



Prevalence of Antibiotic Resistant Genes in *Staphylococcus aureus* Isolated from Bovine Mastitis

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ABSTRACT

A total of 280 *Staph. aureus* strains from a total of 1250 milk samples from buffaloes were tested for 15 antibiotics using disc diffusion method followed by detection of their respective antimicrobial resistant genes through PCR. Among them, the highest prevalence of *Staph. aureus* was found in Peshawar-Mardan division (30%), followed by Malakand (28.5%), Bannu-Dera Ismail Khan division (25%) and Hazara division (16%). Over all the high resistance was found against Lin (96.25%) followed by AMX (82.5%), TET (63.75%), AMP (58.75%), SXT (50%), CHL (48.7%), CLR (36.25%), STR (25%), GEN (17.5%), OFX (15%), LFX (12.5%), AZM (8.75%) while least resistance against GAT (3.375%) and CRO (6.25%). Over all the highest prevalent gene was *bla*TEM (179) followed by *tetA* (147), *tetB* (144), *bla*CMY-2 (142), *sul1* (139), *sul3* (137), *tetC* (130), *aadA* (121), *sul2* (118), *strA/strB* (117) while the least resistant gene was *aadB* (12) and *aac(3)IV* (16).

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Authors' Contributions

SBK, MAK, IA and FAK designed the study. SBK, MAK, SAK executed the experimental work and analyzed the samples. SBK, MAK, HK, SAK helped in data analysis and article drafting.

Key words

Antibiotic resistance, Antibiotic resistant genes, Bovine, Mastitis, *Staphylococcus aureus*.

INTRODUCTION

Mastitis is one of the most important economic diseases of dairy animals. It causes huge economic losses to the national exchequer in terms of morbidity, drop in milk production, reduction of milk quality and veterinary services cost. Different countries have reported different economic losses due to disease including UK, USA and Holland (Hillerton *et al.*, 2005; Huijps *et al.*, 2008; Viguier *et al.*, 2009). There are reports of more than 140 species of different microbes responsible for bovine mastitis. Staphylococci, coliforms and streptococci are most frequently isolated microbes (Watts, 1998; Tenhagen *et al.*, 2006; Piepers *et al.*, 2007; Malinowski *et al.*, 2010; Smulski *et al.*, 2011). *Staphylococcus aureus* associated mastitis is more dangerous and complex than others microbes as the cure rates are comparatively lower. This complexity of *Staph. aureus* is because of their frequent acquisition of antibiotic resistance and biofilm formation

(Cramton *et al.*, 1999). It is thought that biofilm production is the major reason behind recurrent mastitis in dairy animals (Melchior *et al.*, 2006). A rapid increase in spreading of antibiotic resistant staphylococci and other microorganism is caused by merciless and indiscriminate use of antibiotics in animal feed and veterinary practice. An appropriate and proper usage of these antibiotics could minimize this malady of antibiotic resistance. There are certain factors including antibiotic resistant genes responsible for resistance to antibiotics.

Proper and appropriate usage of antibiotics is the need of the hour to overcome this malady of antimicrobial resistance (AMR). Discovery and development of new antibiotics is another alternative to tackle this issue. The prime purpose of the present study was to uncover the prevalence of antibiotic resistance and antibiotic resistant genes in *Staph. aureus* strains isolated from clinically positive animals suffering from mastitis in North West Pakistan.

MATERIALS AND METHODS

A total of 1250 milk samples from buffaloes clinically

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positive for mastitis were collected. Samples were brought to laboratory under hygienic condition at 4°C. Upon arrival to the Laboratory these samples were processed for culturing on tryptose agar followed by identification through colonial, microscopic morphology and tube tests for coagulase and catalase activity. For extraction of genomic DNA, bacterial DNA extraction kit (E.Z.Ne.A, Omega Bio-Tek, USA) was used. Thermostable gene (*nuc*), *mecA* and *blaZ* specific for *S. aureus* were targeted in genomic DNA. PCR conditions and primer sequences are given in Table I.

