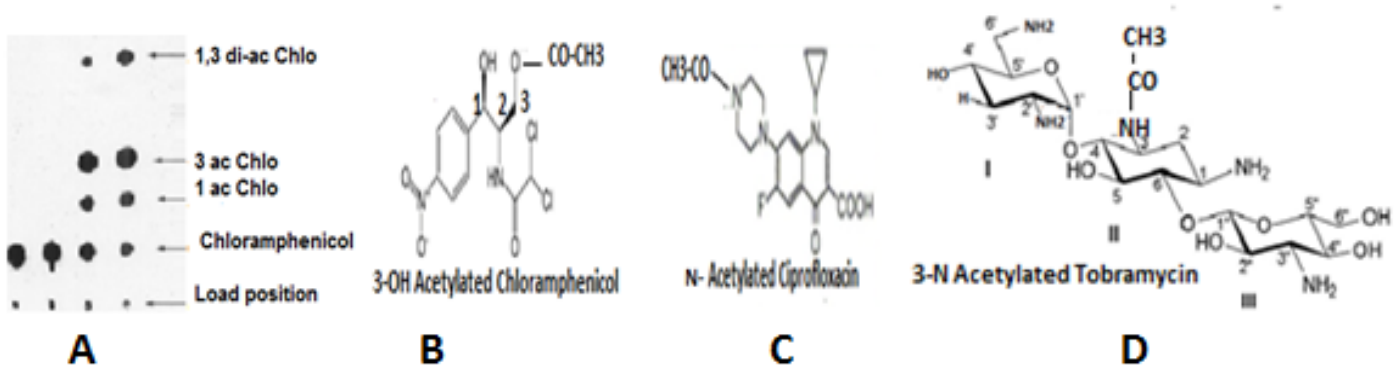

Figure 1. Acetylation of choramphenicol by CATB2 plasmid transfection of HeLA cells with Lypofectamine reagent (panel, A). Lane, 1 free chloramphenicol, lane 2, cell extract with no plasmid, lane 3, 5 µl cell extract and lane 4, 10 µl cell extract with transfected plasmid. Structures of 3-acetylated chloramphenicol (panel-B), N-acetylated ciprofloxacin (panel, C) and tabromycin (panel, D).

Figure 2. Homologies among CAT Enzymes: (A) CatB3, catB4 & CatB8 similarities. (B) catA2 and CatA4 similarity and (C) catB3 *vs*. catA_1 similarity.

*cat*B3 gene was present in many MDR plasmids including 249kb Samonella enterica plasmid pHXY0908 (AN:KM877269) and in high molecular weight P1 plasmid of *Klebsiella pneumoniae* containing mphA (macrolide 2" phosphotransferase; protein Id. WP_000219391), *aad*A2 (ANT3"-1a), *aph*3'-1a (protein Id. WP_000018329), GCN5 acetyl transferase (protein Id. WP_003026803) and phosphonothricin acetyl transferase (protein Id. WP_004152096) including other mdr genes like drug transporter *tet*A/*tet*G, ABC transporter (protein Id. WP_004118832), MFS-type drug efflux proteins (protein Ids. WP_003846917 and WP_000214125) as well as CTX-M-24/KPC-2/VEB-3 types β-Lactamases. Similarly pOZ176 large plasmid (AN:KC543497) contains catB8a, neo (aminoglycoside 3'-O-phosphotransferas; protein Id. AGL46257) and *aac*6'-IId (*aac*A4-type) giving resistance to chloramphenicol, neomycin and amikacin respectively [16,24].

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Table 1. Classification of major AAC(6') acetyl transferase; The accession numders of plasmids, integrons and genomic fragments carrying aac(6')-type genes were demonstrated. The different types of pathogenic MDR bacteria and associated *mdr* genes were also described.

Classification of drug modifying aac(6') N-acetyl transferases						
Gene	**Synoname**	**AA length**	**Accession no.**	**Protein ID**	**Plasmid/ Bacteria**	**Associated MDR Genes**
aacA1	Aac6'	185aa	AB061794	BAB72153	*pCMXR1/ E. coli*	*Sul1, blaCMY-9*
	Aac6'	185aa	AB116723	BAD11027	*InIt-1/ K. pneumoniae*	*blaGES-4*
	Aac6'-Ia	185aa	AB901039	BA048019	*IntI-1/ P. aeruginosa*	*blaIMP-10*
	Aac6'	185aa	NC_014167	YP_0036573222	*pJA144188/ C. resistens*	*aphA, strA/B, sul1*
	Aac6'-Ia	185aa	M18967	AAA98298	*IntI/ Citrobacter diversus*	*nd*
	Aac6'-Ii	182aa	L12710	AAB63533	*IntI/ Enterococcus facium*	*nd*
aacA2	Aac6'-1b	199aa	JN420336	AEB82864	*pNDM-MAR/K. pneumoniae*	*OXA-1, CTXM-15*
aacA3	aac(6')	184aa	KM111260	AIP98294	*IntI-1/ P. aeruginisa*	*nd*
aacA4	aac6'	184aa	EF138817	ABO21792	*IntI-1/P. aeruginosa*	*blaVIM-3, sul1*
	Aac6'	184aa	EF488369	ABP35556	*IntI-1/ E. coli*	*catB3,*
	Aac6'	184aa	HM043570	ADH82126	*IntI-1/ K. pneumoniae*	*cmlA1*
	aac6'	184aa	JN596279	AEZ05102	*IntI-1/ K. oxytoca*	*blaGES11, sul1*
	Aac6'-Ib	201aa	M55547	AAA98404	*IntI-1/ P. aeruginosa*	*nd*
	Aac6'-IIa	184aa	M29695	AAA25688	*IntI/ P. aeruginosa*	*nd*
	Aac6'-1b	210aa	GQ293500	ADC80825	*23kb plasmid*	*cmlA, blaTEM-24*
	Aac6'-IIb	180aa	L06163	AAA25680	*IntI/ P. aeruginosa*	*nd*
	Aac6'-Ic	146aa	M94066	AAA26549	*IntI/ S. marcescens*	*nd*
	Aac6'	172aa	KF977034	AHY00029	*pDW16C2/ K. pneumoniae*	*blaVIM*
	Aac6'	172aa	KP870110	AKC98300	*pRCPEC6335-2/ E. coli*	*blaC-5, blaTEM1*
aacA5	aac6'	158aa	AM749810	CA078542	*IntI-1/ P. aeruginosa*	*blaVIM-2*
	Aac6'	158aa	HQ005291	ADN22946	*IntI-1/ P. aeruginosa*	*blaVIM-2, dhfr*
aacA6	Aac6'-Ib	172aa	AY686225	AAT94163	*IntI-1/ A. xylosoxidans*	*blaVIM-2*
aacA7	Aac6'	152aa	KJ679405	AID65189	*IntI-1/ P. aeruginosa*	*OXA-2, aadA6*
	aac6'-1a	152aa	EF577406	ABQ65124	*In58/ P. aeruginosa*	*blaVIN-2*
	Aac6'	152aa	FJ715943	ACN62402	*IntI-I/P. aeruginosa*	*nd*
	Aac6'	152aa	KJ679406	AID65194	*IntI1/P. aeruginosa*	*blaVIM-2, aacA4*
	Aac6'	152aa	JX486753	AFV31445	*In 58/C. fruendii*	*blaVIM-2, aacA4*
	aac(6')-I1	152aa	KP754008	ALE32150	*In 903/P. aeruginisa*	*Sul1*
	Aac6'	152aa	KJ679405	AID65190	*IntI1/P. aeruginosa*	*blaOXA-2. aadA6*
	Aac6'-I1	152aa	U13880	AAA90937	*IntI-1/ E. aerogenes*	*nd*
aacA8	Aac(6')	172aa	KJ679405	AID65187	*IntI1/P. aeruginosa*	*OXA-2, aacA6/7*
	Aac6'-IIb	180aa	L06163	AAA25680	*Genomic/P. fluorescences*	*ANT(3')-Ia*
	Aac(3')	308aa	AXTL01000004	ESD46433	*Genome/Escherichia coli*	*nd*
äacA(?)	Aac6''-Ic	146aa	M94066	AAA26549	*IntI/ Serratia marcescens*	*nd*
	Aac6'-If	144aa	X55353	CAA39038	*IntI/ Enterobacter cloacae*	*nd*
	Aac6'-Ig	145aa	L09246	AAA21889	*IntI/ Acinetobacter sp*	*nd*
	Acc6'-Ij	146aa	L29045	AAC41392	*IntI/ Acinetobacter sp*	*nd*
	Aac6'-Ik	145aa	L29510	AAA87229	*IntI/ Acinetobacter sp*	*nd*
	Acc6'-Im	173aa	Z54241	CAA91010	*IntI/ Citrobacter freundii*	*nd*
	Acc6'-Is	146aa	AF031327	AAD03491	*IntI/ Acinetobacter sp*	*nd*
	Acc6'-Iq	183aa	AF047556	AAC25500	*IntI/ K. pneumoniae*	*nd*
	Aac6'-Iw	146aa	AF031331	AAD03495	*IntI/ Acinetobacter sp*	*nd*
	Aac6'-Ix	146aa	AF031332	AAD03496	*IntI/ Acinetobacter sp*	*nd*
	Aac6'-Iy	145aa	AF144880	AAF03531	*IntI/ Salmonella enterica*	*nd*
	Aac6'-Iz	153aa	AF140221	AAD52985	*IntI/ S. maltophila*	*nd*
aacA16	Aac6'-Ip	173aa	Z54241	CAA91010	*1153bp IntI/Citrobacter*	*nd*
AacA17	Aac6'-Iq	183aa	AF047556	AAC25500	*plasmid/ K. pneumoniae*	*nd*
aacA28	Aac6'-Iae	183aa	AB104852	BAD14386	*Plasmid/ P. aeruginosa*	*blaIMP, aadA1*
aacA30	Aac6'-I30	184aa	AY289608	AAP43642	*2220bp IntI/S. enterica*	*blaOXA-53*
aacA39	Aac6'-Iai	188aa	EU886977	ACI28880	*pLQ1001/P. aeruginosa*	*aadA1, Sul1*
aacA41	Aac6'-Iaf	183aa	AB462903	BAH66386	*IntI123/P. aeruginosa*	*blaIMP, sul1*
aacA42	Aac6'-33	184aa	GQ337064	ACT99625	*6816bp/P. aeruginosa*	*blaGES,/OXA-2*
aacA43	Aac6'-Ii	182aa	L12710	AAB63533	*IntI/Enterococcus faecium*	*nd*

