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Research Article

Investigation of the Genetic Basis of Tetracycline Resistance in *Staphylococcus aureus* from Pakistan

Farhat Ullah[1], Salman Akbar Malik[2], Jawad Ahmed[3], Farman Ullah[1], Syed Majid Shah[1], Muhammad Ayaz[1], Sajid Hussain[1] and Lubna Khatoon[4]

[1]Kohat University of Science and Technology, Kohat, [2]Quaid-i-Azam University, Islamabad, [3]Khyber Medical University, Peshawar, [4]University of Agriculture, Faisalabad, Pakistan

Abstract

Purpose: *To determine the prevalence and genetic basis of tetracycline resistance in Staphylococcus aureus.*
Methods: *One hundred and thirty (130) clinical isolates of S. aureus were collected from Khyber Teaching Hospital, Peshawar, Pakistan. Susceptibility to antibiotics (doxycycline, tetracycline and minocycline) was determined by Kirby-Bauer disc diffusion method with minimum inhibitory concentration (MIC) evaluated on Muller-Hinton agar as described by the Clinical and Laboratory Standards Institute (CLSI). The tetracycline-resistant strains (TET-R) were screened by polymerase chain reaction (PCR) for the presence of four common tetracycline resistance determinants, viz, tet(K), tet(L), tet(M) and tet(O).*
Results: *Sixty (46.0 %) of these isolates were methicillin-resistant S. aureus (MRSA) while 70 (54.0 %) were methicillin-susceptible S. aureus (MSSA). Seventy four (56.9 %) strains were resistant to tetracycline (TET-R), 30 (23.1 %) to minocycline and 23 (17.7 %) to doxycycline. A majority of the MRSA were resistant to tetracyclines and all the MSSA were sensitive to doxycycline and minocycline. The tet(K) gene was found in 58 isolates and tet(L) in one isolate. No tet(M) and tet(O) were detected.*
Conclusion: *This study indicates that resistance to tetracyclines is mainly by efflux pumps mediated by tet(K) in S. aureus in northwestern Pakistan.*

Keywords: *Staphylococcus aureus, Antimicrobial susceptibility, Antibiotic resistance, Tetracycline, Pakistan*

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Corresponding author:* ***Email**: farhataziz80@hotmail.com;* ***Tel:*** *0092-3339361513, 0092-922-554188*

INTRODUCTION

Tetracyclines are broad spectrum antibiotics used against a wide variety of bacterial infections, including both Gram-positive and Gram-negative. After their discovery in the 1940's, tetracyclines have continuously been used in both humans and animals with no major adverse effects. Tetracyclines inhibit bacterial ribosome from associating with the aminoacyl-tRNA and as a result, protein synthesis is inhibited. The discovery of tetracyclines sparked the development of many chemically altered antibiotics: including semisynthetic drugs such as minocycline and doxycycline [1].

A large number of tetracycline resistance genes have been identified. There are 38 acquired tetracycline resistance genes that are known and all use one of three strategies to render the bacteria resistant [2]. These include (1) efflux proteins, (2) ribosomal protection proteins and (3) enzymatic inactivation of tetracycline.

The majority of these genes (60 %) code for energy-dependent efflux pumps, and different bacterial genera tend to have the same efflux or ribosomal protection genes [3]. This indicates that tetracycline resistance genes can be transferred amongst the bacterial population. In fact, resistance to tetracycline in most bacteria is due to the acquisition of new genes; these genes tend to be associated with mobile elements such as transposons and plasmids [1]. Acne patients often require sequential treatment with tetracycline with several courses, which can last from three months to several years [4]. This long-term antibiotic treatment exerts a long selective pressure on both the targeted propionibacteria and the other microflora of the skin.

Tetacycline resistance genes: *tet*K, *tet*M, *tet*O and *tet*L are four major genes associated with tetracycline resistance amongst Gram-positive bacteria. The *tet*K and *tet*L genes code for efflux proteins; these are energy-dependent membrane-associated proteins which prevent tetracycline from accumulating within the cell [3]. The other two genes, *tet*M and *tet*O, code for ribosomal protection proteins, which reduce the affinity of tetracycline to the ribosome [5].