Fifteen different antibiotics namely Chloramphenicol (CHL) 30µg, Clarithromycin (CLR) 15µg, Levofloxacin (LVX) 5µg, Ofloxacin (OFX) 5µg, Gatifloxacin (GAT) 5µg, Ciprofloxacin (CIP) 5µg, Sulphamethoxazole+Trimethoprim (SXT) 25µg, Ampicillin (AMP) 10µg, Lincomycin (LIN) 2µg, Azithromycin (AZM) 15µg, Ceftriaxone (CRO) 30µg, Amoxicillin (AMX) 20µg, Gentamycin (GEN) 10µg, Strptomycin (STR) 10µg and Tetracyclin (TET) 30µg were used to test sensitivity and resistance in *Staph. aureus* isolates according to disc diffusion method as

Table I.- Targeted genes, their specific primers and PCR conditions.

Name of gene	Name of primer	Primer sequence	Primer concentration (µM)	Annealing Temp. (°C)	Size of product (bp)
<i>nuc</i>	nucF5'	GCGATAGATGGTGATACGGTT	0.1	55	270
	nucR5'	AGCCAAGCCTTGACGAACATAAGC	0.1	55	
<i>mecA</i>	mec1 5'	AAAATCGATGGTAAAGGTTGG	0.25	55	533
	mec2 5'	AGTTCTGCAGTACCGGATTTGC	0.25	55	
<i>blaZ</i>	blaZ15'	AAGAGATTTGCCTATGCTTC	0.20	54	517
	blaZ25'	GCTTGACCACTTTTATCAGC	0.20		
<i>blaTM</i>	GKTEMF ^d	TAACTGGCGAACTACTTAC	0.2	55	247
	GKTEMR ^d	GTCTATTTTCGTTTCATCCATA	0.2		
<i>blaSHV</i>	SHV-F ^j	AGGATTGACTGCCTTTTTG	0.4	55	393
	SHV-R ^j	ATTTGCTGATTTTCGCTCG	0.4		
<i>blaCMY-2</i>	CMYF ^d	GACAGCCTCTTCTCCACA	0.2	55	1000
	CMYR ^d	GGACACGAAGGCTACGTA	0.2		
<i>aadA</i>	4F ^e	GTGGATGGCGGCCTGAAGCC	0.1	63	525
	4R ^e	AATGCCCAGTCGGCAGCG	0.1		
<i>strA/strB</i>	strA-F ^f	ATGGTGGACCCATAAACTCT	0.4	63	893
	strB-R ^f	CGTCTAGGATCGAGACAAAG	0.4		
<i>aac(3)IV</i>	aac4-L ^g	TGCTGGTCCACAGCTCCTTC	0.2	63	653
	aac4-R ^g	CGGATGCAGGAAGATCAA	0.2		
<i>aadA</i>	4F ^e	GTGGATGGCGGCCTGAAGCC	0.1	63	525
	4R ^e	AATGCCCAGTCGGCAGCG	0.1		
<i>tet (A)</i>	TetA-L ^c	GGCGGTCTTCTTCATCATGC	0.1	63	502
	TetA-R ^c	CGGCAGGCAGAGCAAGTAGA	0.1		
<i>tet (B)</i>	TetBGK-F2 ^m	CGCCCAGTGCTGTTGTTGTC	0.2	63	173
	TetBGK-R2 ^m	CGCGTTGAGAAGCTGAGGTG	0.2		
<i>tet (C)</i>	TetC-L ^c	GCTGTAGGCATAGGCTTGGT	0.5	63	888
	TetC-R ^c	GCCGGAAGCGAGAAGAATCA	0.5		
<i>strA/strB</i>	strA-F ^f	ATGGTGGACCCATAAACTCT	0.4	63	893
	strB-R ^f	CGTCTAGGATCGAGACAAAG	0.4		
<i>aac(3)IV</i>	aac4-L ^g	TGCTGGTCCACAGCTCCTTC	0.2	63	653
	aac4-R ^g	CGGATGCAGGAAGATCAA	0.2		
<i>aadB</i>	aadB-L ⁱ	GAGGAGTTGGAATATGGATT	0.2	55	208
	aadB-R ⁱ	CTTCATCGGCATAGTAAAG	0.2		
<i>sul1</i>	sul1-F ^b	CGGCGTGGGCTACCTGAACG	0.2	66	433
	sul1-B ^b	GCCGATCGCGTGAAGTTCCG	0.2		
<i>Sul2</i>	sulII-L ^c	CGGCATCGTCAACATAACCT	0.3	66	721
	sulII-R ^c	TGTGCGGATGAAGTCAGCTC	0.3		
<i>Sul3</i>	sul3-GKa-F ^d	CAACGGAAGTGGGCGTTGTGGA	0.2	66	244
	sul3-GKa-R ^d	GCTGCACCAATTCGCTGAACG	0.2		

already described (Galani *et al.*, 2008). Strains resistant to two or more than two antibiotics are considered multi drug resistant (MDR).