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Table 2. Classification of major AAC(3') acetyl transferase; The accession numders of plasmids, integrons and genomic fragments carrying *aac*(3')-type genes were demonstrated. Protein ids and types of MDR bacteria were also described.

Classification of different amino glycoside 3'-aceteyl transferases (aacC1)						
Aac3' Genes	**Enzyme types**	**No of AA**	**Accession number**	**Protein Id number**	**Plasmid/genomic**	**Name of bacteria**
aacC1	Aac3'-Ia	177aa	X15852	CAA33850	*pR1033(Tn1696*	*Enterobacteriaceae*
	Aac3'-Ia	154aa	AY577724	AAT51721	*3035bp genomic*	*A. baumannii*
	Aac3'-Ia	176aa	L06157	AAA88422	*genomic*	P. aeruginosa
	Aac3'-I	154aa	KJ688704	AID61151	*2684bp genomic*	*K. pneumoniae*
	Aac3'-I	154aa	AJ009820.2	CAA08847	*6436bp pSEM*	*S. enterica*
	Aac3'-Ia	154aa	KR028107	ALD03719	*2427bp genomic*	*A. baumannii*
	Aac3'-I	154aa	JX486753	AFV31447	*3030bp In58 integron*	*C. fruendii*
aacC2	Aac3'-IIa	286aa	JQ364967	AFI72859	*87kb pGuE-NDM*	*E. coli*
	accC2	286aa	X54723	CAA38525	*R plasmid*	*E. coli*
	Aac3'-IId	286aa	EU022314	ABS70977	*Plasmid fragment*	*E. coli*
	Aac3'-IIe	286aa	EU022315	ABS70978	*Plasmid fragment*	*E. coli*
	Aac3'	286aa	JN202624	AFP55521	*pFZ51; 15672bp*	*H. parasiuis*
	Aac3'	311aa	AXUL01000144	ESA99429	*2663 bp genomic*	*K pneuminiae*
	Aac3'	286aa	KJ187752	AJD77170	*pTR2*	*K. pneumoniae*
	Aac3'-III*	286aa	HF545433	CCN79846	*pE66An*	*E. coli*
	Aac3'-III*	286aa	NC_024983	YP_009061951	*pSTm-A54650*	*S. enterica*
	Aac3'	286aa	KP010147	AJN91221	*pECO-HN; 18784bp*	*E. coli*
	Aac3'	286aa	JX988621	AFZ84485	*pNDM-OM*	*K. pneumoniae*
	Aac3'	286aa	HQ840942	AES85952	*pSRC27-H; 50129bp*	*S. enterica*
aacC3	aacC3	286aa	X13543	CAA31895	*Plasmid pWP113a*	*Enterobacteriaceae*
	Aac(3')-III	208aa	JMVN01000059	KDG46702	*5980bp genomic*	*E. coli*
	Aac3'-IIIa	271aa	X55652	CAA39184	*2336bp genomic*	*P. aeruginosa*
	Aac3'-IIIb	245aa	L06160	AAA25682	*2613bp genomic*	*P. aeruginosa*
	Aac3'-IIIc	279aa	L06161	AAA25683	*1234bp genomic*	*P. aeruginosa*
	Aac3'-III	308aa	ADTS01000075	EGB89811	*genomic*	*E. coli*
	Aac3'-III	308aa	AXTL01000004	ESD46483	*6803bp genomic*	*E. coli*
	Aac3'-III	294aa	LO017738	CRH08791	*143kb pRCS57*	*E. coli*
aacC4	Aac3'-IV	261aa	X01385	CAA25642	*1368bp genomic*	*E. coli*
	Aac3'-IV	255aa	MPWC01000123	OKB98731	*genomic*	*K. pneumoniae*
	Aac3'-IV	172aa	AJ009820.2	CAA08845	*pSEM*	*S. enterica*
	AAC3'	206aa	CP015500	ANE70283	*Genome*	*K. pneumoniae*
	Aac3'	227aa	FLCN01000053	SAT62391	*Genomic fragment*	*K. pneumoniae*
aacC5	aac(3')-Vb	269aa	M97172	AAA26548	*1572bp genomic*	*S. marcescens*
aacC6	Aac(3')-VI	299aa	M88012	AAA16194	*2077bp genomic*	*E. cloacae*
	Aac3'-VIa	274aa	nd	WP_031611451	*nd*	*E. coli*
	Aac3'-VI	300aa	nd	WP_053271189	*nd*	*E. coli*
	Aac3'-VI	299aa	nd	WP-020837048	*nd*	*S. enterica*
aacC7	Aac3'-VIIa	288aa	M22999	AAA88552	*1494bp genomic*	*S. rimosus*
	Aac3'-VIIa	288aa	AJ749845	CAG44462	*genomic*	*S. rimosus*
	Aac3'-IIb	286aa	GG657754	EFL26570	*genomic*	*S. himasstatinious*
aacC8	Aac3'-VIIIa	286aa	M55426	AAA26685	*1353bp genomic*	*S. fradiae*
	Aac3'-VIIIb	287aa	nd	WP_063840271	*nd*	*S. ribosidificus*
aacC9	Aac3'-IXa	281aa	M55427	AAA25334	*1149bp genomic*	*M. chalcea*
	Aac3'-IXb	279aa	KB405056	EMF57573	*genomic*	*S. bottropensis*
aacC10	Aac3'X-Ia	284aa	AB028210	BAA78619	*genomic*	*S. griseus*
	Aac3'X-I	284aa	FMCP01000318	SCE16679	*genomic*	*Streptomyces sp*
	Aac3'X-I	284aa	FLTQ01000010	SBU98351	*genomic*	*Streptomyces sp*