Surveillance for detection of various *tet* determinants has been carried out worldwide. In 2006, Jones *et al* found 74.0 % *tet*K and 13.0 % *tet*M in MRSA, and 73.8 % *tet*K and 21.4 % *tet*M in MSSA in a worldwide collection of *S. aureus* [6]. A study conducted by the SENTRY Antimicrobial Surveillance Programme between 1997 and 1999 found 57.0 and 10.0 % resistance to tetracycline in MRSA and MSSA, respectively. They recorded high prevalence of *tet*M in MRSA (76.0 %) and of *tet*K in MSSA (96.0 %) [7].

Tetracyclines are extensively used in Pakistan for skin and throat infections. But little surveillance work has been done to assess the extent of resistance. The aim of this study was to determine the genetic basis of tetracycline resistance in *S. aureus*. This is the first report disclosing genetic basis of tetracycline resistance in *S. aureus* from Pakistan.

EXPERIMENTAL

Bacterial strains

Between April 2005 and May 2006, 130 non-duplicate, consecutive *S. aureus* isolates were collected from in- and out-patients at Khyber Teaching Hospital, a tertiary care hospital in Peshawar. In this study, 130 *S. aureus* were obtained from various sources. There were 69 male and 61 female patients. A majority of the isolates were obtained from wound and burn/skin infections. Identification was carried out by Gram stain, catalase, coagulase and DNAse tests.

Antimicrobial agents susceptibility testing

Susceptibility to antimicrobial agents was determined by Kirby-Bauer disc diffusion and

minimum inhibitory concentration (MIC) method on Muller-Hinton agar (Oxoid, England) as per Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. The tetracycline antibiotics used against the collected strains were: doxycycline (DOX), tetracycline (TET), and minocycline (MN). *E. coli* NCTC 10418 was used as control for susceptibility testing.

Bacterial DNA extraction

Achromopeptidase method was used for DNA extraction. In this method, 50 ul of NaCl-EDTA Tris (NET) buffer (1x TE buffer in PCR water plus 20ul 5M NaCl) was added to 10 ml achromopeptidase solution in a small tube. Thereafter, 2 - 3 fresh colonies (18 – 20 h) of *S. aureus* were added and incubated for 15 - 20 min at 50 ^{o}C. After incubation, the extracted DNA was diluted 10 times in NET buffer for molecular work. The DNA was stored at –4^{o} C and used for up to one month.

Confirmation of MRSA

The phenotypically identified MRSA, using 30 µg cefoxitn disc, were genotypically confirmed by a duplex PCR targeting *mec*A gene responsible for methicillin resistance and a specific region of 16s rDNA region of the *S. aureus* (*nuc* gene). The primers used are stated in Table 1.

Determination of tetracycline resistance genes using PCR

The presence of *tet*K, *tet*M, *tet*L and *tet*O genes responsible for tetracycline resistance was detected by PCR. The primer sequence, amplicon size and reference for the detection of each gene are given in Table 1.

Agarose gel electrophoresis

The amplified products were run on 1.5 % agarose gel in Tris-Acetate EDTA (TAE) buffer for 45 min. The ethidium bromide stained bands were examined under an ultraviolet transilluminator and photographed with a Kodak camera.

Statistical analysis

Statistical analysis was performed by Chi-square test (Minitab 15 software) and *p*-values of ≤ 0.05 were considered significant.