Specific antibiotic resistant genes (ARGs) responsible for or conferring resistance to these antibiotics were targeted using multiplex PCR according to the method already described (Kozak *et al.*, 2009). Details of these ARGs, their primers specifications and PCR conditions are given in Table I.

RESULTS AND DISCUSSION

A total of 280 (22.4%, 280/1250) *Staph. aureus* strains were isolated from the four different divisions of Khyber Pakhtunkhwa province. Among them, the highest prevalence of *Staph. aureus* was found in Peshawar-Mardan division (30%, 85/280), followed by Malakand division (28.5%, 80/280), Bannu- Dera Ismail khan division (25%, 70/280) and Hazara division (16%, 45/280) (Table II). A total of 280 *Staph. aureus* strains were isolated which were tested for 15 antibiotics using disc diffusion method. Overall the high resistance was found against Lin (96.25%) followed by AMX, TET, AMP, SXT, CHL, CLR, STR, GEN, OFX, LFX, AZM while least resistance against GAT (3.375%) and CRO (6.25%) (Table II). About 80% *Staph aureus* were found to have multiple drug resistance. The drugs of choice were GAT and CRO. As for as antibiotic resistant genes are concerned, over all the highest prevalent gene was *blaTEM* followed by

tetA, *tetB*, *blaCMY-2*, *sul1*, *sul3*, *tetC*, *aadA*, *sul2*, *strA/strB* while the least resistant gene was *aadB* and *aac(3) IV* (Table III). It was observed that *tetA* gene were more associated with TET antibiotic followed by *tetB* and *tetC*. Similarly for beta- lactams antibiotic resistance *blaTEM* was found the highest followed by *blaCMY-2* and *blaSHV*. For sulpha drugs *sul1* was found the highest followed by *sul3* and *sul2*. For streptomycin, the highest ARG was *aadA* followed by *strA/strB* and *aac(3)IV*.

Antimicrobial resistance is one of the global and greatest issues after infection. There are reports of different countries regarding antimicrobial resistance in *Staph. aureus*. Malinowski *et al.* (2008) have reported 62.3% resistance to penicillin, 41.7% to tetracycline, 39.4% to lincomycin and 20% to bacitracin and cephalexin. In Turkey, Turutoglu *et al.* (2006) have reported resistance to penicillin, ampicillin and amoxicillin that were 62.1%, 56.3% and 45.6%, respectively. Resistance to gentamicin (56.3%) and trimethoprim/sulfa-methoxazole (45.6%) was also reported in the same study. Kalmus *et al.* (2011) have reported resistance to ampicillin (59.5%) and penicillin (61.4%) in Estonia. In Lithuania, Klimiene *et al.* (2012) have also found resistance to penicillin (76.7%), ampicillin (78.4%) and amoxicillin (81.3%). In China, Gao *et al.* (2012) have reported 96.3% resistance to penicillin and 98.1% to tetracycline, and 100% sensitivity to oxacillin, cefazolin and ciprofloxacin. In Ethiopia, 82.4% resistance to penicillin, 88.2% to clindamycin and 58.8%

Table II.- Prevalence of antibiotic resistance in *Staph. aureus*.

S. No.	Antimicrobials	No. of isolates resistant in different regions				
		Total n= 280 (22.4%)	Malakand division n= 80 (28.5%)	Hazara division n= 45 (16%)	Bannu- Dikhan n= 70 (25%)	Peshawar -Mardan n= 85 (30%)
1	LIN	277(96.25)	80(100)	45(100)	70(100)	70(85)
2	AMX	266(82.5)	78(95)	44(95)	60(80)	60(60)
3	TET	180(63.75)	50(65)	40(90)	37(55)	40(45)
4	AMP	170(58.75)	49(65)	26(70)	34(45)	47(55)
5	SXT	140(50)	32(40)	32(80)	32(40)	36(40)
6	CHL	120(48.75)	48(60)	30(65)	32(40)	25(30)
7	CLR	110(36.25)	45(55)	9(20)	35(50)	18(20)
8	STR	70(25)	20(25)	14(30)	20(30)	13(15)
9	GEN	28(17.5)	10(10)	14(30)	3(10)	18(20)
10	OFX	22(15)	5(5)	15(35)	5(15)	4(5)
11	CIP	22(15)	5(5)	14(30)	5(15)	9(10)
12	LVX	15(12.5)	5(5)	14(30)	3(10)	4(5)
13	AZM	10(8.75)	16(15)	3(5)	0(0)	13(15)
14	CRO	8(6.25)	0(0)	9(20)	0(0)	4(5)
15	GAT	3(3.75)	0(0)	0(0)	3(10)	4(5)