Similarities among the aac(6') isomers (aacA1-aacA8)

Aminoglycoside 6'-N-acetyl transferase actively acetylates 6-N atom of amikacin, kanamycin, and neomycin [25-30]. *BLAST* analysis suggested that *aac*A1 was unique enzyme and had no similarity to *aac*A2/3/4/5/6 but *aac*A3 has similarity to *aac*A4 and *aac*A8 lineages **(Figure 3 and Table 1)**. Two types (184aa and 172aa) of *aac*A4 enzyme contained difference in NH2 terminal 13 amino acids indicating a precursor. Aminoglycoside 6'-N-acetyl transferases (*aac*A1) were located in plasmids and integrons of *E. coli* and other *Enterobacteriaceae* and were identical sequence [31-36]. As for example, plasmid NR79 (8298bp) of *E. coli* had *aac*A1 including *aad*A3, catB2, and sul1 genes. Another 8049bp small plasmid pCMXR1 contains *bla*CMY-9 and sul1 mdr

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genes with *aac*A1. However, in *Pseudomonas aeruginosa*, *aac*A1 gene was located in class I integron In831 with *bla*IMP-10 that involved in imipenem resistance. All *aac*A1 sequences were identical and no mutation was found. *Pseudomonas aeruginosa* class I integron contains *aac*A3 gene (protein Id. AIP98294 and AN:KM111260). A conjugative MDR plasmid, pNDM-MAR (267242bp, AN:JN420336) of *Klebsiella pneumoniae* contains *aac*A4 gene (protein Id. AFB82784) along with deadly *bla*NDM-1 and *bla*CTX-M-15 genes **(Table 4)** [37]. Another *aac*A4 gene (AN:ABP35556) was located in 2731bp class I integron (AN: EF488369) of *E. coli* with closely linked to *catB3* gene [38]. Two *aac*A4 genes (accession nos. AEZ05099) were located in 6061bp small plasmid pINCan01 (AN:JN596279) of *Klebsiella oxytoca* in association with *bla*GES-11 and sul1 *mdr* genes [39]. Two genes for aminoglycoside acetyl transferases (*aac*A5 and *aad*A7) were located in Pseudomonas 2903bp class I integron in association with *bla*VIM-2 and *dhfr* genes, involved in carbapenem and trimethoprim resistance respectively [40-44]. Two aminoglycoside 6'-acetyltransferase type Ib (*aac*6'-1b; 172aa) were cloned from *Achromobacter xylosoxidans* (AN:AY686225) as 3436bp class I integron with also *bla*VIM-2 gene. A 281 amino acids *aac*A8 gene was found with *aac*A7 (152aa) isomers in 3608bp class I integron (AN:KJ679405) of *Pseudomonas aeruginosa* associated with *aad*A6 and *bla*OXA-2. There was no similarity between *aac*A1 *vs. aac*A4 of *Klebsiella pneumoniae* (protein Id. ADH82126) and *Escherichia coli* (Protein Id. BAB72153) or *Pseudomonas aeruginosa* (protein Id. AIP98294) and therefore Pseudomonas enzyme designated as *aac*A3 which was isolated in China **(Figure 2 and Table 1)**. Neither 172aa 6'-N-acetyl transferase (*aac*6'-1b; protein Id. CAF18332) of Morganella morgani has similarity to *aac*A1 and designated as *aac*A4 and such enzyme in both plasmids were identical suggesting horizontal transfer but Klebsiella plasmid (AN:HM043570) was partially sequenced (38-44). The few new aminoglycoside resistance gene, designated *aac*(6')-Iae, encoded a 183-amino-acid protein that shared 57.1% identity with *AAC*(6')-Iq. Such *Escherichia coli* expressing exogenous *aac*(6')-Iae showed resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin but not to arbekacin, gentamycin or streptomycin **(Figure 1 and Tables 1-4)** [45].

Table 3. Localization of cat genes and hybrid aac-aph genes. Popular cat genes in plasmids/integrons of pathogenic MDR bacteria were described with GenBank accession numbers and protein ids. Also activated hybrid aac-aph genes were described including less well known aac2' and aac4' type genes that destroy many aminoglycoside antibiotics.

Types of aac2' and aac4' acetyl transferases						
AAC2'	aac(2')-I	181aa	NC_000962.3	NP_214776	*Genome*	*M. tuberculosis*
	aac(2')-Id	210aa	U72743	AAB41701	*Genomic fragment*	*M. smegmatis*
	Aac2''-IC	181aa	CP012506.2	ALB17378	*Genome*	*M. tuberculosis*
	aac-2'	198aa	CWKH01000002	CVZ16866	*genomic*	*M. neworleansense*
AAC-APH hybrid acetyl transferases-phospho transferase						
AAC-APH	aacA-aphD	479aa	AB682805	BAM15583	*6483bp genomic*	*S. aureus*
	aacA-aphD	479aa	GZU565967	AAA88548	*pSK1*	*S. aureus*
	aacA	479aa	AP003367	BAB47534	*pVRSAp; 25107bp*	*S. aureus*
	Aac6'-aph2'	479aa	M13771	AAA26865	*2120bp integron*	*E. faecalis*
	Aac6'-aph2''	479aa	CP002120	ADL66016	*2924344bp genomic; nt 2111743-2113182*	*S. aureus*
Type of CAT genes or chloramphenicol acetyl transferases						
CAT	catB3	210aa	EF516991	ABP52023	*2655bp integron*	*E. coli*
	catB3	210aa	HX259086	AAD20921	*pHSH2*	*E. coli*
	catB3	210aa	DQ343904	ABC69169	*1406bp integron*	*M. morganii*
	catB3	210aa	HQ170516	ADX02581	*3735bp integron*	*A. media*
	catB3	210aa	KM278198	A*IX48179*	*2208bp integron*	*V. fluminis*
	catB3	210aa	JX885645	AGM38586	*13241bp genomic*	*S. enterica*
	catB3	210aa	KC237285	AGM38599	*9983bp plasmid*	*S. enterica*
	catB3	210aa	KX421096	AOR05996	*253kb plasmid pA32*	*S. enterica*
	catB3	210aa	EF488369	ABP35557	*2731bp integron*	*E. coli*
	catIII	213aa	JN202624	AFP55523	*pFZ51; 15672bp*	*H. parasuis*
	catB4	182aa	AP012056	BAN19548	*pKPX-2*	*K. pneumoniae*
	catB8	210aa	KC543497	AGL46467	*pOZ176*	*P. aeruginosa*
	catA1	219aa	AP012055	BAN19276	*pKPX-1*	*K. pneumoniae*
	catA1	219aa	KJ541071	AIV96857	*pG5A4Y217*	*E. coli*
	cat_2	219aa	LN850163	CRK62815	*pI1-34TF*	*E. coli*
	catB2	210aa	AF047479.2	AAC14737	*pNR79*	*E. coli*

Similarities among aac(3') acetyl transferases;

Aminoglycoside 3'-N/-O–acetyl transferases were isolated from various microorganisms where it was found both in plasmids and chromosomes [46-55]. *aac*C1 enzyme was isolated from 2324bp plasmid R1033 of many *Enterobacteriaceae* and were *aac*(3)'-1a type (AN:CAA33850) [46]. *aac*C2 (EC:2.3.2.81) of *Escherichia coli* was cloned from a Moscow isolate (AN:X54723; Protein Id. CAA38525) [47]. A 310 amino acids length *Escherichia coli aac*3' enzyme (protein Id. ESD46483) was isolated in genome fragments (Accession nos. AXTL01000004 and ADTS01000075). *Pseudomonas aeruginosa aac*(3')-1b enzyme was 176 aa and likely partial