Table 1: PCR primer sequences, amplicon size and PCR conditions

Gene targeted	Primer sequence (5-3)	Amplicon size (bp)	Reference	PCR conditions
mecA	F-CTCAGGTACTGCTATCCACC R-CACTTGGTATATCTTCACC	449	[9]	1 cycle of 95^{0} for 5 min 30 cycles of 95^{0}C for 30 s 55^{0}C for 30 se 72^{0}C for 1 min 1 cycle of 72^{0}C for 5 min
nuc	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	280	[10]	
tet(K)	F-GTAGCGACAATAGGTAATAGT R-GTAGTGACAATAAACCTCCTA	360	[11]	1 cycle of 95^{0} for 5 min 30 cycles of 95^{0}C for 60 s 55^{0}C for 60 s 72^{0}C for 90 s 1 cycle of 72^{0}C for 5 min
tet(L)	F-ATAAATTGTTTCGGGTCGGTAAT R-AACCAGCCAACTAATGACAAGAT	1077	[12]	
tet(M)	F-AGTTTTAGCTCATGTTGATG R-TCCGACTATTTAGACGACGG	1862	[12]	
tet(O)	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	[13]	1 cycle of 94^{0} for 5 min 30 cycles of 94 ^{0}C for 60 s 50^{0}C for 60 s s 72^{0}C for 90 s 1 cycle of 72^{0}C for 5 min

RESULTS

The age range of the patients was 3 months to 80 years with a mean of 28 years. There were 60 (46.15%) isolates that were resistant to cefoxitin, i.e., were MRSA (methicillin resistant *S. aureus*). These MRSA strains were also confirmed by PCR. Among the tetracycline antibiotics tested, 98 (75.4 %) isolates were susceptible to minocycline, 79 (60.8 %) to doxycycline and 55 (42.3 %) to tetracycline. A significant difference was found in the susceptibilities of all the tetracyclines used ($p < 0.05$). Among MRSA, 30 strains (50.0 %) were resistant to minocycline, 23 (38.3 %) to doxycycline and 48 (80.0 %) to tetracycline. All the MSSA strains were sensitive to minocycline and doxycycline, while 26 (37.1 %) were resistant to tetracycline (Table 2).

On screening the 74 isolates resistant to tetracycline for the presence of *tet*K, *tet*O, *tet*M and *tet*L genes, *tet*K gene was found in 58 isolates, made up of 34 among MRSA and 24 among MSSA. The electrophoretic gel photo for *tet*K is given in Figure 1. Only one *tet*L gene was found in MRSA. No *tet*M and *tet*O genes were found in these isolates.

All the *tet*K positive strains were highly resistant to tetracycline, MIC_{50} being 64ml/ml. The MIC distribution curve is shown in Figure 2. Among *tet*K positive *S. aureus*, 40 (67.8 %) were susceptible to doxycycline and 19 (32.2 %) to minocycline.

DISCUSSION

This study was conducted to identify the molecular mechanisms of resistance against tetracycline by *S. aureus* in Pakistan. The results of tetracyclines susceptibility in this study are in agreement with those of others who observed 83 and > 50 % resistance to tetracycline, respectively. [14,15].

Table 2: Susceptibility of the total isolated *S. aureus* and comparison of susceptibility between MRSA and MSSA to tetracyclines

Antiobiotic	*S. aureus n*=130				
	Sensitive N (%)	Intermediate* N (%)	Resistant N (%)	MIC_{50} (ug/ml)	MIC_{90} (ug/ml)
Minocycline	98 (75.38)	02 (01.54)	30 (23.08)	2	64
Doxycycline	79 (60.77)	28 (21.54)	23 (17.69)	4	16
Tetracycline	55 (42.31)	1 (0.77)	74 (56.92)	16	64
	MRSA *n*=60				
Minocycline	28 (46.67)	2 (00.33)	30 (50.00)	8	64
Doxycycline	18 (30.00)	19 (31.67)	23 (38.33)	8	16
Tetracycline	12 (20.00)	0 (0.00)	48 (80.00)	64	128
	MSSA *n*=70				
Minocycline	70 (100)	0 (0.00)	0 (0.00)	2	4
Doxycycline	61 (87.14)	9 (12.86)	0 (0.00)	0.25	1
Tetracycline	43 (61.43)	1 (1.43)	26 (37.14)	1	16