LIN, Lincomycin; AMX, Amoxicillin; TET, Tetracyclin; AMP, Amipicillin; SXT, Sulphamethoxazole-Trimethoprim; CHL, Chloramphenicol; CLR, Clarithromycin; STR, Streptomycin; GEN, Gentamycin; OFX, Ofloxacin; CIP, Ciprofloxacin; LVX, Levofloxacin; AZM, Azithromycin; CRO, Ceftriaxone; GAT, Gatifloxacin.

Table III.- Prevalence of antibiotic resistant genes (ARGs) in *Staph. aureus*.

ARGs	Overall n=280 (%)	Malakand division n=80 (%)	Hazara division n=45 (%)	Bannu-DIKhan n=70 (%)	Peshawar -Mardan n=85 (%)
<i>tetA</i>	52.5	52.5	77.7	52.8	47
<i>tetB</i>	51.4	52.5	75.5	47.1	31.7
<i>tetC</i>	46.4	50	57.7	34.2	49.4
<i>aadA</i>	43.2	31.2	57.7	34.2	30.5
<i>strA/strB</i>	41.7	31.2	53.3	20	28.2
<i>aac(3)IV</i>	5.7	13.7	46.6	18.5	12.9
<i>blaTEM</i>	63.9	100	44	28.5	92.9
<i>blaSHV</i>	42.1	16.2	28.8	38.5	29.4
<i>blaCMY-2</i>	50.7	57.5	84.4	0	32.9
<i>Sul1</i>	49.6	35	80	38.5	32.9
<i>Sul2</i>	42.1	28.7	60	18.5	17.6
<i>Sul3</i>	48.9	35	80	25.7	17.6
<i>aaddB</i>	4.2	0	0	15.7	9.4

to erythromycin while sensitivity to chloramphenicol (58.8%) and nalidixic acid (82.4%) was reported by [Haftu et al. \(2012\)](#). In India [Kumar et al. \(2011\)](#) have found resistance to streptomycin (36.4%), oxytetracycline (33.6%), gentamicin and ampicillin (29.9%), penicillin (28.9%) and chloramphenicol, pristinamycin and ciprofloxacin (26.2%). Resistance to tetracycline in France (3.1%) and Switzerland (5.3%) has been reported by [Sakwinska et al. \(2011\)](#). Very low antibiotic resistance (3%) has been reported in Sweden to kanamycin, tetracycline and penicillin by [Persson et al. \(2011\)](#). The difference in antibiotic resistance in the different countries may be due to use of different antibiotics, difference in antibiotic concentration and geographical variation. The high prevalence of antibiotic resistance to beta-lactams worldwide could be due to their worldwide application against staphylococcal mastitis. It was found that *tetA* gene was more associated with TET antibiotic followed by *tetB* and *tetC* which is in close agreement to the previous study conducted by [Olowe et al. \(2013\)](#). Similarly for beta-lactams antibiotic resistance *blaTEM* was found the highest followed by *blaCMY-2* and *blaSHV* which is partially in agreement and partially in disagreement with the previous study conducted by [Nambram et al. \(2018\)](#). For sulpha drugs *sul1* was found the highest followed by *sul3* and *sul2* which is closely related to the study conducted by [Patrícia et al. \(2005\)](#). For streptomycin, the highest AMRG was *aadA* followed by *strA/strB* and *aac(3)IV* which is a little disagreement with the previous study conducted by [Ramirez and Tolmasky \(2010\)](#).

CONCLUSION

In conclusion, 80% *S. aureus* strains have multiple drug resistance and antibiotic resistant genes which is a matter of great concern. The drugs of choice against *Staph aureus* are CRO and GAT followed by AZM, LFX and OFX. It is the need of the hour to develop alternatives antibiotics and ban unnecessary use of antibiotics to overcome this alarming and challenging situation of antimicrobial resistance.

Statement of conflict of interest

All the authors declare no conflict of interest

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