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(protein Id. AAA88422) [48]. *aac*C3 isomers (*aac*3'-IIIa/b/c) were cloned from *Pseudomonas aeruginosa* (ANs. X55652, L06160 and L06161) [49] and *aac*3'-Vb was sequenced from *Serratia marcescens* genomic fragment and the enzyme had 72% similarity to *aac*3'-Va and gave high label resistance to gentamycin, netimicin and moderate resistance to tobramycin (protein Id. AAA26548) [53]. Similarly, *aac*3'-*VIa* gene (AN:M88012) was cloned from *Enterobacter cloacae* large plasmid with 39% and 48% similarities to *aac*3'-VII and *aac*3'-II respectively [51,52]. Similarly, *aac*3'-*VIa* enzyme was found 299 aa (protein Id. AAA16194). *aac*C7 (protein Id. AAA88552), *aac*C8 (protein Id. AAA26685) and *aac*C9 (protein Id. AAA25334) genes were isolated from *Streptomyces rimosus* (AN:M22999), *Streptomyces fradiae* (AN:M55426) and Micromonospora chalcea (AN:M55427) **(Figure 3 and Table 2)** [53,54]. *AAC*(3)-XI, a new aminoglycoside 3-N-acetyltransferase from *Corynebacterium striatum* was isolated recently [55].

Similarities among 2'' acetyl transferases

An Acinetobacter baumannii *aac*2'' acetyl transferase (286aa; protein Id. AAA21890) was identified [56]. Also a chromosomally encoded aminoglycoside 2'-N-acetyltransferase (*AAC*(2')-Ic) from Mycobacterium tuberculosis was isolated [57-61]. Similarities among *aac*6'-1b-cr enzymes that mediates fluoroquinolone resistance. Aminoglycoside acetyltransferase, *AAC*(6')-Ib reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substituent [62]. *AAC*(6')-Ib-cr differs from *AAC*(6')-Ib, by two specific codon exchanges Trp102→Arg and Asp179→Tyr, which have been found to be necessary and sufficient for the ciprofloxacin resistance phenotype [63]. Among 313 Enterobacteriaceae from the United States, *aac*(6')-Ib-cr was detected in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae* isolates, and 12 (7.5%) of 160 Enterobacter isolates [64]. ESBL *Escherichia coli* were isolated from poultry farm in Switzerland associated with fluoroquinlone resistance due to *aac*6'-Ib-cr acetyl transferase. *Aac*6'-1b-cr enzyme present in pKPS30 plasmid (AN:KF793937; nt. 22991-23509) of *Klebsiella pneumoniae* [65]. Enterobacter aerogenes isolates associated with variable expression of the *aac*(6')-Ib-cr gene was reported giving high label fluoroquinilones resistance [66]. A Korean study indicated the presence of *aac*(6')-1b-cr variant enzyme in *E. coli* and *K. pneumoniae* and few strains simultaneously contained *aac*(6')-1b and *aac*6'-1b-cr with resistant to tobromycin, kanamycin and amikacin **(Figure 1)** [66]. Other mechanisms for fluoroquinolone resistance were target protection by qnrA1/B1/S1 proteins (has high affinity for ciprofloxacin) and active drug efflux by QepA and OqxAB pumps (remove ciprofloxacin from bacterial cytoplasm) [63]. *Salmonella enterica* plasmid also detected *aac*6'-1b-cr gene (AN:KM877269; nt. 119381-119965, complement).

Table 4. Characteristics of large MDR conjugative plasmids carrying multiple *aac* and *cat* genes. Chloramphenicol acetyl transferases, AG acetyl transferases and other mdr genes were clustered in one plasmid and bacteria carrying such plasmid were usually XDR and PDR types. Further, mutations in gyrAB, 16S rRNA, porB and *ABC* genes have not been analyzed here.

Multi-drug resistant genes in Plasmids involving aac and cat genes				
Accession number	**Plasmid Size**	**MDR Genes profiles with one or more aac and cat genes in large bacterial conjugative plasmids**	**GenBank Year**	**Pathogenic bacterial name**
CP015725	210	**aacA4**, blaOXA1, **cat**, arr2, sul1, aph2', mrx, mph2', blaNDM1, ANT3'', dhfr, blaTEM1, RND, ANT3''-Ia, ble, blaCTXm-65, floR(MFS), sul1. **Aac3'-IVa**	2016	*S. enterica*
KF793937	61	tetA, blaOXA-30, **catB3, aac6'-1b-cr,** arr3, sul1, SAPa, QnrB4, blaDHA, mex, mphA(2''), aphA1(3')	2016	*K. pneumoniae*
KM877269	249	Sul1, aphA1, aadA1, cmlA, aadA2, floR, hph, **aac3'-IV, aac6'-1b-cr, ,** blaOXA1, **catB,** arr3, sul1, terA/C/F	2015	*S. enterica*
LO017736	124	tetA, **aac3'-Ib,** blaOXA-1, **aac6'-Ia**, blaCTX-M-15, blaTEM1, ABC	2016	*E. coli*
LO017738	143	tetA, blaTEM1, blaCTX-M-15, **aac3'-III, cat,** bla OXA-1. Dhfr, mrx(2''), mphA, ABC	2016	*E. coli*
LN794248	300	Sul1, strA/B, blaTEM1, tetA, **aac6'-Ib,** blaOXA1, **catB3, aacC3**, tmrB, blaCTXM-15, aadA1, **catA1**	2015	*S. enterica*
KF954759	73	*bla*KPC-3, *str*B, **aac6'**, *chr*B, *ncr*A/Y, *srb*A	2014	*K. pneumoniae*
KX421096	253	OqxB/A RND, sul1, dhfr, fosA3, sul2, **aac3'-Iva**, **aac6'-1b-cr**, blaOXa1, **catB3**, arr3, sul1, TerC/D/A	2016	*S. enterica*
AB61665	47	*bla*IMP--2, **aacA4,** *aad*A2, *tet*A, *bla*CTXm, *sul*1	2012	*K. pneumoniae*
KC354802	41	**aacA4**, *aad*A1, *bla*OXA-9, *bla*TEM-1	2013	*K. pneumoniae*
NC_021087	26	*bla*GIM-1, **aacA4,** *aad*A1, *bla*OXA-2, *sul*1	2015	*E. cloacae*
NG_035843	15	*bla*OXA-30, **catB3**, *arr*-3, *sul*1, *qnr*, *bla*DHA-1	2014	*E. coli*
LN555650	299	terF, sul1, strA, **catB**, blaACC-1, **aacA4**, blaVIM-1	2015	*S. enterica*
JN420336	267	blaNDM1, blaOXA1, **aac6'**, qnrB1, **catB**, blaCTX-M,	2012	*K. pneumoniae*
KC543497	501	Ter2, sul1,, MFS,, blaIMP-9, ble, **catB8, aac6'-IId**	201 6	*P. aerogenosa*
NC_022078	317	ABC, *mer*B, *cat*, *aph**, **aac3'**, *cmr*, *tet*A, *bla*KPC	2015	*K. pneumoniae*
JQ64967	87	aad, sul1, blaNDM1, **aac3'-II**, blaOXA1, **aac6'-1b-cr**	2016	*E. coli*
AP012056	141	**Aac3', aac6', catB4**, tetA, sul2, blaOXA/CTX, strB,	2016	*K. pneumoniae*
CP011634	227	*bla*OXA, *aad**, *bla*TEM, *mer*C, *sul*1, **aac**	2015	*K. oxytoca*
NC_010795	15	Sul1, **aacC2, catB3,** strA, blaROB-1 , aph3'-I	2014	A. *p-pneumoniae*
CP009116	95	Aph, blaTEM, **aac3'**, MFS, dhfr, aad, arr2, blaNDM1	2014	*K. pneumoniae*