**An intermediate: Isolate is one that is inhibited in vitro but therapeutic effect is uncertain*

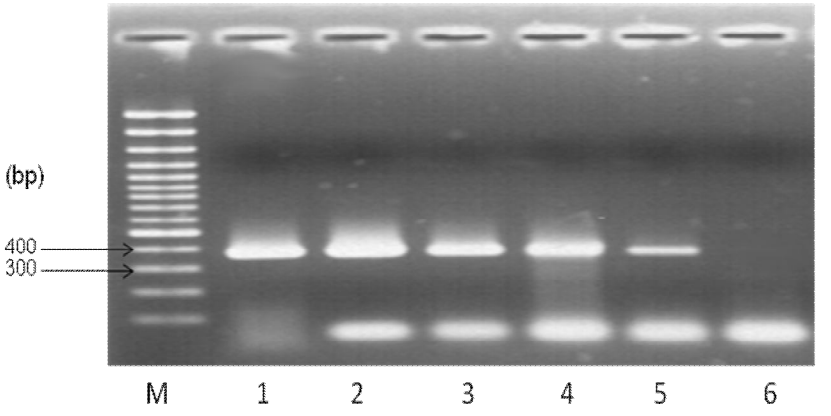

Fig 1. Agarose gel electrophoresis for *tet*K gene. M :Molecular weight marker, Lane 1: Positive control, Lane 2: Isolate (1), Lane 3: Isolate (5), Lane 4: Isolate (8), Lane 5: Isolate (10), Lane 6: Negative control.

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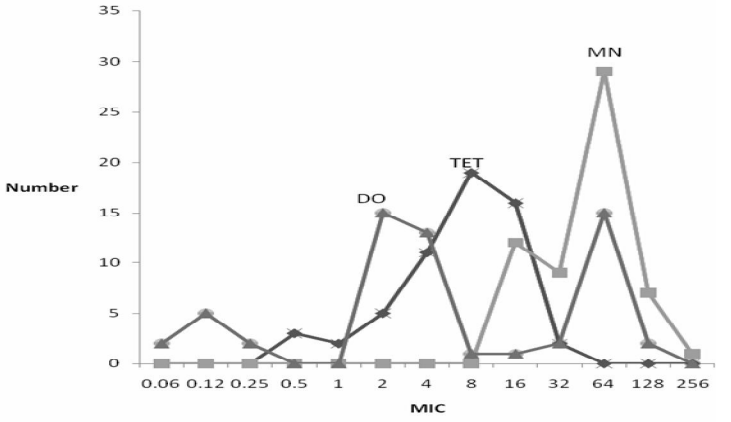

Fig. 2: MIC distribution of doxycycline (DO), tetracycline (TET) and minocycline (MN) among TET-R, *tet*(K) gene positive *S. aureus* (*n*=58)

On screening the isolates for the presence of four commonly found tetracycline resistance genes, 58 (78 %) of the isolates were positive for *tet*(K). The *tet*(K) gene protects bacteria from tetracycline by a resistance mechanism known as tetracycline efflux. This mechanism prevents the accumulation of tetracycline within bacterial cells [1] by the synthesis of a cytoplasmic membrane protein which pumps tetracycline out of the cell at a quicker rate than it enters [3]. This may explain why *tet*(K) gene does not impart resistance to minocycline.

Twenty four of the isolates had raised levels of resistance to minocycline, which was reflected in the MIC_{90} value of 64 ug/ml. The raised level of minocycline resistance may be due to other tetracycline resistance genes, besides *tet*(K) being present within the isolates that were not screened for.

Trzcinski *et al*, in a study conducted in Poland, found 66 % *tet*(K) in tetracycline-resistant MRSA [12]. Schmitz *et al*, in 1997 – 99, collected 400 MRSA and 200 MSSA from 25 university hospitals in Europe in the

international SENTRY program. They detected *tet*(K) in 73 % (292/400) MRSA and 96 % (192/200) MSSA isolates [7]. Huys *et al*, in 2005, in a collection of *S. aureus* from poultry processing plants reported 58 % (22/38) *tet*(K) in South Africa [16]. The level of *tet*(K) genes present in the three studies provides evidence to suggest that the distribution of *tet*(K) genes in *S. aureus* are wide spread. In addition to this, the frequency at which *tet*(K) occurs signifies that in *S. aureus*, the main mechanism of resistance is through tetracycline efflux. This also suggests that when screening *S. aureus* for tetracycline resistance genes, *tet*(K) must always be screened for first.

