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%). Overall 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%). Overall the highest prevalent gene was blaTEM (179) followed by tetA (147), tetB (144), blaCMY-2 (142), sul1 (139), sul2 (137), tetC (130), aadA (121), aadB (118), strA/strB (117) while the least resistant gene was aaddB (12) and aac(3)IV (16).

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...
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 coagulate and catalase activity. For extraction of genomic DNA, bacterial DNA extraction kit (E.Z.Nce.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.

### 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) |
|--------------|----------------|-----------------|---------------------------|----------------------|---------------------|
| nuc          | nucF5'         | GCGATAGAAGGTTGATACCGGTT | 0.1                       | 55                   | 270                 |
|              | nucR5'         | AGCAAGGCTTGGCAAGCTAAAGGC | 0.1                       | 55                   |                     |
| meca         | mec1 5'        | AAATCGATGTTAAAGGTTGG | 0.25                      | 55                   | 533                 |
|              | mec2 5'        | ATGTTCTGCACTACCGGATTGC | 0.25                      | 55                   |                     |
| blaZ         | blaZ15'        | AAGAGATTTGGCCTATGCTTC | 0.20                      | 54                   | 517                 |
|              | blaZ25'        | GCTTGGACCTTTTATCGAC | 0.20                      | 54                   |                     |
| blaTM        | GKTEM9d        | TTAACCTGGCGAAGCTAAC | 0.2                       | 55                   | 247                 |
|              | GKTEM9d        | GTTATTTGCTGTTATCGA | 0.2                       | 55                   |                     |
| blaSHV       | SHV-1F        | AGGATTTGACCTTTTTTG | 0.4                       | 55                   | 393                 |
|              | SHV-1R        | ATTTTGCTGATTGTCG   | 0.4                       | 55                   |                     |
| blaCMY-2     | CMYF9d        | GACAGCCCTTTTATCCACA | 0.2                       | 55                   | 1000                |
|              | CMYR9d        | GGAACACAGGCGCTACGTA | 0.2                       | 55                   |                     |
| aadA         | 4Fe           | GTGGAATGGCGCGGCTGAAGCG | 0.1                       | 63                   | 525                 |
|              | 4R            | ATGCCCAAGTGCGGCGAGCG | 0.1                       | 63                   |                     |
| strA/strB    | strA-1F       | ATGGTGAGGACCTAAAACCT | 0.4                       | 63                   | 893                 |
|              | strB-1R       | CGTCGAAGATCAGACAAAG | 0.4                       | 63                   |                     |
| aac(3)Iv     | aac4-L8       | TGCTGTCAGCCAGCTCTTC | 0.2                       | 63                   | 653                 |
|              | aac4-R8       | CGATGACGAAGATCACA | 0.2                       | 63                   |                     |
| tet (A)      | TetA-Lc       | GGGCGGTCTTCTTCATCATGC | 0.1                       | 63                   | 502                 |
|              | TetA-Rc       | CGCGAGCGAGCAAGCTAGA | 0.1                       | 63                   |                     |
| tet (B)      | TetBGK-F22p    | GGCCTGAGAGAATGCAGGGTG | 0.2                       | 63                   | 173                 |
|              | TetBGK-R22p   | GGCCTTGAAGAATGCTAGTG | 0.2                       | 63                   |                     |
| tet (C)      | TetC-Lc       | GCTGGTGGACAGGCTTGGTG | 0.5                       | 63                   | 888                 |
|              | TetC-Rc       | GCGGAAGAGGCAAGAAGATAA | 0.5                       | 63                   |                     |
| strA/strB    | strA-1F       | ATGGTGAGGACCTAAAACCT | 0.4                       | 63                   | 893                 |
|              | strB-1R       | CGTCGAAGATCAGACAAAG | 0.4                       | 63                   |                     |
| aac(3)Iv     | aac4-L8       | TGCTGTCAGCCAGCTCTTC | 0.2                       | 63                   | 653                 |
|              | aac4-R8       | CGATGACGAAGATCACA | 0.2                       | 63                   |                     |
| aadB         | aadB-Lc       | GAGGATTTGGCACTATGGATT | 0.2                       | 55                   | 208                 |
|              | aadB-Rc       | CTTCATCGGACATGAAAG | 0.2                       | 55                   |                     |
| sul1         | sul1-1F       | CGCGGTTGAGGCTTGGTG | 0.2                       | 66                   | 433                 |
|              | sul1-1B       | GCCGATCGGCTGGTGGCCTGG | 0.2                       | 66                   |                     |
| Sul2         | sul2-Lc       | TGGCGGGATAGGCTGGTGGC | 0.2                       | 66                   | 721                 |
|              | sul2-Rc       | TGGCGGGATAGGCTGGTGGC | 0.3                       | 66                   |                     |
| Sul3         | sul3-GKa-pd   | CAACGGAAGAGGGAATGGTGG | 0.2                       | 66                   | 244                 |
|              | sul3-GKa-Rd   | GCTGACCAAATTCGCTGAGC | 0.2                       | 66                   |                     |
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, overall 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 aaddB 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 sulphura drugs sulI 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 cephalaxin. 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 pencillin, 88.2% to clindamycin and 58.8% resistance to gentamicin were reported.

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

| S. No. | Antimicrobials | 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(43.75)           | 48(60)                        | 30(65)                   | 32(40)                  | 25(30)                      |
| 7     | CLR            | 110(39.25)           | 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, Ampicillin; SXT, Sulphamethoxazole-Trimethoprim; CHL, Chloramphicol; 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 (%) |
|-------------|-------------------|-----------------------------|--------------------------|-----------------------|-------------------------|
| tetA        | 52.5              | 52.5                        | 77.7                     | 52.8                  | 47                      |
| tetB        | 51.4              | 52.5                        | 75.5                     | 47.1                  | 31.7                    |
| tetC        | 46.4              | 50                          | 57.7                     | 34.2                  | 49.4                    |
| aadA        | 43.2              | 31.2                        | 57.7                     | 34.2                  | 30.5                    |
| strA/strB   | 41.7              | 31.2                        | 53.3                     | 20                    | 28.2                    |
| aac(3)IV    | 5.7               | 13.7                        | 46.6                     | 18.5                  | 12.9                    |
| blaTEM      | 63.9              | 100                         | 44                       | 28.5                  | 92.9                    |
| blaSHV      | 42.1              | 16.2                        | 28.8                     | 38.5                  | 29.4                    |
| blaCMY-2    | 50.7              | 57.5                        | 84.4                     | 0                    | 32.9                    |
| Sul1        | 49.6              | 35                          | 80                       | 38.5                  | 32.9                    |
| Sul2        | 42.1              | 28.7                        | 60                       | 18.5                  | 17.6                    |
| Sul3        | 48.9              | 35                          | 80                       | 25.7                  | 17.6                    |
| aaddB       | 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 Patricia 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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