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NC_019889	87	**Aac3'-II**, *bla*NDM1, *sul*1, *Msr*E, *mph*E	2014	*K. pneumoniae*
AP012055	250	*bla*NDM1, *ccd*A/B, *aad*A2, **catA1**, *qac*A1	2013	*K. pneumoniae*
KF250428	151	*bla*IMP-4, **aacA4**, *Mer*C, *cmr*, *flo*^{R}	2013	*K. pneumoniae*
HG530658	223	*bla*ACC-1, *str*A, *aad*A2, **aac3'**	2015	*E. coli*
NC_019375	180	*bla*VIM, **aacA7**, *dhfr*, *ANT3'*, *bla*SHV-5, *sul*1, *aph*3'	2014	*P. stuartii*
KF705205	134	*hph*, *str*A, **aac3'-IV**, *tet*A, *bla*TEM-1	2015	*S. enterica*
NC_022522	168	*bla*CTX-M-25, **aacA4***, *str*B, *str*A, *aad*B, *bla*OXA-21	2014	*S. enterica*
LC055503	160	*bla*SHV-12, **aac6'**, *bla*OXA-10, *aad*A1, *sul*, *bla*DHA	2015	*K. pneumoniae*
HG941719	135	*bla*TEM/CTX/OXA, *aad*A5, *mph*A, **aac6'**, *sul*I, *tet*A	2014	*Escherichia coli*
KJ541071	44	*sul*1, *bla*OXA-2, *aad*A/B, *bla*TEM, **catA1**, *bla*GES-5	2014	*E. coli*
GU256641	110	*sul*2, *str*A, *bla*TEM, *bla*SCO, **aacC2**, *bla*ACC-4	2011	*E. coli*
KJ541681	90	*tetA, sul1, aadA1,* **aac3'-Ia, aac6'-Ib**, *aadA2, blaSHV5*	2015	*K. oxytoca*

Similarities among bifunctional (aac-aph) drugs acetylating enzymes

Discovery of *Enterococcus facium aac*6'-*aph*2'' bifunctional aminoglycoside modifying enzyme in 70 kb plasmid containing transposon Tn5281 and IS256 element was phenomenon [67]. All *E. facium* strains from United Kingdom had showed high level gentamycin resistance with many MDR genes and multiple conjugative plasmids were detected as demonstrated by Pulse-Field Gel Electrophoresis (PFGE) and hybridization studies [68-71]. A French study isolated *Staphylococcus aureus* strains with aminoglycoside resistance and had both *aac*6'-*aph*2'' hybrid and *aph*3'-III genes (phospho transferases) located chromosomally [40]. A turkish study with 358 genetamycin resistant *Staphylococcus aureus* showed 334 *aac-aph* type bifunctional acetylating enzyme [72]. A US-study indicated high level aminoglycoside resistance in *Enterococcus fecalis* due to *aac*6'-*aph*2'' enzyme causing both gentamycin and streptomycin resistance but streptomycin resistance conferred due to other *aph*3'-III/*aph*5''-III type genes [73]. Shigila sonnei genome has *aac*6'-*aph*2'' bifunctional enzyme (protein Id. CSO06978) with similarity to *aac*A4 enzyme (EC:2.3.1.82) of Proteus vulgaris (protein Id. WP_058127929), *Escherichia coli* (protein Id. CRL66321), Acinetobacter johnsoni (protein Id. ALV74709) or *Pseudomonas aeruginosa* (protein Ids. CBI63199, CBL95252 and ALI59095). Entercoccus faecalis chromosome islands carried bi-functional acetyl transferases (*aac*A-*aph*D; protein Id. ANN02929), streptothricin acetyl transferase (Sat4; Protein Id. ANN02919) including many adenyl tramsferases (Protein Ids. ANN02918, ANN02921, ANN02922 and ANN02927), streptomycin phosphotransferase (*aph*A3) and ISA(E) gene that conferred resistant to pleuromnticin, streptrogramin and incosamide antibiotics [74]. Bifunctional aminoglycoside-modifying enzyme like aminoglycoside (6')acetyltransferase-Ie/ aminoglycoside 2″-phosphotransferase-Ia (*AAC*(6')-Ie-*APH*(2″)-Ia) from Gram-positive cocci, (was isolated that conferred resistance to the 4,6-disubstituted aminoglycosides kanamycin, tobramycin, dibekacin, gentamycin, and sisomicin, but not to arbekacin, amikacin, isepamicin, or netilmicin [75]. A recently discovered bifunctional antibiotic-resistance enzyme named *AAC*(3)-Ib/*AAC*(6')-Ib', from *Pseudomonas aeruginosa*, catalyzes the acetylation of aminoglycoside antibiotics. The *AAC*(3)-Ib domain appears to be highly specific to fortimicin A and gentamicin as substrates, while the *AAC*(6')-Ib' domain exhibits a broad substrate spectrum [76-79].

Similarities of drug acetylating enzymes with strA/B and other phospho transferases

strA and strB genes could inactivate the streptomycin by phosphorylation. Phospho-streptomycin could not bind bacterial ribosome and such bacteria could grow at as high as 100 µg/ml streptomycin giving AMR [80]. Sequence analysis suggested there was no similarities between catB3 *vs.* strA/B and *aac*6'/3' *vs.* strA/B. The aminoglycoside phosphotransferase (*aph* gene) phosphorylate the antibiotics so that phosphorylated kanamycin, amikacin and neomycin could not kill the bacteria. There was also no similarity between AG acetyl transferase (*aac*A1/*aac*C1) with 264aa neomycin phosphotransferase (*aph*3'-Ia, Protein Id. CAA23892) and 341aa hygromycin phospho transferase (hygA, protein Id. AHC55481). However, streptomycin phosphotransferases (strA, 278aa and strB, 276aa; protein Ids. AAA26443 and CED95338) have only 28% similarities within 72% and 37% cover respectively [81,82]. Similarity of dug acetylating enzymes with drug adenylation enzymes. The aminoglycoside adenyl transferase [EC:2.7.7.47] was present in many bacterial plasmids of diverse bacterial species of Escherichia (ANs:HG41719, KJ484637, KM377239), Klebsiella (ANs:KF914286 and KF719970), Salmonella (AN:JQ899055) and Acinetobacter (AN: KM401411), but also present in some bacterial chromosome as in *Salmonella enterica* [83].

Antibiotic adenyl transferase (~263aa) adenylate drugs at 6-N position and conferred bacteria resistant to aminoglycoside antibiotics like streptomycin and amikacin [84]. Such enzymes seem Rel-Spo-like super family and do not have much similarity to the AG 3'/6' N-acetyl transferases and catB3 enzyme. Interestingly, adenyl transferases have similarity across the species and also notably exists as different isomers (*aad*A1 to *aad*A17) with 50-80% sequence similarities among itself [85,86].

Similarities of drug acetylating enzymes with β-lactamases

Beta-lactamases are very diverged enzymes with at least twenty distinct isomers including TEM, OXA, NDM1 and CTX-M [1]. Metallo β-lactamases (VIM, IMP, NDM1, SPM, GIM, DIM) are very deadly as resistant to imipenem and beta-lactamase inhibitors cavulinate and sulbactam [10]. *BLA*ST analysis found no similarities between AG acetyl transferases and β-lactamases [87].

Conjugative plasmids have multiple drug acetylating enzymes

GenBank (www.ncbi.nlm.nih.gov) search indicated **(Table 4)** that each single conjugative plasmid carried multiple drug

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acetylating enzymes (cat, *aac*) in association with many beta-lactamase genes as well as strA/B, sul1/2 genes (10). As for example, *Salmonella enterica* plasmids (pIncH12 and pHXY40908) accquired four (*aac*6'-1b, catB3, *aaC*3, catA1) and three (*aac*3'-IV, *aac*6'-1b-cr, catB3) drug acetylating enzymes (accession nos. LN794248, KM877269). **Table 4** profoundly indicated how seriously multiple mdr genes had gathered in plasmids of many common pathogens that no drug would work to cure such bacterial infections.