No traces of *tet*(O) were found in the current study and these findings correlate with those of Bismuth *et al*. This suggests that this gene is rare in *S. aureus*, unlike *tet*(K). In addition, there is very little evidence to indicate the occurrence of *tet*(L) in *Staphylococci* species, as indicated by both Bismuth *et al* and Trzcinski *et al*. As a result, this study as qell as previous studies indicate that *tet*(K) is widely distributed, and therefore, is more likely to be found in *S. aureus*.

CONCLUSION

The findings of this study indicate the mechanism of resistance to tetracyclines in *S. aureus* isolates from Pakistan could be through the production of efflux pumps, encoded mostly by *tet*(K).

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REFERENCES

1. *Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 2001; 65(2): 232-260*
2. *Roberts MC. Update on acquired tetracycline resistance genes. FEMS Microbiol Lett 2005; 245(2): 195-203.*
3. *Speer BS, Shoemaker NB, Salyers AA. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin Microbiol Rev 1992; 5(4): 387-399.*
4. *Coates P, Vyakrnam S, Eady EA, Jones CE, Cove JH, Cunliffe WJ. Prevalence of antibiotic-resistant propionibacteria on the skin of acne patients: 10-year surveillance data and snapshot distribution study. Br J Dermatol 2002; 146(5): 840-848.*
5. *Bismuth R, Zilhao R, Sakamoto H, Guesdon JL,Courvalin P. Gene heterogeneity for tetracycline resistance in Staphylococcus spp. Antimicrob Agents Chemother 1990; 34(8): 1611-1614.*
6. *Jones CH, Tuckman M, Howe AY, Orlowski M, Mullen S, Chan K, Bradford PA. Diagnostic PCR analysis of the occurrence of methicillin and tetracycline resistance genes among Staphylococcus aureus isolates from phase 3 clinical trials of tigecycline for complicated skin and skin structure infections. Antimicrob Agents Chemother 2006; 50(2): 505-510.*
7. *Schmitz FJ, Krey A, Sadurski R, Verhoef J, Milatovic D, Fluit AC. Resistance to tetracycline and distribution of tetracycline resistance genes in European Staphylococcus aureus isolates. J Antimicrob Chemother 2001; 47(2): 239-240.*
8. *CLSI. Clinical and Laboratory Standards Institute (CLSI): Performance Standard for Antimicrobial Susceptibility Testing. 16th Informational supplement. CLSI document 2006; M100-S16. Wayne, PA 2006.*
9. *Bignardi GE, Woodford N, Chapman A, Johnson AP, Speller DC. Detection of the mec-A gene and phenotypic detection of resistance in Staphylococcus aureus isolates with borderline or low-level methicillin resistance. J Antimicrob Chemother 1996; 37(1): 53-63.*
10. *Brakstad OG, Aasbakk K, Maeland JA. Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol 1992; 30(7): 1654-1660.*
11. *Strommenger B, Kettlitz C, Werner G, Witte W. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. J Clin Microbiol 2003; 41(9): 4089-4094.*
12. *Trzcinski K, Cooper BS, Hryniewicz W,Dowson CG. Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother 2000; 45(6): 763-770.*
13. *Ng LK, Martin I, Alfa M, Mulvey M. Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes 2001; 15(4): 209-215.*
14. *Sekiguchi J, Fujino T, Saruta K, Kawano F, Takami J, Miyazaki H, Kuratsuji T, Yoshikura H, Kirikae T. Spread of erythromycin-, tetracycline-, and aminoglycoside-resistant genes in methicillin-resistant Staphylococcus aureus clinical isolates in a Kumamoto*

Hospital. Jpn J Infect Dis 2003; 56(3): 133-137.
15. Saderi H, Owlia P, Habibi M. Mupirocin resistance among Iranian isolates of Staphylococcus aureus. Med Sci Monit 2008; 14(10): BR210-213.
16. Huys G, D'Haene K, Van Eldere J, von Holy A, Swings J. Molecular diversity and characterization of tetracycline-resistant Staphylococcus aureus isolates from a poultry processing plant. Appl Environ Microbiol 2005; 71(1): 574-579.