Chromosomal localization of drug acetyl transferases

A 143aa long acetyl transferase was isolated from *A. baumannii* (AN:JFWP02000007; nt. 103508-103939) with similarity to *aac*6'-like enzyme (protein Id. EXT17036). *Acinetobacter* genomospecies *aac*6'-Ir enzyme (protein Id. WP_063840327) has 79% similarity to *aac*6'-Is (protein Id. AHD03491) but *aac*6'-It, *aac*6'-Iw, *aac*6'-Ix and *aac*6'-Iu have 83%, 85%, 90% and 94% similarities to *aac*6'-Is respectively (protein Ids. WP_063840329, WP_005296085, ALB75422 and WP_063840330) [88,89]. Aeromonas hydrophila genome (AN:LNUR01000009; nt. 843037-843495) has an unique 152aa 6'-acetyl transferase with limited similarity (47%) to *aac*6'-Ih of *A baumannii* (protein Id. ALB75422). *Pesudomonas saponiphila* genome (AN:FNTJ01000001; nt. 257741-258187) also has148aa long 6'-acetyl transferase (protein Id. SEB43247) with 51% similarity to *A. baumannii aac*6'-Ih enzyme (protein Id. ALB75422).

Chromosomally located *AAC*(2')-Ic of Mycobacterium tuberculosis catalyzes the coenzyme A (CoA)-dependent acetylation of the 2' hydroxyl or amino group of a broad spectrum of aminoglycosides. The *aac*(2')-Ic gene was cloned and expressed in *Escherichia coli*, and was purified. Recombinant *AAC*(2')-Ic was a soluble protein of 20,000 Da and acetylated all aminoglycosides substrates tested *in vitro*, including therapeutically important antibiotics like tobramycin, amikacin, kanamycin and ribostamycin. MRSA bacteria were initially discovered as methicillin resistant microbe named *Staphylococcus aureus* gram positive circular bacteria that notoriously known as for skin infections. The methicillin resistance gene (mecA) encodes a methicillin-resistant penicillin-binding protein and activated with mobile genetic element, the st*aph*ylococcal cassette chromosome mec (SCCmec), of many MRSA isolates which also associated with *bla*, *aac*, *aad*, ANT and sul1/2 types MDR genes. Such multi-drug resistant bacteria are susceptible only to glycopeptides antibiotics such as colistin and tigicycline [90,91]. Mutations among CAT enzymes

catB3 gene has no similarity to chloramphenicol drug efflux gene cmlA (protein Id. AKG90151), or acetyl transferases (*aac*3'-IV; Protein Id. AKG90173) or kanamycin phosphotransferases (protein Id. AKG90144) or hygromycin phosphotransferase (protein Id. AKG90172). No mutations were reported among the class I integron mediated catB3 genes of many Enterobacteriaceae (see, ANs: EF660562; ABP35557; ADF59078; AGJ70489). However, 217 aa long Proteus mirabilis CAT enzymes (protein Ids. WP_049194799 *vs.* WP_049197252) had two point mutations (V24A, N130D) and 201 aa long CAT enzymes in *Escherichia coli* had two point mutations (Y16N, D195N) and 9aa NH2-terminal substitutions (protein Ids. WP_050436713 *vs.* WP_050558894)

Mutation among *aac*A1 type enzymes

We see seven mutations in *Aeromonas hydrophila aac*6'-I enzymes (ANT67440 *vs.* KWR67119) with 95% similarities. However, 26 mutations in *Aeromonas piscicola aac*6' enzyme (protein Id. KWR67119) at the NH2 -termianl 60 amino acid region (WP_065401184 *vs.* KWR67119). A 194aa long *aac*6'-Ia enzyme of Wohlfahrtiimonas chitiniclastica has 9aa signalling peptide at the NH2 terminus and very similar to *aac*A1 enzyme of *E. coli* plasmid pCMXR1 (AN:AB061794) except one point mutation (V84I). But 185 aa *E. coli aac*A1 enzyme (protein Id. BAB72153) has only 55% similarity with 185 aa long *Klebsiella pneumoniae aac*6'-Iai enzyme (protein Id. WP_032495046). Such unusual *aac*A43 enzyme has two different mutations in *A. baumannii* ((I2S, K141N; protein Id. WP_024437351) and P. aeruginosa (R20Q, R95K; protein Id. WP_071846376).

Several mutations in many Enterobacteriaceae *aac*A4 enzymes (*aac*6'-1b type) were reported with respect to *Escherichia coli* enzyme (protein Id. ABP35556). Different point mutations were found in *Pseudomonas aeruginosa* (A75G; protein Id. WP_071846301), (S83G; protein Id. WP_071846385) and (T132A, K133R; protein Id. WP_071593232). Similarly in *E. coli aac*A4 enzyme, a single mutation (Q101L; protein Id. WP_069985732) was reported and also in *Enterobacter hormachei* (R181C; protein Id. WP_07220113) and in *Pseudomonas putida* (Q49R; protein Id. WP_071984682). A 197aa long *aac*A4 enzyme (protein Id. AKJ19116) in P. aeruginosa plasmid pMRVIM0713 (AN:KP975076) had extended 13 aa and two mutations (M1V and S102L) were reported. Such NH2-terminal fusion were found frequently as in P. aeruginosa integron-mediated 203aa long *aac*6'-1b enzyme (protein Id. *AAC*46343; AN:U59183) and also in 210 aa long enzyme (protein Id. CBI63203; AN:FN554980). A chromosome mediated *Acinetobacter baumannii* 216aa long *aac*A4 enzyme (protein Id. EKA73751) with similar M1V and S102L mutations was also reported **(Figure 3)**.

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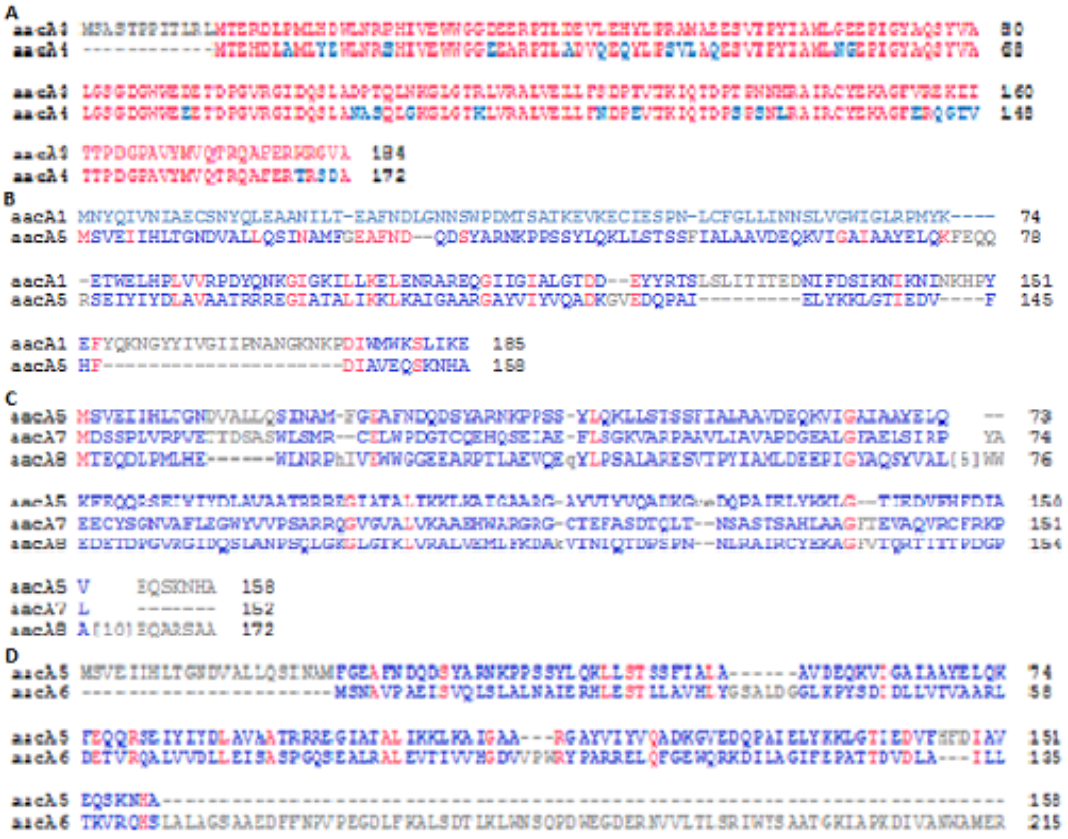

Figure 3. Similarities among aacA1 enzymes: (A) aacA1 *vs.* AaacA4 with terminal truncation with point mutations. (B) aacA1 *vs.* aacA5 with internal deletions and no similarity. (C) No similarities among aacA5, aacA6 and aacA8 and (D) no similarity between aacA5 *vs.* aacA6.

A 183aa long *aac*A16 N(6')-acetyl transferase (protein Id. WP_001109644) has only 60% similarity with *aac*A43 enzyme of many Enterobacteriaceae (protein Ids. WP_063840279; WP_024437351). Such enzyme has 60% similarity with the *Citrobacter freundii aac*6'-I1 enzyme (protein Id. CAA91010; AN:Z542441) with most divergent at the NH2-terminus. As such enzymes have only 55-65% similarity with the *aac*A1 enzymes (protein Id. BAB72153), their association in *aac*6'-I class was not therefore justified (>60 mutations).

Mutations among *aac*C1 type enzymes

*Aac*C1 enzyme of Enterobacteriaceae is 177 amino acids (protein Id. CAA33850) and has similarity to Acinetobacter baumannii gentamycin N-3'-acetyl transferases having different mutations reported in different isolates: In one isolate (protein Ids. WP_031950771, EXD70625 and WP_000441892) with three mutations (R98K, P102A, T175P). In *Pseudomonas aeruginosa*, *aac*C1 enzyme has many mutations reported. As for example, one isolate has S23R, K74R, D83E mutations (protein Id. WP_052158612); in another K38Q, D83E, E165D mutations (protein Id. WP_063840256) and in another (protein Id. ALE32149) five mutations (K38Q, D83E, R98K, P102A, and E165D) with few common mutations. However, less conserved enzymes have 71-73% similarities (protein Ids. ABN10340 and CRQ60998). *Salmonella enterica aac*C1 enzyme (protein Id. WP_032491356) has three mutations with two very common (S104I, R98K and P102A) and in another, two mutations were detected (P152T, S81I; protein Id: WP_032491356). An *A. baumannii* and *P. aeruginosa AAC*(3)-I enzyme have P152T common mutation (protein Ids: WP_052133400 and WP_063840258).

E. coli 158aa long *AAC*3'-acetyl transferase has two mutations (A53G, R234Q) that induces apramycin resistance (protein Id. WP_064756331) and also in *K. pneumoniae* (W5L, A53G; protein Id. WP_064735602). But in another *E. coli* mutant three mutations (A53G, H241R, G246E ; protein Id. WP_072833186) and in another four mutations (A53G, K177N, L178C, D182E; protein Id. WP_072739411) were reported. *AAC*(3)-I enzyme of *Serratia marcescens* (178aa; protein Id. OCO95380) and *Enterobacter cloacae* (178aa; protein Id. WP_032663836) have only 73% similarity with 22aa signalling protein at the NH2 terminus suggesting those enzymes are diverged *aac*3'-Ia type. Even both enzymes are 178aa long with 88% identity, have 22 mutations demonstrating drug acetyl transferases are indeed involved rapidly similar to β-lactamases **(Figure 4)**.

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Figure 4. Sequence alignment of different AAC3' drug acetyl transferases. ESD46483 (*E. coli* chromosomal aacC1 protein, 308aa, AN:AXTL01000004); AAA21890 (*A. baumannii* aacC2 protein, 286aa; AN:M62833); CAD27711 (*E. coli* aacC4 protein, 261aa, pHK11-apra vector; AN:AJ438947); CAA33850 (*Enterobacteriaceae* plasmid mediated aacC1 protein, 177aa: AN:X15852); CAA38525 (*E. coli* aacC2 protein, 286aa; AN:X54723); CAA39184 (P. aeruginosa aacC3, 271aa; AN:X55652); AAA26682 (P. aeruginosa aacC3b protein, 245aa; AN:L06160); AAA25683 (P. aeruginosa aacC3c protein, 279aa; AN:L06161); AAA26548 (*C. marcescens* aacC5b protein, 269aa; AN:M97172); AAA16194 (*E. cloacae* aacC6 protein, 299aa;AN:M88012); AAA88552 (*S. rimosus* aacC7 protein, 288aa, AN:M22999); AAA26685 (*S. fradiae* aacC8 protein, 286aa; AN:M55426); AAA25334 (*M. Chalcea* aacC9 protein, 281aa; AN:M55427); BAA78619 (*S. greseus kan* gene. 284aa, AN:AB028210).

The 286 amino acids length *Escherichia coli aac*C2 (protein Id. CAA38525) was found similar to *aac*(3')-IIc enzyme of *E. coli* (protein Id. WP_063840266) but no similarity to *aac*C1 enzyme. *Escherichia coli aac*C2 enzyme (protein Id. AFI72859) had four (L14F, H275Q, E276K) and ten mutations (T11L, R12Q, K78E, P84L, A162T, N194D, E204D, A268P, A274V, Q278E) with the similar *aac*3'-IId (protein Id. ABS70977) and *aac*3'-IIe (protein Id. ABS70978) enzymes respectively. Among the Enterobacteriace

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*aac*3'-II enzymes several mutations were reported (T87A, A112T, T132S, A245V; see Protein Ids. WP_00988063; WP_051421733; KTQ31168). In *Salmonella enterica* enzyme 4bp deletion and T132S mutation were reported (protein Id. WP_060588432). No mutation was found between *K. pneumoniae* and *E. coli aac*C2 enzymes (AGP03376 *vs.* ODH13880). Other *aac*(3')-II enzymes reported in *Klebsiella pneumoniae* and *Acinetobacter baumannii* were shown very similar mutations indicating a horizontal transfer of such genes from *E. coli* plasmid by conjugation. The mutations in *K. pneumoniae* (protein Ids. AGP03376 and WP_031944095) were; L11I, Q12R, R70L, T79A, R183W, S193R, D204E, T270A, V277A, E279Q, and C280R. The mutations in *A. baumannii* (protein Ids. WP_057690920 *vs.* WP_002063884) were E142K, G184V, D186X but with *Escherichia coli* plasmid-mediated *aac*C2 enzyme (protein Id. CAA38525) 14 mutations were reported (L11I, Q12R, R70L, T79A, K135E, R183W, V184G, Y186D, S193R, D204E, T270A, V277A, E279Q, and C280R). In Enterobacter sp strain 50858885 similar mutations were reported as follows: L11I, Q12R, R70L, K78E, K135E, T79A, P84L, K135E, R183W, S193R, T270A, V277A, E279Q and C280R. Although *Shigella flexneri* (protein Id. ADY02606) had very similar mutations but much more mutations (only 76% similarity) were found in *Salmonella enterica* enzyme (protein Id. WP_061873001) and 85% similarity to *Sinorhizobium melilti* (protein Id. WP_003525983) *aac*3'-II enzyme.

However, numerous mutations were reported in *aac*(3')-III enzymes (EC:2.3.1.81) of *E. coli* conjugative MDR-plasmid pRCS57 (143225bp; AN:LO017738) as follows: L11I, Q12R, R70L, T79A, K135E, R183W, S193R, D204E, T270A, V277A, E279Q, and C280R (protein Id. CRH08791). Such plasmid has also had mrx macrolide resistant protein, mphR repressor, *bla*TEM-1 and *tet*A *tet*racycline efflux protein as well as Tnp and Tra genes including many IS-elements. Interestingly. a 308aa *aac*3'-III-like *E. coli* enzyme (protein Id. EGB89811) had extended 9aa at the NH2 terminus and very similar to other Enterobacteriaceae 294aa *aac*3'-III enzyme (protein Id. WP_013023858).

An 172aa long *aac*C4 enzymes of *E. coli* (protein Id. ACS75040) and P. aeruginosa (protein Id. AGG23542) were found two mutations R107Q, D170S and M54L, D170S respectively. *Stenotrophomonas maltophila* had D170M point mutation (protein Id. ABN48565; AN:EF210035). *Acinetobacter baumannii* genome had reported 210aa 38aa N-terminal extended *aac*A4 enzyme with D170S, O171V mutations. *Citrobacter freundii* plasmid pMRVIM1012 had 34aa N-terminal extended *aac*C4 enzyme with L90S, D170S mutations. *Achromobacter xylosoxidans* 19.8kb plasmid mediated 210aa *aac*A4 enzyme (protein Id. BAV17747) had also similar mutations (L90S, D170S). Similar mutation further reported in *Serratia marcescens* class-3 integron 188aa long *aac*C4 enzyme likely due to 16aa extension (protein Id. AAL10408) and also in Vibrio cholerae 192aa *aac*A4 enzyme (protein Id. AAM52493), suggesting similar integron/plasmid involved in conjugation to transfer *aac*A4 genes. Certainly such extended enzymes had not proved by the protein product analysis and reflects wrong reporting as judged by plasmid mediated shorter active enzymes reported **(Table 1)**.

Other *Escherichia coli* extended *aac*3'-IV enzymes (258aa) have several point mutations (Y188H, R236Q; WP_064770919 *vs.* WP_064756331) and (A53G, A216G; WP_064769430 *vs.* WP_064769895) and (A53G, A241R, G247E; WP_064769430 *vs.* WP_072833186). A genomic clone of S. enterica may code 266 aa enzyme with NH2 terminal extension of 8aa and M1V and common A53G mutations (AN:LHLZ01000022; protein Id. KNT82816). Similarly, in *K. pneumoniae* genome (AN: MPWC0100123 and JMXV01000021) two *aac*C4 enzymes (protein Ids. OKB98731 and KDJ63161) were predicted with 255 and 254 aa long and had common A53G point mutation.

*Aac*3'-VI enzyme of *E. coli* differs with single point mutations at T132S and A244V of E. cloaceae *aac*C6 enzyme (protein Ids. WP_053271189 *vs.* AAA16194) and insertions in *Salmonella enterica* genome may code for different acetyl transferases (protein Ids. KNK91744 and EHC71407).

Drug acetylating enzymes in expression vectors

Cat gene was introduced in many DNA vectors: As for example BAC vector pHL931 (protein Id. ALV82398); Gateway vector pB4cCGGW for plant (protein Id. BAV44483); pDONRpEX18Gm expression vector (protein Id. AJW82929); orf selection vector pSOS (protein Id. ABK62679) and so many to state. *Aac*C1 gene (177aa) was cloned in varieties of expression vectors like pMQ175 (AN:FJ380062), pCVD001 (AN:KM017942), pSX (AN:JN703735), pUCP24 (AN:HM368668), and also in association with Beta-lactamase gene like pEX18gmGW (AN:KM880127), pLOXGen4 (AN:AJ401048); and in association with cat gene like pMpGWB236 (ANLC057515), pJM101 (AN:KX782328); and in association with AG phospho transferase gene like pBG51 (AN:KT192133) and pVZ324 (AN:AF100177). *Aac*A4 gene (267aa) was also cloned in suicidal vector pSUI3 (AN:KX863720) and in BAC vector pHL931 (AN:KT362048) and in cloning vector pHK11-apra (AN: AJ438947) 263aa long *aac*A4-type gene was dissimilar at amino and carboxy terminals. Wide spread use of mdr genes in plasmids should be controlled as recombinant drug resistant bacterial isolates might be released into environment.

DISCUSSION

We see wide spread presence of *AAC* enzymes in plasmids and chromosomes of household bacteria. Recently Hasani et al. evaluated aminoglycoside resistance in 87 *Acinetobacter baumannii* strains isolated from four hospitals of Iran and was found *aac*(3')-Ia predominant sequence group (SG) including ANT(2')-Ia and. *APH*(3')-Via. *APH*(3'-Ia related to resistance against amikacin and kanamycin, whereas ANT(2')-Ia was related to the resistance for gentamycin and tobramycin in SG2 and tobramycin

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resistance was correlated with *aac*(6')-Ib [92]. Many cellular N-acetyl transferases (NAT2) are known but are different than CAT and AAC enzymes.

Corynebacterium striatum BM4687 was resistant to gentamicin and tobramycin but susceptible to kanamycin A and amikacin. A novel 3-N-acetyltransferase type XI was purified and sequenced with 60% amino acid identity with acety ltransferases [93]. The purified protein acetylated dibekacin to the amine at the C-3 position Many chromosomal enzymes designated as *aac*A9, *aac*A16, *aac*A30, *aac*A41, *aac*A43 etc. were reported but further needed for placement [94]. Further, an unique bi-functional acetylating enzyme, *aac*(6')-Ie-*aph*2''-I was detected in *Staphylococci cassette* chromosome [95] as well as Enterococcei clinical isolates in China [93] and Campilobacter isolates in USA.

CONCLUSION

Thus it was concluded that drug acetyl transferases were highly diversified. Cat gene although had minimum divergence but 3'- and 6'- acetyl transferases (*aac*A1/C1) were arose very highly similar to very diversified β-lactamase (*bla*) genes (Chakraborty, 2016). Functional analysis of aminoglycoside acetyl transferases mutants, however less explored. Because such genes were associated with MBLs and acrAB/CD or mexAB/XY diverged tripartite proton drug efflux genes, Never the less, cmlA2 chloramphenicol transporter was also detected in 23kb plasmids like pRYC103T24 (AN:GQ293500; protein Id. ADC80829) of *E. coli* indicating chloramphenicol highly contaminated in nature and mdr gene evolution was maximum. 3-D structures and critical active site changes with better drug acetylating motifs and enlarged drugs selectivity must be addressed to design new drug against superbugs. AMR had reached an alarming label worldwide and all mdr genes must be assessed carefully at the molecular level. More importantly, three rings of aminoglycosides with many –OH and -NH2 groups of acetylation were designated as (I=1'-6', upper), (II=1-6, middle) and (III=1''-6'', lower) but the number of acetylating preferences would be determined carefully. Although drug phospho transferases and adenyl transferases could act very similarly to various O- and N-atom of the drugs but such enzymes had no similarity to *aac*C1 or *aac*A1 type enzymes indicating drug modifying enzymes indeed strongly diversified [50]. Presently, no de-acetylase enzyme was reported in MDR plasmids. Thus super conjugative plasmids with deacetylase genes could be used as a control measure to combat drug modifying superbugs that were contaminated highly in air and water. We are studying acetyl transferases in multi-drug resistant bacteria from Kolkata Ganga River and data indicates multiple isomers are present. In essence, mdr genes like drug acetyl transferases have created a very serious problem in human health and safety. It appears new drug development should be accelerated but alternate strategies like development of phyto-antibiotics, gene medicines (antisense, ribozyme, dicer-casper, miRNA) and DNA nanotechnology applications also should adapted in R&D research of India and other Asian countries with population burden.

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